University of Alberta

The mechanism of action of cidofovir and *(S)*-9-(3-hydroxy-2-phosphonomethoxypropyl)adenine against viral polymerases

by

Wendy Colleen Magee

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Virology

Medical Microbiology and Immunology

Fall 2009
Edmonton, Alberta

Examining Committee

Dr. David H. Evans, Department of Medical Microbiology and Immunology

Dr. D. Lorne J. Tyrrell, Department of Medical Microbiology and Immunology

Dr. Tom Hobman, Department of Cell Biology

Dr. Atul Humar, Department of Medicine

Dr. Matthias Götte, Department of Microbiology and Immunology, McGill University

DEDICATION

To Dr. James Campbell, whose enthusiasm for microbiology encouraged me to pursue the field and to Dr. Ken Roy, who taught me the value of learning the principles and theories behind my experiments.

ABSTRACT

The nucleoside phosphonates cidofovir (CDV) and (*S*)-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine [*(S)*-HPMPA] are analogs of dCMP and dAMP, respectively. Collectively these drugs are effective inhibitors of a wide range of DNA viruses, RNA viruses, and retroviruses. Because they are nucleotide analogs, the drugs are thought to target viral polymerases and inhibit viral genome replication. However, the precise mechanism by which these drugs block viral growth remains unclear. We have studied the mechanism of action of these antivirals against three viral polymerases, vaccinia virus DNA polymerase and the reverse transcriptases from human immunodeficiency virus type 1 (HIV-1) and Moloney murine leukemia virus (MMLV). *In vitro* experiments using the active intracellular metabolites of CDV and *(S)*-HPMPA, CDV diphosphate (CDVpp) and *(S)*-HPMPA diphosphate [*(S)*-HPMPApp], respectively, showed that the drugs are substrates for each enzyme and can be incorporated into DNA without causing chain termination, although the rate of DNA elongation catalyzed by the vaccinia virus and MMLV polymerases is slowed. We have also found that incorporation of CDV or *(S)*-HPMPA blocked the 3′-to-5′ proofreading exonuclease activity of the vaccinia virus DNA polymerase. In addition, we determined that when these drugs are incorporated into a template DNA strand, they inhibited replication across the drug lesion. These results indicate that although CDV and *(S)*-HPMPA can inhibit some enzymes when incorporated into the primer strand, the main effects of drug action occur when they are incorporated into the template strand. Our findings point to a new avenue of targeted drug design, one in which nucleoside or nucleotide analogues are efficient

substrates for the viral nucleic acid polymerase, do not inhibit primer strand elongation, but exert their effects in subsequent rounds of nucleic acid synthesis.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. David Evans, for his support and guidance and my committee members, Dr. Tom Hobman and Dr. Lorne Tyrrell, for their encouragement and helpful suggestions during the completion of this study. I would also like to thank the members of the Evans lab, past and present; in particular Li Ge, Heather Jenkins, Chad Irwin and Dr. James Lin for the preparation of vaccinia virus DNA polymerase, and Danielle Lavallee and Lindsay Ross for technical assistance. Several people, including Dr. Graciela Andrei (Rega Institute for Medical Research), Dr. Michele Barry (Department of Medical Microbiology and Immunology), Dr. Craig Brunetti (Trent University), Dr. Matthias Götte (McGill), Dr. Karl Hostetler (University of California, San Diego), and Dr. Éva Nagy (University of Guelph) generously provided many of the reagents used during the course of these studies. I also thank Sriram Kammila (Faculty of Pharmacy and Pharmaceutical Sciences) for the immunization and handling of mice used in experiments described in the Appendix, Ted Birse (HIV Outpatient Program, Alberta Health Services) for answering all of my questions related to the clinical use of antiviral agents, Dr. Neeraj Shakya and Dr. Naveen Srivastav (Department of Laboratory Medicine and Pathology) for drawing all of the chemical structures in this thesis and Logan Banadyga and Stephanie Campbell for helpful discussion. Finally, I would like to thank the administrative and support staff in the Department of Medical Microbiology and Immunology for all of their help, in particular Anne Giles, Debbie Doudiet, Tabitha Vasquez, San Vinh, Alma Luces and Jadwiga Zurek.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3TC	(-)-β-2′,3′-dideoxy-3′-thiacytidine; lamivudine
α	alpha
A	adenosine
Å	angstrom
AIDS	acquired immune deficiency syndrome
AMP	adenosine monophosphate
araC	cytosine arabinoside
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AZT	3′-azido-3′-deoxythymidine; azidothymidine; zidovudine
β	beta
bp	base pair(s)
BGMK	buffalo green monkey kidney
BSA	bovine serum albumin
°C	degrees Celsius
CDV	cidofovir
CDVp	cidofovir monophosphate
CDVp-choline	cidofovir phosphocholine
CDVpp	cidofovir diphosphate
CFA	complete Freund's adjuvant
cm^2	centimeter(s) squared
CMV	cytomegalovirus
cpm	counts per minute
CPTS	copper phthalocyanine 3,4′,4″,4‴-tetrasulfonic acid tetrasodium salt
δ	delta
D4T	2′,3′-didehydro-2′,3′-dideoxythymidine; stavudine
dA	deoxyadenosine
dAMP	deoxyadenosine monophosphate
DAPI	4′,6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate

dC	deoxycytidine
dCMP	deoxycytidine monophosphate
dCTP	deoxycytidine triphosphate
ddAMP	dideoxyadenosine monophosphate
ddATP	dideoxyadenosine triphosphate
ddI	2′,3′-dideoxyinosine; didanosine
ddNTP	dideoxynucleoside triphosphate
dG	deoxyguanosine
dGMP	deoxyguanosine monophosphate
dGTP	deoxyguanosine triphosphate
DHBV	duck hepatitis B virus
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNMP	deoxynucleoside monophosphate
dNTP	deoxynucleoside triphosphate
dpm	decay per minute
dT	deoxythymidine
dTTP	deoxythymidine triphosphate
ε	epsilon
EBV	Epstein Barr virus
EC_{50}	50% effective concentration
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
(-)FTC	(-)-β-2′,3′-dideoxy-5-fluoro-3′-thiacytidine; emtricitabine
γ	gamma
x *g*	times gravity
G	guanosine
GST	glutathione-S-transferase
GTP	guanosine triphosphate
h	hour(s)

HBV	hepatitis B virus
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HDP	hexadecyloxypropyl
His_6	hexahistidine
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
hOAT1	human organic anion transporter 1
HPMP	N-*(S)*-(3-hydroxy-2-phosphonomethoxypropyl)
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
IC_{50}	50% inhibitory concentration
IFA	incomplete Freund's adjuvant
IgG	immunoglobulin G
IPTG	isopropyl-β-D-1-thiogalactopyranoside
K	reaction rate constant
kb	kilobase(s)
k_{cat}	turnover number
K_d	dissociation constant
kDa	kiloDalton(s)
K_i	inhibition constant
K_m	Michaelis-Menten constant
KSHV	Kaposi's sarcoma-associated herpesvirus
LB	Luria-Bertani
M	molar
µg	microgram(s)
µl	microliter(s)
µM	micromolar
mCi	milliCurie(s)

MEM	minimum essential medium
mg	milligram(s)
min	minute(s)
min^{-1}	per minute
ml	milliliter(s)
mm	millimeter(s)
mM	millimolar
MMLV	Moloney murine leukemia virus
mmol	millimole(s)
MMSV	Moloney murine sarcoma virus
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
msec	millisecond(s)
N	normal
ng	nanogram(s)
nm	nanometer(s)
nM	nanomolar
NMR	nuclear magnetic resonance
NNRTI	non-nucleoside reverse transcriptase inhibitor
NNRTI-BP	non-nucleoside reverse transcriptase inhibitor binding pocket
NS5B	nonstructural protein 5B
NTP	nucleoside triphosphate
%	percent
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline containing 0.1% Tween 20
PCR	polymerase chain reaction
pfu	plaque forming units
PME	phosphonomethoxyethyl
PMEA	9-(2-phosphonomethoxyethyl)adenine; adefovir
pmol	picomole(s)

$pmol^{-1}$	per picomole
±	plus or minus
PMP	phosphonomethoxypropyl
PMPA	9-(2-phosphonomethoxypropyl)adenine; tenofovir
′	prime
RNA	ribonucleic acid
RNase H	ribonuclease H
RSV	respiratory syncytial virus
RT	reverse transcriptase
SARS	severe acute respiratory syndrome
(S)-DHPA	*(S)*-9-(2,3-dihydroxypropyl)adenine
SDS	sodium dodecyl sulfate
sec^{-1}	per second
(S)-HPMPA	*(S)*-9-(3-hydroxy-2-phosphonomethoxypropyl)adenine
(S)-HPMPAp	*(S)*-9-(3-hydroxy-2-phosphonomethoxypropyl)adenine monophosphate
(S)-HPMPApp	*(S)*-9-(3-hydroxy-2-phosphonomethoxypropyl)adenine diphosphate
(S)-HPMPC	*(S)*-1-[3-hydroxy-2-(phosphonylmethoxypropyl)]cytosine
SIV	simian immunodeficiency virus
TdT	terminal deoxynucleotidyl transferase
TMB	tetramethylbenzidine
U	units
UDG	uracil-DNA glycosylase
UV	ultraviolet
V	volt(s)
V_{max}	maximum velocity
VZV	varicella zoster virus
v/v	volume per volume
W	watt(s)
WR	strain Western Reserve
w/v	weight per volume

CHAPTER ONE

INTRODUCTION

1.1 ANTIVIRAL AGENTS

Research into the discovery and development of new antiviral agents has increased over the past several years, motivated by an increase in chronic viral infections, like those caused by hepatitis B virus (HBV) and hepatitis C virus (HCV), the emergence of novel viruses causing human disease like the severe acute respiratory syndrome (SARS) coronavirus, the potential re-emergence of viral infections previously brought under control, like smallpox virus and measles virus infections, as well as the increase in viral resistance to current antiviral agents. As viruses replicate within host cells and utilize host cell machinery during their replication cycles, any antiviral agent needs to balance therapeutic efficacy with minimal toxicity to the host. There are several stages of the virus life cycle that can be targeted by antiviral drugs, including entry into the host cell, genome replication, transcription, and maturation. At present, there are several agents approved in North America for the treatment of viral infections (Table 1.1). These drugs target a number of virus life cycle stages and have diverse mechanisms of action. This thesis will focus on those compounds that target enzymes responsible for the replication of viral genomes. Both those compounds that are currently approved for use as well as examples of those that are in development will be discussed.

Antiviral Agents Targeting Enzymes That Replicate the Viral Genome

The majority of the antiviral agents that are approved for use are those that target the virus-encoded enzymes responsible for the replication of the viral genome. The properties of these enzymes differ depending on the nucleic acid composition of the genome and the life cycle of the virus. These enzymes include DNA-dependent DNA polymerases, RNA-dependent RNA polymerases, and RNA- and DNA-dependent DNA polymerases [reverse transcriptases (RT)]. Although these genome-replicating enzymes are diverse, antiviral agents that target them can be divided into two broad categories, the

Table 1.1 Currently approved antiviral agents[1]

Antiviral Agent	Virus[2]
Nucleoside/Nucleotide Analogs	
Acyclovir/Valaciclovir	HSV, VZV
Abacavir	HIV-1
Adefovir	HBV
Cidofovir	Adenovirus, Polyomavirus, CMV, HSV, VZV
Didanosine	HIV-1
Emtricitabine	HIV-1
Entecavir	HBV
Famciclovir	HSV, VZV
Ganciclovir/Valganciclovir	CMV, HSV, VZV
Lamivudine	HIV-1, HBV
Ribavirin	HCV[3], RSV
Stavudine	HIV-1
Telbivudine	HBV
Tenofovir	HIV-1, HBV
Zidovudine	HIV-1
Non-Nucleoside Analog Polymerase Inhibitors	
Efavirienz	HIV-1
Etravirine (TMC-125)	HIV-1
Foscarnet	CMV, HSV, VZV
Nevirapine	HIV-1
Integrase Inhibitors	
Raltegravir	HIV-1
Protease Inhibitors	
Amprenavir	HIV-1
Atazanavir	HIV-1
Darunavir	HIV-1
Indinavir	HIV-1
Lopinavir	HIV-1
Ritonavir	HIV-1
Saquinavir	HIV-1
Tipranavir	HIV-1
Fusion Inhibitors	
Enfuvirtide	HIV-1
Co-Receptor Inhibitors	
Maraviroc	HIV-1
Ion Channel Inhibitors	
Amantadine	Influenza[4]
Rimantadine	Influenza[4]
Neuraminidase Inhibitors	
Oseltamivir	Influenza
Zanamivir	Influenza

[1] Adapted from Gilbert *et al.*, 2009

[2] Virus abbreviations: CMV – cytomegalovirus, HIV-1 – human immunodeficiency virus type 1, HBV – hepatitis B virus, HCV – hepatitis C virus, HSV- herpes simplex virus, RSV – respiratory syncytial virus, VZV – varicella zoster virus

[3] In combination with interferon

[4] Not recommended due to the high prevalence of resistance

non-nucleoside polymerase inhibitors and the nucleoside/nucleotide analog polymerase inhibitors.

Non-nucleoside Analog Inhibitors

Non-nucleoside analog polymerase inhibitors are a structurally diverse group of compounds that are unrelated to nucleosides and nucleotides. One of the first compounds of this class to be described was foscarnet, an analog of pyrophosphate (Helgstrand *et al.*, 1978). Foscarnet blocks pyrophosphate exchange during DNA synthesis and shows efficacy against the herpesviruses herpes simplex virus (HSV), cytomegalovirus (CMV), varicella zoster virus (VZV), and Epstein Barr virus (EBV) as well as human immunodeficiency virus type 1 (HIV-1) (Crumpacker, 1992). Other compounds in this class of antiviral agents have been developed as specific inhibitors of HIV-1 RT. There are currently three of these non-nucleoside reverse transcriptase inhibitors (NNRTIs) licensed for use (Table 1.1). However, many other inhibitors are being developed for the treatment of this and other virus infections, like HCV, herpesviruses, measles virus and respiratory syncytial virus (RSV) (Huang *et al.*, 2006; Jochmans, 2008; Manns *et al.*, 2007; Oien *et al.*, 2002; Sudo *et al.*, 2005; White *et al.*, 2007). Because of their structural diversity, these inhibitors have diverse of mechanisms of inhibition of polymerases.

The NNRTIs bind to a site on the HIV-1 RT 66 kDa (p66) subunit termed the NNRTI binding pocket (NNRTI-BP). This hydrophobic binding pocket is approximately 10 Å away from the active site of the enzyme, and the entrance to the pocket is composed of residues that also make up the interface between the p66 and 51 kDa (p51) RT subunits (Sluis-Cremer *et al.*, 2004). Interestingly, the NNRTI-BP does not exist in the absence of the inhibitor (Ding *et al.*, 1995), nor does it exist in the smaller HIV-1 RT p51 subunit (Kohlstaedt *et al.*, 1992). Further, NNRTIs do not inhibit the closely related human immunodeficiency virus type-2 (HIV-2) RT (Le Grice, 1993). The binding of an NNRTI to HIV-1 RT induces the NNRTI-BP but does not result in a significant change in the secondary structure surrounding this pocket; however, significant changes do occur in the relative positions of the enzyme's secondary structural elements and in the orientations of some of the side chain residues (Ding *et al.*, 1995). These changes are

thought to alter the geometry of the active site, as well as the association of p66 and p51 (Sluis-Cremer *et al.*, 2004).

Based on pre-steady state analyses, the NNRTI nevirapine has been shown to inhibit the chemical reaction step in DNA polymerization; catalysis still occurs, but at a slower rate (Rittinger *et al.*, 1995; Spence *et al.*, 1995). In contrast, the binding of the nucleotide substrate and the nucleotide-induced conformational change of the enzyme are not inhibited (Spence *et al.*, 1995). As such, nevirapine exhibits non-competitive inhibition with respect to the nucleotide and primer-template substrates (Spence *et al.*, 1995). These results were confirmed and extended to the NNRTI efavirenz by Xia *et al.* (2007), who also suggested that the slow rate of catalysis was due to an indirect effect of these NNRTIs on the amino acids involved in positioning the active site, rather than on a direct effect on phosphodiester bond formation.

The NNRTIs efavirenz and etravirine, but not nevirapine, have also been shown to inhibit the late stages of HIV-1 replication (Figueiredo *et al.*, 2006). Efavirenz and etravirine were shown to enhance the intracellular processing of the Gag and Gag-Pol polyproteins resulting in decreased viral particle production. This enhanced processing of the polyproteins was correlated with increased HIV-1 p66 homodimerization induced by these NNRTIs. These authors suggest that the binding of efavirenz or etravirine to the HIV-1 RT, as part of the Gag-Pol polyprotein, results in enhanced interaction between these polyproteins, leading to premature activation of the HIV-1 protease, which in turn results in fewer unprocessed polyproteins in the cell available for virion assembly and budding (Figueiredo *et al.*, 2006). Nevirapine is ineffective in this inhibition because it has only a weak capacity to enhance p66 homodimerization (Figueiredo *et al.*, 2006). This modification of the inter-subunit interactions of HIV-1 RT has also been suggested as a mechanism of action of NNRTIs on HIV-1 RT activity (Sluis-Cremer *et al.*, 2004). However, experiments have shown that although the various NNRTIs have differential effects on HIV-1 RT dimerization, they all mediate similar effects on single nucleotide incorporation, suggesting that the effects on subunit association do not contribute to the inhibition of DNA synthesis (Xia *et al.*, 2007).

CP-94,707 is another NNRTI that was discovered using a high throughput screening analysis. This compound differs from nevirapine and efavirenz in that its binding pocket only partially overlaps the NNRTI-BP and is therefore ~3.5 Å closer to the active site (Pata *et al.*, 2004). The conformation of the CP-94,707 binding pocket closely resembles the unliganded HIV-1 RT conformation. However, the active site of the bound enzyme is distorted and there is an additional conformational change that occurs in the nucleotide binding pocket that has not been observed previously with the binding of other NNRTIs (Pata *et al.*, 2004). This conformational change is a result of the reorientation of residue tyrosine 115 to a position that is incompatible with dNTP binding. This latter change was suggested to be an additional factor in the inhibition of HIV-1 RT activity (Pata *et al.*, 2004).

KM-1 is another novel NNRTI (Wang *et al.*, 2004). Although the binding site of this inhibitor has not been determined, KM-1 has been shown to weaken the binding affinity of HIV-1 RT for DNA and displace DNA from the enzyme, suggesting that the binding site overlaps with the primer-template binding site. These authors also suggest that KM-1 distorts the HIV-1 RT conformation and misaligns DNA at the active site (Wang *et al.*, 2004).

Indolopyridones are non-nucleoside inhibitors of RT with a unique mechanism of action (Jochmans *et al.*, 2006). These compounds, typified by indolopyridone-1 (INDOPY-1), act neither as NNRTIs nor as nucleoside/nucleotide RT inhibitors. Further, unlike other NNRTIs, INDOPY-1 is active against the lentiviruses HIV-1, HIV-2 and simian immunodeficiency virus (SIV), but is inactive against Moloney murine sarcoma virus (MMSV) and other RNA and DNA viruses (Jochmans *et al.*, 2006). INDOPY-1 exhibits competitive inhibition of HIV-1 RT with respect to nucleotide substrate and reversibly inhibits HIV-1 RT by binding to the active site of the enzyme, thus preventing the binding and incorporation of next templated nucleotide. This binding is not dependent on base-like complementarities, but rather on structural features at, and/or in close proximity to, the 3′-end of the primer and occurs preferentially following incorporation of pyrimidines (Jochmans *et al.*, 2006).

Non-nucleoside inhibitors of the HCV nonstructural protein 5B (NS5B) RNA-dependent RNA polymerase have also been described. These compounds tend to be allosteric inhibitors of the enzyme that cause conformational changes in the structure of HCV NS5B upon binding (Huang *et al.*, 2006; Manns *et al.*, 2007).

Benzo-1,2,4-thiadiazine and 1,5-benzodiazepine compounds inhibit the RNA synthesis activity of NS5B non-competitively with respect to the nucleotide substrate and interfere with the initiation of RNA synthesis rather than with elongation (Dhanak *et al.*, 2002; Gu *et al.*, 2003; Nyanguile *et al.*, 2008). Benzimidazole derivatives also act to inhibit the enzyme non-competitively with respect to nucleotide substrate and inhibit NS5B prior to the elongation phase of RNA synthesis (Tomei *et al.*, 2003). A novel group of benzylidene HCV NS5B inhibitors also inhibit the RNA synthesis initiation reaction and appear to form a reversible covalent bond with the enzyme (Lee *et al.*, 2006).

A pyranoindole class of compounds has also been shown to be allosteric inhibitors of the HCV NS5B (Howe *et al.*, 2004; Howe *et al.*, 2006). These compounds inhibit the enzyme non-competitively with respect to nucleotide and RNA template and appear to block the transition from RNA synthesis initiation to elongation (Howe *et al.*, 2006). One of these compounds, HCV-371, showed a high selectivity for the HCV NS5B polymerase versus cellular enzymes and exhibited efficacy in an HCV subgenomic replicon system with no apparent cytotoxicity (Howe *et al.*, 2004). Unfortunately however, no significant antiviral activity was found during a phase 1b efficacy study (Laporte *et al.*, 2008). However, a newer pyrano[3,4-*b*]indole-based derivative does show antiviral activity *in vivo* in a chimeric mouse model (Laporte *et al.*, 2008).

The crystal structures of other non-nucleoside NS5B polymerase inhibitors, in complex with the enzyme have also been described (Biswal *et al.*, 2005; Biswal *et al.*, 2006; Love *et al.*, 2003; Wang *et al.*, 2003). On the basis of these structural studies, several mechanisms of action for these inhibitors have been suggested, including perturbations of the enzyme structure, such as the NS5B allosteric GTP binding site; disruption of oligomerization; interference with RNA binding; and interference with conformational changes required for RNA synthesis.

Nucleoside/Nucleotide Analog Polymerase Inhibitors

Nucleoside and nucleotide analog polymerase inhibitors were among the first compounds described for the treatment of viral infections. Most of these inhibitors have been developed to act as competitive substrates for viral DNA and RNA polymerases with respect to the natural substrates, deoxynucleoside triphosphates (dNTPs) and nucleoside triphosphates (NTPs). Specificity for inhibition of viral nucleic acid synthesis relative to cellular DNA synthesis occurs because of the selective phosphorylation of these compounds in virus infected cells and/or the increased inhibition of viral enzymes relative to the cellular enzymes by the phosphorylated metabolites. These inhibitors can be divided into two groups: those that are obligate chain terminators and those that have the potential to be incorporated into the growing nucleotide chain and promote further elongation. The difference between these two types of compounds is whether they possess a hydroxyl moiety in a position equivalent to that of the 3′-hydroxyl group of a natural nucleotide.

Obligate Chain Terminators

One of the first antiviral agents developed and licensed for use was acyclovir for the treatment of herpesvirus infections. This compound is an acyclic analog of guanosine, and because it lacks a 3′-hydroxyl group, it is an obligate chain terminator (Figure 1.1). Acyclovir has low oral bioavailability, and as such, an oral prodrug derivative of this compound, valaciclovir, has also been developed and approved for use. Bioavailability of valaciclovir approximates that achieved with intravenous acyclovir therapy (Soul-Lawton *et al.*, 1995). Acyclovir is converted to its 5′-monophosphate derivative by the thymidine kinases of herpes simplex virus type 1 (HSV-1) and VZV, and subsequent conversion to the 5′-triphosphate derivative is catalyzed by cellular enzymes (Biron and Elion, 1980; Elion *et al.*, 1977). The metabolic activation of acyclovir occurs to only a limited extent in uninfected cells (Biron and Elion, 1980; Elion *et al.*, 1977; Furman *et al.*, 1981). The incorporation of acyclovir 5′-monophosphate into DNA, followed by dead-end complex formation after the binding of the next templated nucleotide results in the selective inhibition of the HSV-1 and VZV DNA polymerases (Abele *et al.*, 1988; Derse *et al.*,

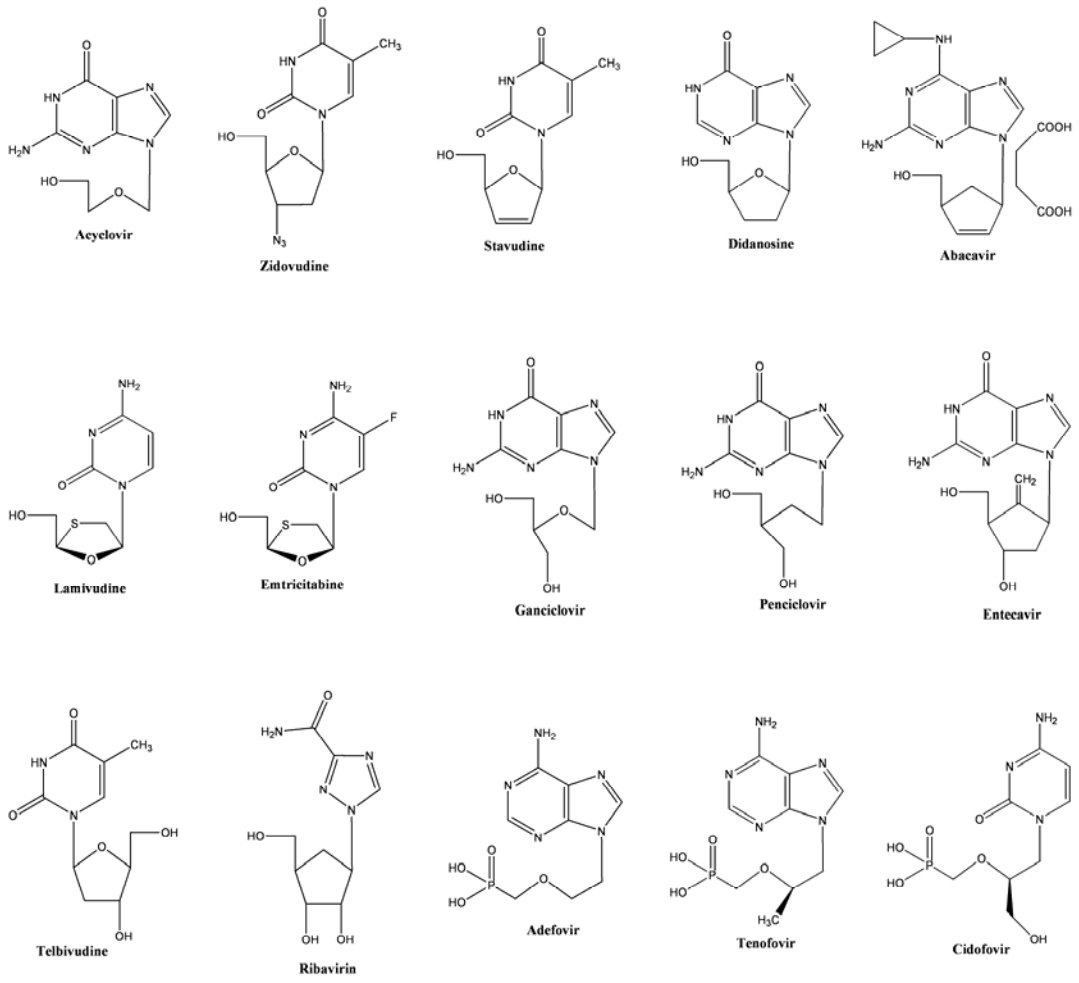

Figure 1.1 Structures of the nucleoside/nucleotide analog polymerase inhibitors currently approved in North America for the treatment of viral infections.

1981; Reardon and Spector, 1989). Further, once incorporated, acyclovir monophosphate cannot be excised by the HSV-1 DNA polymerase 3′-to-5′ proofreading exonuclease activity (Derse *et al.*, 1981).

Several obligate chain terminators have also been developed for the treatment of HIV-1 infections. Zidovudine [3′-azido-3′-deoxythymidine (AZT); Azidothymidine] (Figure 1.1) was the first antiviral compound described to inhibit HIV-1 replication (Mitsuya *et al.*, 1985). This drug is phosphorylated by cellular enzymes and selectively inhibits HIV-1 RT (Furman *et al.*, 1986). The incorporation of AZT 5′-monophosphate into DNA by HIV-1 RT results in chain termination (Huang *et al.*, 1990). Stavudine (2′,3′-didehydro-2′,3′-dideoxythymidine; D4T) (Figure 1.1) is also strongly and selectively inhibitory to the RT of HIV-1 and the DNA chain termination activity of this compound has been shown to be equipotent to that of AZT (Balzarini *et al.*, 1989; Huang *et al.*, 1992).

Didanosine (2′,3′-dideoxyinosine, ddI) (Figure 1.1), is metabolized by cellular enzymes to yield the active intracellular metabolite, 2′,3′-dideoxyadenosine triphosphate (ddATP) (Ahluwalia *et al.*, 1987). Didanosine selectively inhibits HIV-1 replication by the incorporation of 2′,3′-dideoxyadenosine monophosphate (ddAMP) into DNA by HIV-1 RT and subsequent chain termination (Mitsuya and Broder, 1986; Mitsuya *et al.*, 1987), although ddATP also potently inhibits human DNA polymerase γ, leading to mitochondrial toxicity (Martin *et al.*, 1994).

Abacavir [(-)-(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol] (Figure 1.1) undergoes a unique metabolism to yield carbovir [1*R*,4S)-9-[4-(hydroxymethyl)-2-cyclopenten-1-yl]guanine] 5′-triphosphate (Daluge *et al.*, 1997; Faletto *et al.*, 1997). Carbovir 5′-triphosphate is a potent and selective inhibitor of HIV-1 RT (Daluge *et al.*, 1997) and is inefficiently used as a substrate by human DNA polymerase γ, leading to the least mitochondrial toxicity of the approved antiretroviral nucleoside analogs (Johnson *et al.*, 2001).

Lamivudine [(-)-β-2′,3′-dideoxy-3′-thiacytidine; 3TC] and its fluorinated derivative emtricitabine [(-)-β-2′,3′-dideoxy-5-fluoro-3′-thiacytidine; (-)FTC] are nucleoside analogs with an unnatural (-)-β-L configuration (Figure 1.1). Lamivudine 5′-

triphosphate is a selective inhibitor and substrate for HIV-1 RT and can be incorporated into DNA by this enzyme where it acts as a chain terminator (Gray *et al.*, 1995; Hart *et al.*, 1992). Emtricitabine 5′-triphosphate is also used as a substrate by HIV-1 RT and is incorporated into DNA three- to ten-fold more efficiently than is lamivudine 5′-triphosphate (Feng *et al.*, 2004; Feng *et al.*, 1999). Lamivudine has also been approved for the treatment of HBV infections. Its 5′-triphosphate derivative is used as a substrate by the hepadnavirus DNA polymerase and its incorporation results in chain termination (Severini *et al.*, 1995).

Nucleoside Analogs Possessing a 3′-Hydroxyl Group

Ganciclovir and penciclovir are guanosine analogs that are related to acyclovir, and like acyclovir, prodrugs (valganciclovir and famciclovir, respectively) were developed to improve oral bioavailability (Cocohoba and McNicholl, 2002; Hodge *et al.*, 1989). The major difference between these compounds and acyclovir is that the former contain a hydroxymethyl group that is equivalent to the 3′-hydroxyl group of natural nucleosides (Figure 1.1). However, both of these compounds act as functional chain terminators. The first step of ganciclovir and penciclovir phosphorylation is catalyzed by viral kinases; subsequent steps are catalyzed by cellular enzymes, leading to the production of the active triphosphorylated metabolites in virus infected cells (Biron *et al.*, 1985; Hodge and Perkins, 1989; Earnshaw *et al.*, 1992; Koyano *et al.*, 1996; Sullivan *et al.*, 1992). Although ganciclovir can be incorporated internally into DNA (Cheng *et al.*, 1983), subsequent work showed that when DNA polymerases from human CMV (HCMV) and HSV-1 incorporate ganciclovir, the incorporation of one additional nucleotide results in stalling (Reid *et al.*, 1988). This stalling is thought to be due to a greatly reduced V_{max} and increased K_m in the DNA polymerase after the formation of the ganciclovir + 1 structure, effectively resulting in chain termination (Reardon, 1989). Penciclovir monophosphate can be incorporated into DNA by herpes simplex virus type 2 (HSV-2) DNA polymerase but chain termination occurs after the incorporation of a few subsequent nucleotides (Earnshaw *et al.*, 1992).

Entecavir is approved for use in the treatment of HBV. A novel 2′-deoxyguanosine (dG) analog, entecavir is a functional chain terminator (Figure 1.1). After activation by cellular enzymes and incorporation into DNA, entecavir causes pausing of the multifunctional HBV polymerase at sites two to three residues downstream from dG incorporation sites (Levine *et al.*, 2002; Seifer *et al.*, 1998). Termination was shown to be less efficient past single dG residues than termination after closely spaced dG residues (Seifer *et al.*, 1998). Modeling studies have indicated that chain termination is caused by steric strain, resulting in a distortion of the DNA that deteriorates the efficiency of dNTP incorporation (Langley *et al.*, 2007). This mechanism has also been shown to apply to the inhibition of HIV-1 RT by entecavir (Domaoal *et al.*, 2008). Although this drug was originally thought to be selective for HBV (Innaimo *et al.*, 1997), subsequent data indicated that entecavir is also clinically effective against HIV-1 (McMahon *et al.*, 2007). Tchesnokov *et al.* (2008) found that entecavir interferes with DNA replication by HIV-1 in three ways: pausing after incorporation of entecavir at position N, strong pausing at position N + 3, and pausing when entecavir is located in the template strand. Based on kinetic analyses, the delayed chain termination at position N + 3 was determined to be the dominant mechanism of action (Tchesnokov *et al.*, 2008). These data are supported by a model of HIV-1 RT inhibition by entecavir, which showed that entecavir at the + 5 position (i.e. five nucleotides upstream of the nucleotide incorporation site) causes a great distortion in the DNA strand (Domaoal *et al.*, 2008).

Telbivudine (β-L-thymidine) is another antivial agent approved for the treatment of hepatitis B virus. Like lamivudine and emtricitabine, its structure has an unnatural β-L configuration (Figure 1.1). Unlike these two drugs however, telbivudine possesses a 3′-hydroxyl group. Cellular enzymes phosphorylate telbivudine to its active 5′-triphosphate metabolite (Hernandez-Santiago *et al.*, 2002), which selectively inhibits the hepadnavirus DNA polymerase (Bryant *et al.*, 2001). However, the mechanism by which polymerase activity is inhibited remains unclear at present, as it is not known whether this compound acts as a functional chain terminator or can be incorporated into DNA and support further elongation.

Nucleoside analogs have also been developed to treat HCV infections. 2′-*C*-methyladenosine and 2′-*O*-methylcytidine inhibit HCV replication, and their 5′-triphosphates inhibit RNA synthesis catalyzed by HCV NS5B (Carroll *et al.*, 2003; Migliaccio *et al.*, 2003). Although these compounds possess a 3′-hydroxyl group, they terminate RNA elongation after incorporation (Carroll *et al.*, 2003). Modeling studies have indicated that termination is due to steric hindrance between the 2′-methyl groups and the incoming NTP (Migliaccio *et al.*, 2003).

Ribavirin is a synthetic ribonucleoside analog of adenosine (A) or guanosine (G) (Figure 1.1) that has shown *in vitro* activity against a wide range of RNA and DNA viruses (Huffman *et al.*, 1973; Hruska *et al.*, 1980; Sidwell *et al.*, 1972; Streeter *et al.*, 1973). This compound has been used effectively in the treatment of Lassa fever and RSV and has been approved for the treatment of HCV in combination with interferon α (Groothuis *et al.*, 1990; Hall *et al.*, 1983; Manns *et al.*, 2007; McCormick *et al.*, 1986). Ribavirin has also been used to treat measles virus infections, although there are conflicting reports as to whether this drug is effective against this infection *in vivo* (Barnard, 2004). The mechanism of action of ribavirin is unclear, but several activities have been proposed. These include the inhibition of inosine monophosphate dehydrogenase by ribavirin monophosphate, leading to a decrease in cellular GTP pools; inhibition of 5′ cap formation of viral mRNAs by ribavirin triphosphate; inhibition of viral RNA-dependent RNA polymerases by ribavirin triphosphate; and the induction of error catastrophe in RNA viruses due to the mutagenic properties of ribavirin triphosphate (Gilbert and Knight, 1986; Parker, 2005), however the impact of this latter mechanism *in vivo* has been questioned (Heck *et al.*, 2008; Summers and Litwin, 2006). Ribavirin also has immunomodulatory properties and has been shown to enhance the T helper type 1 immune response *in vivo* (Fang *et al.*, 2000). This property has been suggested to comprise the mechanism of action of this drug against HCV because ribavirin monotherapy does not effectively treat HCV but does augment the action of interferon α (Manns *et al.*, 2007).

Another group of nucleoside analogs that are in development are those that induce mutations in the viral genome. This process was first described by Loeb *et al.* (1999),

who showed that when HIV-1 is passaged in the presence of 5-hydroxydeoxycytidine, an analog of deoxycytidine that base pairs with dAMP, there was an accumulation of G to A substitutions and a sustained loss in viral titers after several sequential passages (Loeb *et al.*, 1999). These observations led to the development of novel mutagenic compound, 5-aza-5,6-dihydro-2′-deoxycytidine (KP-1212) (Harris *et al.*, 2005). KP-1212 has a modified cytosine ring and was designed to form base pairs with multiple bases (Murakami *et al.*, 2005). Serial passaging of HIV-1 in the presence of KP-1212 increases the mutation rate by 50 to 100% and causes ablation of the virus after 8 to 13 passages (Harris *et al.*, 2005). Although there is concern that mutagenic nucleoside analogs could also be incorporated into cellular DNA, no genotoxic effects of KP-1212 have been observed (Harris *et al.*, 2005).

Acyclic Nucleoside Phosphonates

The acyclic nucleoside phosphonates are a class of antiviral agents that are nucleotide, rather than nucleoside, analogs. As a class, these drugs have shown activity against a range of DNA viruses, RNA viruses, and retroviruses (De Clercq and Holý, 2005; De Clercq *et al.*, 1986; Wyles *et al.*, 2009). The structure of these drugs consists of an acyclic nucleoside moiety attached to a phosphonate group through a phosphate-carbon-oxygen bond. This type of bond is not cleaved by cellular esterases, making these compounds more stable than those that contain the phosphate-oxygen-carbon bond of a phosphate group (De Clercq, 1997; De Clercq and Holý, 2005). The phosphonate group on this class of compounds acts as a phosphate mimic, making these drugs analogs of deoxynucleoside monophosphates (dNMPs). This structure means that only two phosphorylation steps are required to activate these drugs to their active diphosphoryl metabolites, which then act as analogs of dNTP.

Several acyclic nucleoside phosphonates have been described, and these generally fall into three categories: the phosphonomethoxyethyl (PME) purine and pyrimidine derivatives, the phosphonomethoxypropyl (PMP) purine and pyrimidine derivates, and the N-*(S)*-(3-hydroxy-2-phosphonomethoxypropyl) (HPMP) purine and pyrimidine derivatives. The PME and PMP derivatives are obligate chain terminators because they

lack a hydroxyl group that can act as a 3′-hydroxyl, as found in a natural dNTP structure, whereas the HPMP derivatives do possess a 3′-hydroxyl group in the correct position and can theoretically be incorporated into DNA and extended (De Clercq and Holý, 2005; De Clercq and Neyts, 2009). Three of these nucleoside phosphonates, 9-(2-phosphonomethoxyethyl)adenine (PMEA, adefovir), 9-(2-phosphonomethoxypropyl)adenine (PMPA, tenofovir), and *(S)*-1-[3-hydroxy-2-(phosphonylmethoxypropyl)]cytosine [*(S)*-HPMPC, cidofovir], have been approved for use in the treatment of viral infections (Table 1.1; Figure 1.1). Adefovir has been approved for the treatment of HBV in a prodrug form (adefovir dipivoxil; Hepsera), although it also shows efficacy against herpesviruses and retroviruses, including HIV-1 (De Clercq and Holý, 2005). Adefovir is phosphorylated to its active intracellular metabolite adefovir diphosphate in two steps by the cellular enzyme AMP kinase (Merta *et al.*, 1992). This active metabolite is unable to support DNA synthesis catalyzed by HSV-1 DNA polymerase in the absence of dATP (Merta *et al.*, 1990), most likely because it acts as a chain terminator once incorporated into DNA (Birkus *et al.*, 1999; Cihlar and Chen, 1997; Kramata *et al.*, 1996). Adefovir diphosphate selectively inhibits HBV DNA polymerase when compared to cellular DNA polymerases. The K_i/K_m value for this substrate for HBV DNA polymerase is 0.3, whereas these same values are 0.4, 12.6, and 1.3 for human DNA polymerases α, β, and γ, respectively (Cherrington *et al.*, 1995; Xiong *et al.*, 1998).

Tenofovir has activity against HBV and retroviruses and has also been approved in a prodrug form (tenofovir disoproxil fumatate; Viread) for use in the treatment of HIV-1 and HBV infections (De Clercq and Holý, 2005). Tenofovir is phosphorylated by the cellular enzymes adenylate kinase and nucleoside diphosphate kinase (Robbins *et al.*, 1995) and is selective for the inhibition of HIV-1 RT (K_i/K_m value of 0.33 for a DNA template and 0.24 for an RNA template) and HBV DNA polymerase (K_i/K_m value of 0.47) (Cherrington *et al.*, 1995; Delaney *et al.*, 2006). In contrast, the K_i/K_m values of this substrate for the human DNA polymerases α, β, and γ are 2.0, 14.5, and 82.6, respectively (Cherrington *et al.*, 1995).

Cidofovir has been approved for use in the treatment of CMV retinitis in AIDS patients. This compound also shows activity against a wide range of DNA viruses including polyomavirus, papillomavirus, adenovirus, other herpesviruses [HSV-1, HSV-2, VZV, EBV, Kaposi's sarcoma-associated herpesvirus (KSHV)], and poxviruses (De Clercq and Holý, 2005).

Cidofovir

Cidofovir (CDV), an analog of deoxycytidine monophosphate (dCMP) (Figure 1.2), was the first of the nucleoside phosphonates licensed for use and was approved in the United States in 1996 (U.S. Food and Drug Administration, 1996). As described above, cidofovir has been approved for the treatment of CMV-retinitis in AIDS patients. However, it has also been used off-label to treat a variety of other virus infections, including those caused by the poxviruses orf (Geerinck *et al.*, 2001; Weston and Storch, 2003) and molluscum contagiosum (Davies *et al.*, 1999; Ibarra *et al.*, 2000; Meadows *et al.*, 1997; Toro *et al.*, 2000; Zabawski and Cockerell, 1999), the herpesvirus Epstein Barr virus (Yoshizaki *et al.*, 2008), adenovirus (Gordon *et al.*, 1996), polyomavirus (Dodge, 1999), and human papillomavirus (Calista, 2000; DeRossi and Laudenbach, 2004).

Cidofovir is taken up into cells by fluid-phase endocytosis (Connelly *et al.*, 1993) and the intracellular metabolism of CDV yields three major metabolites: cidofovir monophosphate (CDVp), cidofovir diphosphate (CDVpp), and cidofovir phosphocholine (CDVp-choline) (Aduma *et al.*, 1995; Ho *et al.*, 1992). The production of these metabolites is unchanged by infection with HSV-1 (Ho *et al.*, 1992) or with cowpox virus (Smee *et al.*, 2002), indicating that cellular enzymes catalyze the metabolism of this drug. Pyrimidine nucleoside monophosphate kinase has been shown to catalyze the first phosphorylation step, and one of three enzymes, pyruvate kinase, creatine kinase or nucleoside diphosphate kinase, catalyze the second step (Cihlar and Chen, 1996). The formation of CDVp-choline from CDVpp is catalyzed by choline phosphate cytidyltransferase (Cundy, 1999); CDVp-choline is an analog of cytidine 5′-diphosphocholine, a phospholipid synthesis intermediate (Cihlar *et al.*, 2001), and acts as a slow-release reservoir for CDVpp. The fact that cellular enzymes are used to catalyze

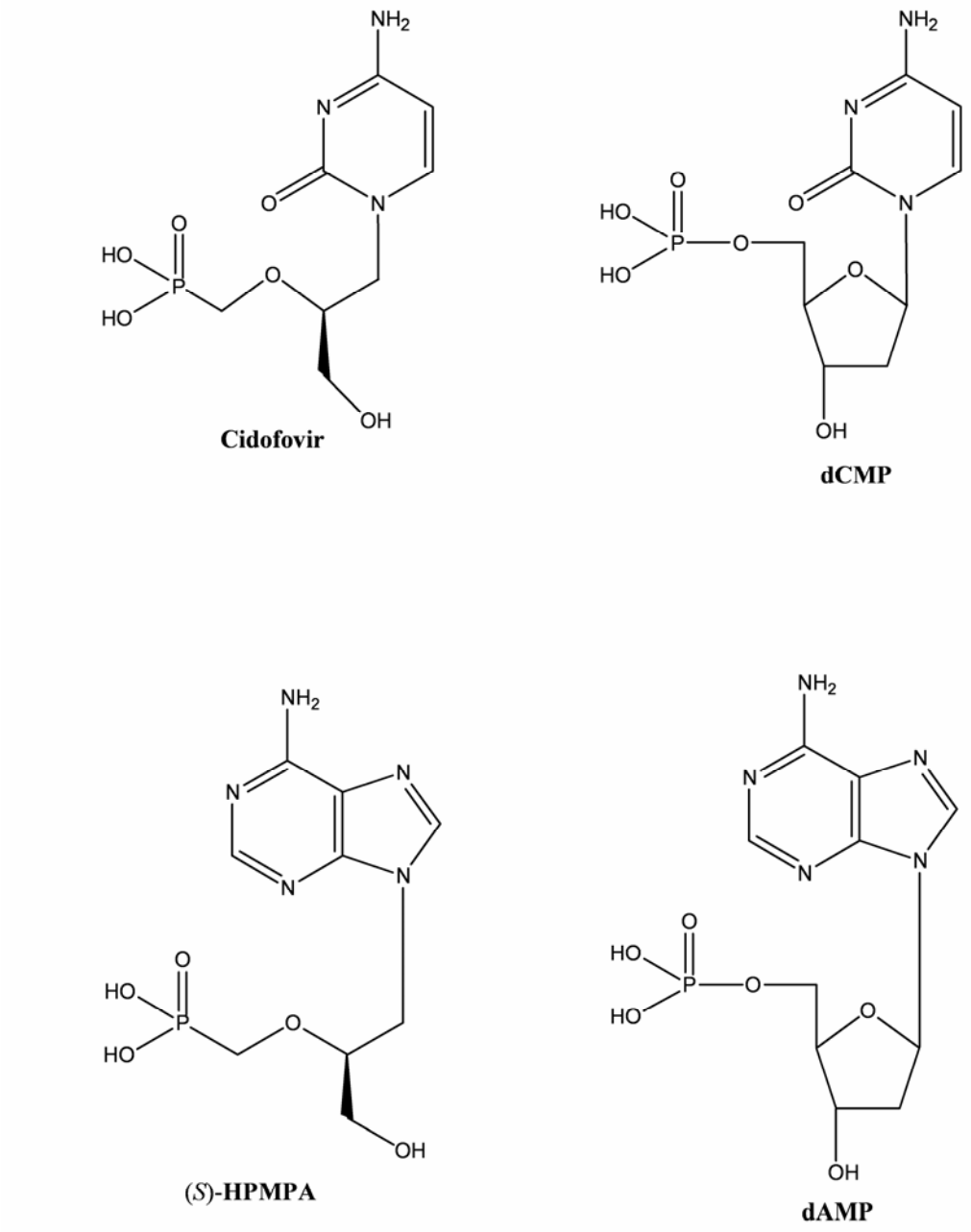

Figure 1.2 The structures of CDV and *(S)*-HPMPA. Cidofovir is an acyclic analog of deoxycytidine monophosphate (dCMP) (top) and *(S)*-HPMPA is an acyclic analog of deoxyadenosine monophosphate (dAMP) (bottom). The acyclic backbone structures of the two compounds are identical; only the structures of the base moieties differ.

these steps means that viral enzymes are not required, thus expanding the range of potential viral targets to include kinase-deficient and mutant viruses.

Another advantage of CDV is its long lasting antiviral effect. Pretreatment of cells with CDV for 12 to 24 hours induces an effective antiviral state, which is maintained for greater than seven days. The long half lives of the metabolites of CDV (6, 17 to 65, and 87 hours for CDVp, CDVpp, and CDVp-choline, respectively) (Aduma *et al.*, 1995; Ho *et al.*, 1992) account for this long-lasting antiviral effect of the drug and have implications for dosing schedules and its use as a prophylactic.

Aside from the numerous advantages of CDV, the drug has two main disadvantages. First, CDV has poor oral bioavailability. The oral bioavailability has been estimated to be less than 5% (Wachsman *et al.*, 1996) and is most likely due to its dianionic charge at physiological pH, resulting in a limited ability to cross the intestinal epithelia (Cundy, 1999). As a result, CDV is currently administered intravenously. Second, CDV exhibits dose-limiting nephrotoxicity (Lalezari *et al.*, 1995), a result of the fact that CDV is a high-affinity substrate for the human organic anion transporter 1 (hOAT1) (Cihlar *et al.*, 1999). This transporter has been localized to the basolateral membrane of human renal proximal tubules (Pastor-Anglada *et al.*, 2005). The uptake of CDV across the basolateral tubular cell membrane has been shown to be more efficient than its subsequent secretion into the tubular lumen resulting in drug accumulation in the renal tubules (Cihlar *et al.*, 1999). Hydration and the use of probenecid, an inhibitor of organic ion transport, have been recommended to reduce the risk of nephrotoxicity (Gilead Sciences, 2003). A group of lipid prodrugs of CDV has been developed that appears to eliminate these drawbacks. These ether lipid ester derivatives were designed to resemble natural lipids that are absorbed intact from the small intestine, circulate in the plasma as the intact prodrug, and be slowly converted to CDV in the tissues (Ciesla *et al.*, 2003; Painter and Hostetler, 2004). These derivatives are highly orally bioavailable and do not concentrate in the kidney (Ciesla *et al.*, 2003). A further advantage of these lipid prodrugs of CDV is that they have increased antiviral activity relative to the parent compound (Beadle *et al.*, 2002; Kern *et al.*, 2002). This increase in activity appears to be a result of a rapid cellular uptake of these prodrugs based on their interaction with the cell

membrane relative to the parent compound (Aldern *et al.*, 2003). The intracellular half life of CDVpp in cells treated with one of these prodrugs was also increased to about 10 days, caused by the presence of a large pool of the prodrug in the cell membrane that is then metabolized to release CDV, which is then further metabolized to CDVpp (Aldern *et al.*, 2003).

Cidofovir diphosphate, an analog of deoxycytidine triphosphate (dCTP), is predicted to target viral DNA polymerases. CDVpp has been shown to be a competitive inhibitor of HCMV, HSV-1, and HSV-2 DNA polymerases with respect to dCTP (Ho *et al.*, 1992; Xiong *et al.*, 1996). CDVpp shows greater affinity for viral enzymes relative to cellular enzymes, leading to a selective inhibition of viral DNA polymerases. CDVpp is a poor inhibitor of human DNA polymerases β and γ (K_i/K_m values are 121 and 1424, respectively) (Cherrington *et al.*, 1994), a moderate inhibitor of human DNA polymerase α and HCMV DNA polymerase (K_i/K_m values are 10.8 and 9.2, respectively) (Ho *et al.*, 1992; Xiong *et al.*, 1996), and a strong inhibitor of HSV-1 and HSV-2 DNA polymerases (K_i/K_m values are 2.8 and 3.8, respectively) (Ho *et al.*, 1992). CDVpp has also been shown to inhibit HIV-1 RT, with a K_i/K_m value of 4.6 for a DNA template and 5.9 for an RNA template (Cherrington *et al.*, 1996). However, subsequent work has indicated that CDVpp is not active against HIV-1 RT (Frangeul *et al.*, 2008). In determining the IC_{50} of CDVpp, these authors showed that HIV-1 RT activity is not inhibited by concentrations of this drug up to 2 mM; however, this enzyme could use CDVpp as a substrate and inefficiently incorporate it into DNA.

In experiments investigating the interaction of CDVpp with HCMV DNA polymerase, Xiong *et al.* (1997) showed that CDVpp can be used as an alternative substrate for this enzyme, and is faithfully incorporated opposite dGMP. Once incorporated, a single drug molecule does not cause chain termination, although it does slow elongation. In contrast, when two consecutive molecules are incorporated into DNA, or when two molecules of CDV separated by a natural nucleotide are incorporated, elongation is dramatically reduced or terminated. Further, when present at the 3′-terminus of the primer strand, CDV is not excised by the 3′-to-5′ proofreading exonuclease activity of HCMV DNA polymerase. In addition, when present in the template strand, pausing

was observed at positions immediately prior to, at, and immediately after the CDV molecule. However, CDV in the template strand is not an absolute block to DNA synthesis as full-length extension past the drug lesion was also observed (Xiong *et al.*, 1997).

Further evidence that CDVpp targets DNA polymerases has come from studies of viruses resistant to CDV, including HSV-1, HCMV, and vaccinia virus (Andrei *et al.*, 2006; Andrei *et al.*, 2000; Becker *et al.*, 2008; Chou *et al.*, 2003; Cihlar *et al.*, 1998; Gilbert *et al.*, 2002; Kornbluth *et al.*, 2006). These resistant viruses have been both selected *in vitro* by prolonged passage in the presence of drug or found in clinical isolates. Mutations conferring resistance to CDV have been mapped to the viral DNA polymerase genes, both in the polymerase and in the 3′-to-5′ proofreading exonuclease domains. In addition, CDVpp was shown to be less inhibitory to the DNA polymerase purified from a CDV-resistant cowpox virus than to the polymerase purified from the wild type cowpox (Smee *et al.*, 2002). However, specific amino acid differences between the polymerases were not determined in the latter case.

_(S)_-HPMPA

(S)-9-(3-hydroxy-2-phosphonomethoxypropyl)adenine [*(S)*-HPMPA] was the first of the acyclic nucleoside phosphonates described and is an analog of deoxyadenosine monophosphate (dAMP) (Figure 1.2) (De Clercq *et al.*, 1986). This compound originated as a merger of two other antiviral agents, phosphonoformate and *(S)*-9-(2,3-dihydroxypropyl)adenine [*(S)*-DHPA] (De Clercq and Holý, 2005; De Clercq *et al.*, 1987) and is related to CDV. *(S)*-HPMPA and CDV have the same backbone structure and differ only in the structure of the base moiety. *(S)*-HPMPA has activity against a number of different viruses, including the DNA viruses HSV-1, HSV-2, VZV, HCMV, vaccinia virus, orf virus, and human adenoviruses (Dal Pozzo *et al.*, 2007; De Clercq *et al.*, 1986). *(S)*-HPMPA was also shown to be inhibitory to the transformation of mouse embryo cells by the retrovirus MMSV (De Clercq *et al.*, 1986) and have activity against duck hepatitis B virus (DHBV) (Yokota *et al.*, 1990a). In contrast, in the original descriptions of this compound, it was determined that *(S)*-HPMPA was not active against

RNA viruses or against HIV-1 (De Clercq *et al.*, 1986; Pauwels *et al.*, 1988). Subsequent work has shown that alkoxyalkyl ester derivatives of *(S)*-HPMPA do exhibit efficacy against HIV-1, HCV, and HBV (Hostetler *et al.*, 2006; Wyles *et al.*, 2009). These lipid derivatives were also more effective than unmodified *(S)*-HPMPA against vaccinia virus, cowpox virus, and HCMV (Beadle *et al.*, 2006), similar to that seen with the prodrug derivatives of CDV. The activity spectrum of *(S)*-HPMPA has also been expanded to include parasites like trypanosomes (Kaminsky *et al.*, 1996; Kaminsky *et al.*, 1994), *Schistosomiasis mansoni* (Botros *et al.*, 2003), and *Plasmodium* species (de Vries *et al.*, 1991; Smeijsters *et al.*, 1999; Smeijsters *et al.*, 1996).

Like CDV, *(S)*-HPMPA is taken up into cells by endocytosis (Palú *et al.*, 1991). The metabolites formed after the uptake of this drug are *(S)*-HPMPA monophosphate [*(S)*-HPMPAp] and *(S)*-HPMPA diphosphate [*(S)*-HPMPApp]; no phosphocholine adduct is observed (Votruba *et al.*, 1987). The phosphorylation pattern of *(S)*-HPMPA is similar in uninfected cells and in cells infected with HSV-1 (Votruba *et al.*, 1987), indicating that cellular enzymes phosphorylate this drug. AMP kinase has been shown to catalyze the two-step phosphorylation of *(S)*-HPMPA to *(S)*-HPMPApp (Merta *et al.*, 1992). Creatine kinase is also able to convert *(S)*-HPMPAp to *(S)*-HPMPApp (Merta *et al.*, 1992). Although the half lives of these metabolites have not been determined, the antiviral state induced by pretreatment of cells with *(S)*-HPMPA is much shorter (24 to 72 hours) than that observed with CDV (Aduma *et al.*, 1995). This shorter antiviral state is most likely due to the lack of a long-lasting reservoir like CDVp-choline.

The active intracellular metabolite of *(S)*-HPMPA, *(S)*-HPMPApp, is an analog of deoxyadenosine triphosphate (dATP), and as such, like CDVpp, is thought to target viral polymerases. *(S)*-HPMPA has been shown to inhibit the DNA synthesis of HSV-1, HBV, and DHBV (Votruba *et al.*, 1987; Yokota *et al.*, 1990a; Yokota *et al.*, 1990b). *(S)*-HPMPApp is a competitive inhibitor of HSV-1 and adenovirus DNA polymerases with respect to dATP (Merta *et al.*, 1990; Mul *et al.*, 1989). Interestingly, *(S)*-HPMPApp is not as selective for viral DNA polymerases as is CDVpp. The K_i/K_m values for HSV-1 and adenovirus DNA polymerases are 1.9 and 0.58, respectively (Merta *et al.*, 1990; Mul *et al.*, 1989). In comparison, these values for human DNA polymerase α, rat DNA

polymerase α, human DNA polymerase β, rat DNA polymerase δ, and rat DNA polymerase ε are 2.3, 3.1 >170, 0.3 and 0.07 (Kramata *et al.*, 1996; Merta *et al.*, 1990). The low K_i/K_m values obtained with the cellular enzymes are most likely the cause of the cytotoxic effects of *(S)*-HPMPA seen at higher drug doses (Bronson *et al.*, 1989; Veselý *et al.*, 1990; Votruba *et al.*, 1987). *Plasmodium* α-like and γ-like DNA polymerases are also competitively inhibited by *(S)*-HPMPApp with respect to dATP; K_i/K_m values were determined to be 3.9 and 0.04, respectively (de Vries *et al.*, 1991). However, resistance to *(S)*-HPMPA maps to substitutions in polymerase δ (Smeijsters *et al.*, 2000).

(S)-HPMPApp can be used by HSV-1 DNA polymerase as an alternative substrate for dATP (Merta *et al.*, 1990). Although the incorporation of *(S)*-HPMPA into DNA by this or the adenovirus enzyme has not been investigated, the rat cellular DNA polymerases α, δ, and ε were shown to use *(S)*-HPMPApp as a substrate and incorporate two to four consecutive *(S)*-HPMPA molecules into a growing DNA strand (Birkus *et al.*, 2004; Kramata *et al.*, 1996). Further, both DNA polymerases δ and ε could excise *(S)*-HPMPA from a primer terminus, with more efficient removal of the drug by polymerase ε (Birkus *et al.*, 2004). Interestingly, although *Plasmodium* DNA polymerases are inhibited by *(S)*-HPMPApp (de Vries *et al.*, 1991), the polymerase activity of a crude extract of *Plasmodium* was not increased by the addition of *(S)*-HPMPApp to a reaction lacking dATP (Smeijsters *et al.*, 1994). Based on these results, the authors suggested that *(S)*-HPMPApp is not an alternative substrate for dATP in *Plasmodium*.

As also seen with CDVpp, additional support for the hypothesis that *(S)*-HPMPApp acts as a nucleotide analog and targets nucleic acid polymerases comes from analyses of viruses resistant to *(S)*-HPMPA. The passage of HSV-1 in increasing concentrations of *(S)*-HPMPA resulted in the production of resistant viruses; the resistance was mapped to the viral DNA polymerase gene (Andrei *et al.*, 2000). In addition, HSV-1 and vaccinia virus selected for resistance to CDV by the same methods showed cross-resistance to *(S)*-HPMPA (Andrei *et al.*, 2006; Andrei *et al.*, 2000). And as described above, *Plasmodium* resistance to *(S)*-HPMPA was also mapped to a DNA polymerase gene of this parasite (Smeijsters *et al.*, 2000).

1.2 MODEL SYSTEMS FOR STUDYING CDV and *(S)*-HPMPA

CDV and *(S)*-HPMPA exhibit a wide spectrum of antiviral activity, including against DNA viruses, retroviruses, and more recently, RNA viruses (De Clercq and Holý, 2005; De Clercq *et al.*, 1986; Wyles *et al.*, 2009). The initial studies on the mechanism of action of this group of nucleoside phosphonates examined the interaction of CDVpp with HCMV DNA polymerase (Xiong *et al.*, 1997; Xiong *et al.*, 1996). For the purposes of this study, two additional model systems were chosen, a second DNA polymerase (from vaccinia virus) and reverse transcriptase [from HIV-1 and Moloney murine leukemia virus (MMLV)]. These model systems were chosen to determine if the mechanism of action of CDV and *(S)*-HPMPA is similar for these diverse viral enzymes.

Poxviruses

The *Poxviridae* are a family of large, double stranded DNA viruses that replicate in the cytoplasm of infected cells (reviewed in Moss, 2007). This family consists of two subfamilies, the *Chordopoxvirinae* and the *Entomopoxvirinae*, that infect vertebrates and insects, respectively. Within the *Chordopoxvirinae*, there are eight genera, shown in Table 1.2, and a number of members are able to cause infections in humans. One of the most well-known members of the poxvirus family is variola virus, the causative agent of smallpox. The World Health Organization declared smallpox eradicated in 1980 (World Health Organization, 1980), and eradication was achieved through a large-scale vaccination campaign using vaccinia virus, the prototypic poxvirus. Routine vaccination of the public was discontinued shortly thereafter.

Recently there has been a renewed interest in the treatment of human poxvirus infections. The events of September 11, 2001 and the subsequent anthrax attacks in the United States have raised concerns that variola virus could be used as a biological weapon (Henderson *et al.*, 1999; Whitley, 2003). A smallpox attack could be potentially devastating, since the vaccine was shown to not confer lifelong immunity (Henderson *et al.*, 1999), and global travel could spread the virus quickly. Although vaccination performed early in the incubation period can attenuate or prevent smallpox disease (Breman and Henderson, 2002; Henderson *et al.*, 1999), the vaccine is contraindicated in

Table 1.2 *Poxviridae* genera[1]

Genus	Examples
Chordopoxvirinae Subfamily	
Orthopoxvirus	Vaccinia, Variola, Monkeypox, Cowpox, Ectromelia
Parapoxvirus	Orf
Avipoxvirus	Fowlpox
Capripoxvirus	Goatpox
Leporipoxvirus	Myxoma, Shope fibroma
Suipoxvirus	Swinepox
Molluscipoxvirus	Molluscum contagiosum
Yatapoxvirus	Tanapox
Unassigned	Crocodilepox, Dolphinpox
Entomopoxvirinae Subfamily	
Entomopoxvirus A	*Melontha melontha*
Entomopoxvirus B	*Amsacta moorei*
Entomopoxvirus C	*Chrionimus luridus*

[1] Adapted from Moss, 2007

a number of people including those with eczema, immunodeficiencies or immunosupression, as well as the household contacts of these individuals (Parrino and Graham, 2006). In addition, smallpox vaccination itself is associated with a number of adverse events, ranging in severity from mild to lethal (Bray, 2003).

Monkeypox is another poxvirus that can cause infections in humans. This zoonotic infection causes a smallpox-type illness and in general results in very few human infections each year. However, recurrent outbreaks of this disease in the Democratic Republic of Congo (Esposito and Fenner, 2001; Hutin *et al.*, 2001; Meyer *et al.*, 2002) and its appearance in the United States in 2003 (Centers for Disease Control and Prevention, 2003), suggest that this rare infection, as well as other zoonotic poxvirus infections (Lewis-Jones, 2004), could become much more common.

The poxviruses orf and molluscum contagiosum generally cause mild, self-limiting disease in humans. However, in immunocompromised individuals, these diseases can be much more extensive and require treatment for resolution (Birthistle and Carrington, 1997; Georgiades *et al.*, 2005).

All of these examples illustrate the need for effective treatment regimens for poxvirus infections. Prior to the eradication of smallpox, marboran (1-methylisatin 3-thiosemicarazone), cytosine arabinoside, and adenine arabinoside were used in the treatment of this disease; however, contemporary clinical and field trials indicated that none of these compounds were effective (Dennis *et al.*, 1974; Heiner *et al.*, 1971; Koplan *et al.*, 1975; Monsur *et al.*, 1975; Rao *et al.*, 1966). ST-246, a small molecule inhibitor of orthopoxvirus extracellular virus formation (Smith *et al.*, 2009; Yang *et al.*, 2005) and CMX001, an alkoxyalkyl ester derivative of CDV, are currently undergoing clinical trials in the United States (www.ClinicalTrials.gov), but at present there are no licensed antiviral agents for the treatment of poxvirus infections.

Vaccinia Virus DNA Polymerase

The vaccinia virus DNA polymerase is a single polypeptide chain that possesses both 5′-to-3′ polymerase and 3′-to-5′ proofreading exonuclease activity (Challberg and Englund, 1979). The 116 kDa protein is a member of the B family of DNA polymerases

and is a product of the E9L gene (Ito and Braithwaite, 1991; McDonald and Traktman, 1994a). Although little is known about the structure of vaccinia virus DNA polymerase, the exonuclease domain is predicted to reside in the amino-terminus and the polymerase domain in the carboxy-terminus, based on similarities to other B family members.

Vaccinia virus DNA polymerase is an early gene product (McDonald *et al.*, 1992) and is responsible for the replication of the viral genome. This enzyme is also involved in catalyzing the recombination that occurs with high frequency in vaccinia virus infected cells (Colinas *et al.*, 1990; Willer *et al.*, 1999; Yao and Evans, 2001). Although vaccinia virus DNA polymerase is capable of catalyzing polymerase and exonuclease activity in the absence of any other protein, this catalysis is highly distributive under physiological conditions (McDonald and Traktman, 1994b). In vaccinia virus infected cells however, a highly processive form of the enzyme exists. This processivity is conferred on the enzyme by its association with a heterodimeric processivity factor, composed of the A20 and D4 vaccinia gene products (Stanitsa *et al.*, 2006).

Vaccinia virus and its DNA polymerase have been used as a model system for variola virus (Kern *et al.*, 2009; Neyts and De Clercq, 2001; Neyts *et al.*, 2002; Prichard *et al.*, 2006; Sauerbrei *et al.*, 2006; Silverman *et al.*, 2008). The DNA polymerases from these two viruses are highly related, and it is expected that compounds that inhibit vaccinia virus DNA polymerase will also inhibit variola virus DNA polymerase.

Retroviruses

The *Retroviridae* are a large family of viruses that infect vertebrates. This family is distinguished by their unique lifecycle (reviewed in Goff, 2007). Retroviruses have RNA genomes, which are copied into double-stranded DNA by a virus-encoded reverse transcriptase. This double-stranded copy of the genome is then integrated into the host cell DNA to form a provirus. The provirus then acts as a template for the transcription of viral RNAs by cellular RNA polymerase to produce mRNAs and full-length genome copies. There are seven genera of retroviruses (Table 1.3). The alpharetroviruses, betaretroviruses, and gammaretroviruses are simple retroviruses in that they encode only the Gag, Pro, Pol, and Env gene products. In contrast, the deltaretroviruses,

Table 1.3 *Retroviridae* genera[1]

Genus	Examples
Alpharetrovirus	Rous sarcoma virus, Avian leukosis virus
Betaretrovirus	Mouse mammary tumor virus
Gammaretrovirus	Moloney murine leukemia virus, Moloney murine sarcoma virus
Deltaretrovirus	Human T-lymphotropic virus type 1 (HTLV-1), Human T-lymphotropic virus type 2 (HTLV-2)
Epsilonretrovirus	Walleye dermal sarcoma virus
Lentivirus	Human immunodeficiency virus type 1 (HIV-1), Human immunodeficiency virus type 2 (HIV-2), Simian immunodeficiency virus (SIV)
Spumavirus	Human foamy virus

[1] Adapted from Goff, 2007

epsilonretroviruses, lentiviruses, and spumaviruses are complex retroviruses that encode these same gene products in addition to a number other gene products with a range of regulatory functions.

The most studied retrovirus is HIV-1. This virus was isolated after a series of unusual outbreaks of Kaposi's sarcoma, *Pneumocystis carinii* pneumonia, chronic HSV, and other opportunistic infections led to the description of an acquired immune deficiency syndrome (AIDS) (Centers for Disease Control, 1981; Gottlieb *et al.*, 1981; Masur *et al.*, 1981; Siegal *et al.*, 1981). This syndrome appeared to be caused by an infectious agent, and a T-lymphotropic retrovirus was subsequently identified and named HIV (Barré-Sinoussi *et al.*, 1983; Coffin *et al.*, 1986; Gallo *et al.*, 1984; Gallo *et al.*, 1983). It was estimated that as of 2007, 33 million people were living with HIV and an estimated 2 million deaths were attributed to AIDS in that year (Joint United Nations Programme on HIV/AIDS, 2008). The high prevalence rate of HIV-1 infections has spurred tremendous interest in the development of anti-HIV-1 drugs. In fact, most of the antiviral agents currently approved for use are those that have been approved for the treatment of HIV-1 infections (Table 1.1).

Reverse Transcriptase

Reverse transcriptase is the enzyme responsible for copying the retrovirus single stranded RNA genome into a double stranded integration-competent DNA form. To accomplish this step in the life cycle, reverse transcriptases possess three activities: RNA-dependent DNA polymerase activity, DNA-dependent DNA polymerase activity and RNaseH activity. The polymerase open reading frame of most retroviruses encodes protease, RT, and integrase gene products (Prasad, 1993). The polymerase gene is translated as a fusion protein with Gag by low-level readthrough or translational frameshift (Prasad, 1993). The processing of this polyprotein to form active RT is thought to take place during release of virions from infected cells and is catalyzed by the protease.

The structures of RTs from different retroviruses vary (reviewed in Prasad, 1993). The MMLV RT is a monomeric enzyme of 75 kDa (Das and Georgiadis, 2004). The

reverse transcriptase and RNaseH domains of this protein are not functionally interdependent as these domains can be expressed separately and still retain activity (Prasad, 1993). This enzyme has been used extensively as a molecular biology tool to produce DNA from RNA samples (Gerard *et al.*, 1997).

In contrast, HIV-1 RT is a heterodimer of 66 and 51 kDa subunits (Le Grice, 1993). The p51 subunit is a product of carboxy-terminal processing of the p66 subunit between amino acid residues phenylalanine 440 and tyrosine 441 by the HIV-1 protease; the subunits therefore have the same amino-termini but different carboxy-termini (Prasad, 1993). Interestingly, although the two subunits share amino acid sequences and the overall folding of the protein subdomains are similar, the spatial arrangements of these subdomains are different (Jacobo-Molina *et al.*, 1993). The p51 subunit does not have a DNA binding cleft and has little or no RT activity (Prasad, 1993). In addition, p51 has no RNAseH activity as this domain resides in carboxy-terminus. The HIV-1 RT heterodimer therefore has one polymerase active site, one RNaseH active site, one tRNA binding site, and one NNRTI binding site (Kohlstaedt *et al.*, 1993). Further, unlike the MMLV RT, the polymerase and RNaseH are interdependent (Prasad, 1993).

As the retrovirus RT plays a key role in the life cycle of the virus, it is a key target in the development of antiretroviral agents. As described above, most of the antiviral agents currently approved for use are those that have been approved for the treatment of HIV-1 infections (Table 1.1); the majority of these target the RT.

1.3 PURPOSE OF STUDY

The purpose of this study was to investigate the mechanism of action of the antiviral agents cidofovir and *(S)*-HPMPA. This study was initially focused on examining the interaction of CDVpp with vaccinia virus DNA polymerase to determine if the inhibition of this enzyme was similar to that previously observed for the inhibition of HCMV DNA polymerase. The study focus was then broadened to include a determination of the mechanism of inhibition of vaccinia DNA polymerase by *(S)*-HPMPApp, a compound structurally similar to CDVpp and differing only in the base moiety. This second group of experiments was undertaken to examine the biochemical

basis for the increased antiviral activity against poxviruses seen with *(S)*-HPMPA relative to CDV. Finally, this study was expanded to investigate the interactions of CDVpp and *(S)*-HPMPApp with the reverse transcriptases from the retroviruses MMLV and HIV-1. These latter experiments were motivated by data showing that both CDV and *(S)*-HPMPA, or their prodrugs, have a much wider range of antiviral activity than originally thought and by the hypothesis that the mechanism of action that we observed for these drugs against vaccinia DNA polymerase are more broadly applicable.

1.4 REFERENCES

Abele, G., B. Eriksson, J. Harmemberg, and B. Wahren. 1988. Inhibition of varicella-zoster virus-induced DNA polymerase by a new guanosine analog, 9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine triphosphate. Antimicrob. Agents Chemother. **32:** 1137-1142.

Aduma, P., M. C. Connelly, R. V. Srinivas, and A. Fridland. 1995. Metabolic diversity and antiviral activities of acyclic nucleoside phosphonates. Mol. Pharmacol. **47:** 816-822.

Ahluwalia, G., D. A. Cooney, H. Mitsuya, A. Fridland, K. P. Flora, Z. Hao, M. Dalal, S. Broder, and D. G. Johns. 1987. Initial studies on the cellular pharmacology of 2',3'-dideoxyinosine, an inhibitor of HIV infectivity. Biochem. Pharmacol. **36:** 3797-3800.

Aldern, K. A., S. L. Ciesla, K. L. Winegarden, and K. Y. Hostetler. 2003. Increased antiviral activity of 1-*O*-hexadecyloxypropyl-[2-^{14}C]cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism. Mol. Pharmacol. **63:** 678-681.

Andrei, G., D. B. Gammon, P. Fiten, E. De Clercq, G. Opdenakker, R. Snoeck, and D. H. Evans. 2006. Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice. J. Virol. **80:** 9391-9401.

Andrei, G., R. Snoeck, E. De Clercq, R. Esnouf, P. Fiten, and G. Opdenakker. 2000. Resistance of herpes simplex virus type 1 against different phosphonylmethoxyalkyl derivatives of purines and pyrimidines due to specific mutations in the viral DNA polymerase gene. J. Gen. Virol. **81:** 639-648.

Balzarini, J., P. Herdewijn, and E. De Clercq. 1989. Differential patterns of intracellular metabolism of 2',3'-didehydro-2',3'-dideoxythymidine and 3'-azido-2',3'-

dideoxythymidine, two potent anti-human immunodeficiency virus compounds. J. Biol. Chem. **264:** 6127-6133.

Barnard, D. L. 2004. Inhibitors of measles virus. Antiviral Chem. Chemother. **15:** 111-119.

Barré-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. C. Dauguet, C. Axler-Blin, F. Vézinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220:** 868-871.

Beadle, J. R., C. Hartline, K. A. Aldern, N. Rodriguez, E. Harden, E. R. Kern, and K. Y. Hostetler. 2002. Alkoxyalkyl esters of cidofovir and cyclic cidofovir exhibit multiple-log enhancement of antiviral activity against cytomegalovirus and herpesvirus replication in vitro. Antimicrob. Agents Chemother. **46:** 2381-2386.

Beadle, J. R., W. B. Wan, S. L. Ciesla, K. A. Keith, C. Hartline, E. R. Kern, and K. Y. Hostetler. 2006. Synthesis and antiviral evaluation of alkoxyalkyl derivatives of 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)adenine against cytomegalovirus and orthopoxviruses. J. Med. Chem. **49:** 2010-2015.

Becker, M. N., M. Obraztsova, E. R. Kern, D. C. Quenelle, K. A. Keith, M. N. Prichard, M. Luo, and R. W. Moyer. 2008. Isolation and characterization of cidofovir resistant vaccinia viruses. Virology J. **5:** 58.

Birkus, G., D. Rejman, M. Otmar, I. Votruba, I. Rosenberg, and A. Holy. 2004. The substrate activity of (*S*)-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine diphosphate toward DNA polymerases α, δ and ε. Antiviral Chem. Chemother. **15:** 23-33.

Birkus, G., I. Votruba, A. Holý, and B. Otová. 1999. 9-[2-(phosphonomethoxu)ethyl]adenine diphosphate (PMEApp) as a substrate toward replicative DNA polymerases α, δ, ε, and ε*. Biochem. Pharmacol. **58:** 487-492.

Biron, K. K., and G. B. Elion. 1980. In vitro susceptibility of varicella-zoster virus to acyclovir. Antimicrob. Agents Chemother. **18:** 443-447.

Biron, K. K., S. C. Stanat, J. B. Sorrell, J. A. Fyfe, P. M. Keller, C. U. Lambe, and D. J. Nelson. 1985. Metabolic activation of the nucleoside analog 9-{[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl}guanine in human diploid fibroblasts infected with human cytomegalovirus. Proc. Natl. Acad. Sci. USA **82:** 2473-2477.

Birthistle, K., and D. Carrington. 1997. Molluscum contagiosum virus. J. Infect. **34:** 21-28.

Biswal, B. K., M. M. Cherney, M. Wang, L. Chen, C. G. Yannopoulos, D. Bilimoria,

O. Nicolas, J. Bedard, and M. N. G. James. 2005. Crystal structures of the RNA-dependent RNA polymerase genotype 2a of hepatitis C virus reveal two conformations and suggest mechanisms of inhibition by non-nucleoside inhibitors. J. Biol. Chem. **280:** 18202-18210.

Biswal, B. K., M. Wang, M. M. Cherney, L. Chan, C. G. Yannopoulos, D. Bilimoria, J. Bedard, and M. N. G. James. 2006. Non-nucleoside inhibitors binding to hepatitis C virus NS5B polymerase reveal a novel mechanism of inhibition. J. Mol. Biol. **361:** 33-45.

Botros, S., S. William, O. Hammam, Z. Zídek, and A. Holý. 2003. Activity of 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine against *Schistosomiasis mansoni* in mice. Antimicrob. Agents Chemother. **47:** 3853-3858.

Bray, M. 2003. Pathogenesis and potential antiviral therapy of complications of smallpox vaccination. Antiviral Res. **58:** 101-114.

Breman, J. G., and D. A. Henderson. 2002. Diagnosis and management of smallpox. New Engl. J. Med. **346:** 1300-1308.

Bronson, J.J., I. Ghazzouli, M.J.M. Hitchcock, R.R. Webb II, E.R. Kern, J.C. Martin. 1989. Synthesis and antiviral activity of nucleotide analogues bearing the (*S*)-(3-hydroxy-2-phosphonylmethoxy)propyl moiety attached to adenine, guanine, and cytosine, p. 88-102. *In* J. C. Martin (ed.), Nucleotide Analogues as Antiviral Agents. American Chemical Society, Washington, DC.

Bryant, M. L., E. G. Bridges, L. Placidi, A. Faraj, A.-G. Loi, C. Pierra, D. Dukhan, G. Gosselin, J.-L. Imbach, B. Hernandez, A. Juodawlkis, B. Tennant, B. Korba, P. Cote, P. Marion, E. Cretton-Scott, R. F. Schinazi, and J.-P. Sommadossi. 2001. Antiviral L-nucleosides specific for hepatitis B virus infection. Antimicrob. Agents Chemother. **45:** 229-235.

Calista, D. 2000. Resolution of recalcitrant human papillomavirus gingival infection with topical cidofovir. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. **90:** 713-715.

Carroll, S. S., J. E. Tomassini, M. Bosserman, K. Getty, M. W. Stahlhut, A. B. Eldrup, B. Bhat, D. Hall, A. L. Simcoe, R. LaFemina, C. A. Rutkowski, B. Wolanski, Z. Yang, G. Migliaccio, R. De Francesco, L. C. Kuo, M. MacCoss, and D. B. Olsen. 2003. Inhibition of hepatitis C virus RNA replication by 2'-modified nucleoside analogs. J. Biol. Chem. **278:** 11979-11984.

Centers for Disease Control. 1981. Kaposi's sarcoma and *Pneumocystis* pneumonia among homosexual men - New York City and California. MMWR **30:** 305-308.

Centers for Disease Control and Prevention. 2003. Multistate outbreak of monkeypox

- Illinois, Indiana, and Wisconsin, 2003. MMWR **52:** 537-540.

Challberg, M. D., and P. T. Englund. 1979. Purification and properties of the deoxyribonucleic acid polymerase induced by vaccinia virus. J. Biol. Chem. **254:** 7812-7819.

Cheng, Y., S. P. Grill, G. E. Dutschman, K. Nakayama, and K. F. Bastow. 1983. Metabolism of 9-(1,3-dihydroxy-2-propoxymethyl)guanine, a new anti-herpes virus compound, in herpes simplex virus-infected cells. J. Biol. Chem. **258:** 12460-12464.

Cherrington, J. M., S. J. Allen, B. H. McKee, and M. S. Chen. 1994. Kinetic analysis of the interaction between the diphosphate of (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine, ddCTP, AZTTP, and FIAUTP with human DNA polymerases β and γ. Biochem. Pharmacol. **48:** 1986-1988.

Cherrington, J. M., S. J. W. Allen, N. Bischofberger, and M. S. Chen. 1995. Kinetic interaction of the diphosphates of 9-(2-phosphonylmethoxyethyl)adenine and other anti-HIV active purine congeners with HIV reverse transcriptase and human DNA polymerases α, β andγ. Antiviral Chem. Chemother. **6:** 217-221.

Cherrington, J. M., M. D. Fuller, A. S. Mulato, S. J. W. Allen, S. C. Kunder, M. A. Ussery, Z. Lesnikowski, R. F. Schinazi, J.-P. Sommadossi, and M. S. Chen. 1996. Comparative kinetic analyses of interactions of inhibitors with Rauscher murine leukemia virus and human immunodeficiency virus reverse transcriptases. Antimicrob. Agents Chemother. **40:** 1270-1273.

Chou, S., N. S. Lurain, K. D. Thompson, R. C. Miner, and W. L. Drew. 2003. Viral DNA polymerase mutations associated with drug resistance in human cytomegalovirus. J. Infect. Dis. **188:** 32-39.

Ciesla, S. L., J. Trahan, W. B. Wan, J. R. Beadle, K. A. Aldern, G. R. Painter, and K. Y. Hostetler. 2003. Esterification of cidofovir with alkoxyalkanols increases oral bioavailability and dimishes drug accumulation in the kidney. Antiviral Res. **59:** 163-171.

Cihlar, T., and M. S. Chen. 1996. Identification of enzymes catalyzing two-step phosphorylation of cidofovir and the effect of cytomegalovirus infection on their activities in host cells. Mol. Pharmacol. **50:** 1502-1510.

Cihlar, T., and M. S. Chen. 1997. Incorporation of selected nucleoside phosphonates and anti-human immunodeficiency virus nucleotide analogues into DNA by human DNA polymerases α, β, and γ. Antiviral Chem. Chemother. **8:** 187-195.

Cihlar, T., M. D. Fuller, A. S. Mulato, and J. M. Cherrington. 1998. A point mutation in the human cytomegalovirus DNA polymerase gene selected *in vitro* by cidofovir

confers a slow replication phenotype in cell culture. Virology **248:** 382-393.

Cihlar, T., E. S. Ho, D. C. Lin, and A. S. Mulato. 2001. Human renal organic anion transporter 1 (hOAT1) and its role in the nephrotoxocity of antiviral nucleotide analogs. Nucleosides Nucleotides Nucleic Acids **20:** 641-648.

Cihlar, T., D. C. Lin, J. B. Pritchard, M. D. Fuller, D. B. Mendel, and D. H. Sweet. 1999. The antiviral nucleotide analogs cidofovir and adefovir are novel substrates for human and rat renal organic anion transporter 1. Mol. Pharmacol. **56:** 570-580.

Cocohoba, J. M., and I. R. McNicholl. 2002. Valganciclovir: an advance in cytomegalovirus therapeutics. Ann. Pharmacother. **36:** 1075-1079.

Coffin, J., A. Haase, J. A. Levy, L. Montagnier, S. Oroszlan, N. Teich , H. Temin, K. Toyoshima, H. Varmus, P. Vogt, and R. Weiss. 1986. Human immunodeficiency viruses. Science **232:** 697.

Colinas, R. J., R. C. Condit, and E. Paoletti. 1990. Extrachromosomal recombination in vaccinia-infected cells requires a functional DNA polymerase participating at a level other than DNA replication. Virus Res. **18:** 49-70.

Connelly, M. C., B. L. Robbins, and A. Fridland. 1993. Mechanism of uptake of the phosphonate analog (*S*)-1-(3-hydroyx-2-phosphonylmethoxypropyl)cytosine (HPMPC) in Vero cells. Biochem. Pharmacol. **46:** 1053-1057.

Crumpacker, C. S. 1992 . Mechanism of action of foscarnet against viral polymerases. Am. J. Med. **92:** 3S-7S.

Cundy, K. C. 1999. Clinical pharmacokinetics of the antiviral nucleotide analogues cidofovir and adefovir. Clin. Pharmacokinet. **36:** 127-143.

Dal Pozzo, F., G. Andrei, I. Lebeau, J. R. Beadle, K. Y. Hostetler, E. De Clercq, and R. Snoeck. 2007. In vitro evaluation of the anti-orf virus activity of alkoxyalkyl esters of CDV, cCDV and (*S*)-HPMPA. Antiviral Res. **75:** 52-57.

Daluge, S. M., S. S. Good, M. B. Faletto, W. H. Miller, M. H. St. Clair, L. R. Boone, M. Tisdale, N. R. Parry, J. E. Reardon, R. E. Dornsife, D. R. Averett, and T. A. Krenitsky. 1997. 1592U89, a novel carbocyclic nucleoside analog with potent, selective anti-human immunodeficiency virus activity. Antimicrob. Agents Chemother. **41:** 1082-1093.

Das, D., and M. M. Georgiadis. 2004. The crystal structure of the monomeric reverse transcriptase from Moloney murine leukemia virus. Structure **12:** 819-829.

Davies, E. G., A. Thrasher, K. Lacey, and J. Harper. 1999. Topical cidofovir for severe molluscum contagiosum. Lancet **353:** 2042.

De Clercq, E. 1997. Acyclic nucleoside phosphonates in the chemotherapy of DNA virus and retrovirus infections. Intervirology **40:** 295-303.

De Clercq, E., and A. Holý. 2005. Acyclic nucleoside phosphonates: a key class of antiviral drugs. Nat. Rev. Drug Discov. **4:** 928-940.

De Clercq, E., A. Holý, I. Rosenberg, T. Sakuma, J. Balzarini, and P. C. Maudgal. 1986. A novel selective broad-spectrum anti-DNA virus agent. Nature **323:** 464-467.

De Clercq, E., and J. Neyts. 2009. Antiviral agents acting as DNA or RNA chain terminators. Handb. Exp. Pharmacol. **189:** 53-84.

De Clercq, E., T. Sakuma, M. Baba, R. Pauwels, J. Balzarini, I. Rosenberg, and A. Holý. 1987. Antiviral activity of phosphonylmethoxyalkyl derivatives of purine and pyrimidines. Antiviral Res. **8:** 261-272.

de Vries, E., J. G. Stam, F. F. J. Franssen, H. Nieuwenhuijs, P. Chavalitshewinkoon, E. de Clercq, J. P. Overdulve, and P. C. van der Vliet. 1991. Inhibition of the growth of *Plasmodium falciparum* and *Plasmodium berghei* by the DNA polymerase inhibitor HPMPA. Mol. Biochem. Parasitol. **47:** 43-50.

Delaney IV, W. E., A. S. Ray, H. Yang, X. Qi, S. Xiong, Y. Zhu, and M. D. Miller. 2006. Intracellular metabolism and in vitro activity of tenofovir against hepatitis B virus. Antimicrob. Agents Chemother. **50:** 2471-2477.

Dennis, D. T., E. B. Doberstyn, S. Awoke, Jr. G. L. Royer, and H. E. Renis. 1974. Failure of cytosine arabinoside in treating smallpox. A double-blind study. Lancet **2:** 377-379.

DeRossi, S. S., and J. Laudenbach. 2004. The management of oral human papillomavirus with topical cidofovir: a case report. Cutis **73:** 191-193.

Derse, D., Y.-C. Cheng, P. A. Furman, M. H. St. Clair, and G. B. Elion. 1981. Inhibition of purified human and herpes simplex virus-induced DNA polymerases by 9-(2-hydroxyethoxymethyl)guanine triphosphate: effects on primer-template function. J. Biol. Chem. **256:** 11447-11451.

Dhanak, D., K. J. Duffy, V. K. Johnston, J. Lin-Goerke, M. Darcy, A. N. Shaw, B. Gu, C. Silverman, A. T. Gates, M. R. Nonnemacher, D. L. Earnshaw, D. J. Casper, A. Kaura, A. Baker, C. Greenwood, L. L. Gutshall, D. Maley, A. DelVecchio, R. Macarron, G. A. Hofmann, Z. Alnoah, H. Y. Cheng, G. Chan, S. Khandekar, R. M.

Keenan, and R. T. Sarisky. 2002. Identification and biological characterization of heterocyclic inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. J. Biol. Chem. **277:** 38322-38327.

Ding, J., K. Das, H. Moereels, L. Koymans, K. Andries, P. A. J. Janssen, S. H. Hughes, and E. Arnold. 1995. Structure of HIV-1 RT/TIBO R 86183 complex reveals similarity in the binding of diverse nonnucleoside inhibitors. Nat. Struct. Biol. **2:** 407-415.

Dodge, R. T. 1999. A case study: the use of cidofovir for the management of progressive multifocal leukoencephalopathy. J. Assoc. Nurses AIDS Care **10:** 70-74.

Domaoal, R. A., M. McMahon, C. L. Thio, C. M. Bailey, J. Tirado-Rives, A. Obikhod, M. Detorio, K. L. Rapp, R. F. Siliciano, R. F. Schinazi, and K. S. Anderson. 2008. Pre-steady-state kinetic studies establish entecavir 5'-triphosphate as a substrate for HIV-1 reverse transcriptase. J. Biol. Chem. **283:** 5452-5459.

Earnshaw, D. L., T. H. Bacon, S. J. Darlison, K. Edmonds, R. M. Perkins, and R. A. V. Hodge. 1992. Mode of antiviral action of penciclovir in MRC-5 cells infected with herpes simplex virus type 1 (HSV-1), HSV-2, and varicella-zoster virus. Antimicrob. Agents Chemother. **36:** 2747-2757.

Elion, G. B., P. A. Furman, J. A. Fyfe, P. De Miranda, L. Beauchamp, and H. J. Schaeffer. 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc. Natl. Acad. Sci. USA **74:** 5716-5720.

Esposito, J.J., F. Fenner. 2001. Poxviruses, p. 2885-2921. *In* D. M. Knipe, P., M. Howley (eds.), Fields' Virology. Lippnicott Williams & Wilkins, Philadelphia.

Faletto, M. B., W. H. Miller, E. P. Garvey, M. H. St. Clair, S. M. Daluge, and S. S. Good. 1997. Unique intracellular activation of the potent anti-human immunodeficiency virus agent 1592U89. Antimicrob. Agents Chemother. **41:** 1099-1107.

Fang, S.-H., L.-H. Hwang, D.-S. Chen, and B.-L. Chiang. 2000. Ribavirin enhancement of hepatitis C virus core antigen-specific type 1 T helper cell response correlates with the increased IL-12 level. J. Hepatol. **33:** 791-798.

Feng, J. Y., E. Murakami, S. M. Zorca, A. A. Johnson, K. A. Johnson, R. Schinazi, P. A. Furman, and K. S. Anderson. 2004. Relationship between antiviral activity and host toxicity: comparison of the incorporation efficiencies of 2',3'-dideoxy-5-fluoro-3'-thiacytidine-triphosphate analogs by human immunodeficiency virus type 1 reverse transcriptase and human mitochondrial DNA polymerase. Antimicrob. Agents Chemother. **48:** 1300-1306.

Feng, J. Y., J. Shi, R. F. Schinazi, and K. S. Anderson. 1999. Mechanistic studies

show that (-)-FTC-TP is a better inhibitor of HIV-1 reverse transcriptase than 3TC-TP. FASEB J. **13:** 1511-1517.

Figueiredo, A., K. L. Moore, J. Mak, N. Sluis-Cremer, M.-P. de Bethune, and G. Tachedjian. 2006. Potent nonnucleoside reverse transcriptase inhibitors target HIV-1 Gag-Pol. PLoS Pathogens **2:** e119.

Frangeul, A., C. Bussetta, J. Deval, K. Barral, K. Alvarez, and B. Canard. 2008. Gln151 of HIV-1 reverse transcriptase acts as a steric gate towards clinically relevant acyclic phosphonate nucleotide analogues. Antiviral Ther. **13:** 115-124.

Furman, P. A., P. De Miranda, M. H. St. Clair, and G. B. Elion. 1981. Metabolism of acyclovir in virus-infected and uninfected cells. Antimicrob. Agents Chemother. **20:** 518-524.

Furman, P. A., J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry. 1986. Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA **83:** 8333-8337.

Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, G. White, P. Foster, and P. D. Markham. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science **224:** 500-503.

Gallo, R. C., P. S. Sarin, E. P. Gelmann, M. Robert-Guroff, E. Richardson, V. S. Kalyanaraman, D. Mann, G. D. Sidhu, R. E. Stahl, S. Zolla-Pazner, J. Leibowitch, and M. Popovic. 1983. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). Science **220:** 865-867.

Geerinck, K., G. Lukito, R. Snoeck, R. De Vos, E. De Clercq, Y. Vanrenterghem, H. Degreef, and B. Maes. 2001. A case of human orf in an immunocompromised patient treated successfully with cidofovir cream. J. Med. Virol. **64:** 543-549.

Georgiades, G., A. Katsarou, and K. Dimitroglou. 2005. Human orf (ecthyma contagiosum). J. Hand Surg. **30B:** 409-411.

Gerard, G. F., D. K. Fox, M. Nathan, and J. M. D'Alessio. 1997. Reverse transcriptase: the use of cloned Moloney murine leukemia virus reverse transcriptase to synthesize DNA from RNA. Mol. Biotechnol. **8:** 61-77.

Gilbert, B. E., and V. Knight. 1986. Biochemistry and clinical applications of ribavirin. Antimicrob. Agents Chemother. **30:** 201-205.

Gilbert, C., J. Bestman-Smith, and G. Boivin. 2002. Resistance of herpesvirus to antiviral drugs: clinical impacts and molecular mechanisms. Drug Resist. Updat. **5:** 88-114.

Gilbert, D. N., R. C. Moellering Jr., G. M. Eliopoulos, H. F. Chambers, and M. S. Saag. 2009. The Sanford Guide to Antimicrobial Therapy 2009, 39th ed. Antimicrobial Therapy, Inc., Sperryville, VA.

Gilead Sciences. 2003. Vistide, p. 1428-1432. *In* Physicians' Desk Reference. Thomson, Montvale, NJ.

Goff, S.P. 2007. *Retroviridae*: the retroviruses and their replication, p. 1999-2069. *In* D. M. Knipe, P. M. Howley (eds.), Fields Virology, fifth edition. Lippincott Williams & Wilkins, a Wolters Kluwer Business, Philadelphia.

Gordon, Y. J., L. Naesens, E. De Clercq, P. C. Maudgal, and M. Veckeneer. 1996. Treatment of adenoviral conjunctivitis with topical cidofovir. Cornea **15:** 546.

Gottlieb, M. S., R. Schroff, H. M. Schanker, J. D. Weisman, P. T. Fan, R. A. Wolf, and A. Saxon. 1981. *Pneumocystis carinii* pneumonia and mucosal candidiasis in previously healthy homosexual men. New Engl. J. Med. **305:** 1425-1431.

Gray, N. M., C. L. P. Marr, C. R. Penn, J. M. Cameron, and R. C. Bethell. 1995. The intracellular phosphorylation of (-)-2'-deoxy-3'-thiacytidine (3TC) and the incorporation of 3TC 5'-monophosphate into DNA by HIV-1 reverse transcriptase and human DNA polymerase γ. Biochem. Pharmacol. **50:** 1043-1051.

Groothuis, J. R., K. A. Woodin, R. Katz, A. D. Robertson, J. T. McBride, C. B. Hall, B. C. McWilliams, and B. A. Lauer. 1990. Early ribavirin treatment of respiratory syncytial viral infection in high-risk children. J. Pediatr. **117:** 792-298.

Gu, B., V. K. Johnston, L. L. Gutshall, T. T. Nguyen, R. R. Gontarek, M. G. Darcy, R. Tedesco, D. Dhanak, K. J. Duffy, C. C. Kao, and R. T. Sarisky. 2003. Arresting initiation of hepatitis C virus RNA synthesis using heterocyclic derivatives. J. Biol. Chem. **278:** 16602-16607.

Hall, C. B., J. T. McBride, E. E. Walsh, D. M. Bell, C. L. Gala, S. Hildreth, L. G. T. Eyck, and W. J. Hall. 1983. Aerosolized ribavirin treatment of infants with respiratory syncytial viral infection. A randomized double-blind study. New Engl. J. Med. **308:** 1443-1447.

Harris, K. S., W. Brabant, S. Styrchak, A. Gall, and R. Daifuku. 2005. KP-1212/1461, a nucleoside designed for the treatment of HIV by viral mutagenesis. Antiviral Res. **67:** 1-9.

Hart, G. J., D. C. Orr, C. R. Penn, H. T. Figueiredo, N. M. Gray, R. E. Boehme, and J. M. Cameron. 1992. Effects of (-)-2'-deoxy-3'-thiacytidine (3TC) 5'-triphosphate on human immunodeficiency virus reverse transcriptase and mammalian DNA polymerases alpha, beta, and gamma. Antimicrob. Agents Chemother. **36:** 1688-1694.

Heck, J. A., A. M. I. Lam, N. Narayanan, and D. N. Frick. 2008. Effects of mutagenic and chain-terminating nucleotide analogs on enzymes isolated from hepatitis C virus strains of various genotypes. Antimicrob. Agents Chemother. **52:** 1901-1911.

Heiner, G. G., N. Fatima, P. K. Russell, A. T. Haase, N. Ahmad, N. Mohammed, D. B. Thomas, T. M. Mack, M. M. Khan, G. L. Knatterud, R. L. Anthony, and Jr. F. R. McCrumb. 1971. Field trials of methisazone as a prophylactic agent aginst smallpox. Am. J. Epidemiol. **94:** 435-449.

Helgstrand, E., B. Eriksson, N. G. Johansson, B. Lannerö, A. Larsson, A. Misiorny, J. O. Norén, B. Sjöberg, K. Stenberg, G. Stening, S. Stridh, B. Öberg, S. Alenius, and L. Philipson. 1978. Trisodium phosphonoformate, a new antiviral compound. Science **201:** 819-821.

Henderson, D. A., T. V. Inglesby, J. G. Bartlett, M. S. Ascher, E. Eitzen, P. B. Jahrling, J. Hauer, M. Layton, J. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. Perl, P. K. Russell, and K. Tonat. 1999. Smallpox as a biological weapon: medical and public health management. JAMA **281:** 2127-2137.

Hernandez-Santiago, B., L. Placidi, E. Cretton-Scott, A. Faraj, E. G. Bridges, M. L. Bryant, J. Rodriguez-Orengo, J. L. Imbach, G. Gosselin, C. Pierra, D. Dukhan, and J. P. Sommadossi. 2002. Pharmacology of β-L-thymidine and β-L-2'-deoxycytidine in HepG2 cells and primary human hepatocytes: relevance to chemotherapeutic efficacy against hepatitis B virus. Antimicrob. Agents Chemother. **46:** 1728-1733.

Ho, H.-T., K. L. Woods, J. J. Bronson, H. De Boeck, J. C. Martin, and M. J. M. Hitchcock. 1992. Intracellular metabolism of the antiherpes agent (S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine. Mol. Pharmacol. **41:** 197-202.

Hodge, R. A. V., and R. M. Perkins. 1989. Mode of action of 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine (BRL 39123) against hereps simplex virus in MRC-5 cells. Antimicrob. Agents Chemother. **33:** 223-229.

Hodge, R. A. V., D. Sutton, M. R. Boyd, M. R. Harnden, and R. L. Jarvest. 1989. Selection of an oral prodrug (BRL 42810; Famciclovir) for the antiherpesvirus agent BRL 39123 [9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine; penciclovir]. Antimicrob. Agents Chemother. **33:** 1765-1773.

Holý, A., and I. Rosenberg. 1987. Synthesis of isomeric and enantiomeric O-

phosphonylmethyl derivatives of 9-(2,3-dihydroxypropyl)adenine. Collect. Czech. Chem. Commun. **52:** 2775-2791.

Hostetler, K. Y., K. A. Aldern, W. B. Wan, S. L. Ciesla, and J. R. Beadle. 2006. Alkoxyalkyl esters of (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine are potent inhibitors of the replication of wild-type and drug-resistant human immunodeficiency virus type 1 in vitro. Antimicrob. Agents Chemother. **50:** 2857-2859.

Howe, A. Y., J. Bloom, C. J. Baldick , C. A. Benetatos, H. Cheng, J. S. Christensen, S. K. Chunduru, G. A. Coburn, B. Feld, A. Gopalsamy, W. P. Gorczyca, S. Herrmann, S. Johann, X. Jiang, M. L. Kimberland, G. Krisnamurthy, M. Olson, M. Orlowski, S. Swanberg, I. Thompson , M. Thorn, A. Del Vecchio, D. C. Young, M. van Zeijl, J. W. Ellingboe, J. Upeslacis, M. Collett, T. S. Mansour, and J. F. O'Connell. 2004. Novel nonnucleoside inhibitor of hepatitis C virus RNA-dependent RNA polymerase. Antimicrob. Agents Chemother. **48:** 4813-4821.

Howe, A. Y., H. Cheng, I. Thompson, S. K. Chunduru, S. Herrmann, J. O'Connell, A. Agarwal, R. Chopra, and A. M. Del Vecchio. 2006. Molecular mechanism of a thumb domain hepatitis C virus nonnucleoside RNA-dependent RNA polymerase inhibitor. Antimicrob. Agents Chemother. **50:** 4103-4113.

Hruska, J. F., J. M. Bernstein, Jr. R. G. Doulas, and C. B. Hall. 1980. Effects of ribavirin on respiratory syncytial virus in vitro. Antimicrob. Agents Chemother. **17:** 770-775.

Huang, P., D. Farquhar, and W. Plunkett. 1990. Selective action of 3'-azido-3'-deoxythymidine 5'-triphosphate on viral reverse transcriptases and human DNA polymerases. J. Biol. Chem. **265:** 11914-11918.

Huang, P., D. Farquhar, and W. Plunkett. 1992. Selective action of 2',3'-didehydro-2',3'-dideoxythymidine triphosphate on human immunodeficiency virus reverse transcriptase and human DNA polymerases. J. Biol. Chem. **267:** 2817-2822.

Huang, Z., M. G. Murray, and J. A. Secrist III. 2006. Recent development of therapeutics for chronic HCV infection. Antiviral Res. **71:** 351-362.

Huffman, J. H., R. W. Sidwell, G. P. Khare, J. T. Witkowski, L. B. Allen, and R. K. Robins. 1973. In vitro effect of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) on deoxyribonucleic acid and ribonucleic acid viruses. Antimicrob. Agents Chemother. **3:** 235-241.

Hutin, Y. J. F., R. J. Williams, P. Malfait, R. Pebody, V. N. Loparev, S. L. Ropp, M. Rodriguez, J. C. Knight, F. K. Tshioko, A. S. Khan, M. V. Szczeniowski, and J. J. Esposito. 2001. Outbreak of human monkeypox, Democratic Republic of Congo, 1996-

1997. Emerg. Infect. Dis. **7:** 434-438.

Ibarra, V., J. R. Blanco, J. A. Oteo , and L. Rosel. 2000. Efficacy of cidofovir in the treatment of recalcitrant molluscum contagiosum in an AIDS patient. Acta Derm. Venereol. **80:** 315-316.

Innaimo, S. F., M. Seifer, G. S. Bisacchi, D. N. Standring, R. Zahler, and R. J. Colonno. 1997. Identification of BMS-200475 as a potent and selective inhibitor of hepatitis B virus. Antimicrob. Agents Chemother. **41:** 1444-1448.

Ito, J., and D. K. Braithwaite. 1991. Compilation and alignment of DNA polymerase sequences. Nucleic Acids Res. **19:** 4045-4057.

Jacobo-Molina, A., J. Ding, R. G. Nanni, Jr. A. D. Clark, X. Lu, C. Tantillo, R. L. Williams, G. Kamer, A. L. Ferris, P. Clark, A. Hizi, S. H. Hughes, and E. Arnold. 1993. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. Proc. Natl. Acad. Sci. USA **90:** 6320-6324.

Jochmans, D. 2008. Novel HIV-1 reverse transcriptase inhibitors. Virus Res. **134:** 171-185.

Jochmans, D., J. Deval, B. Kesteleyn , H. Van Marck, E. Bettens, I. De Baere, P. Dehertogh, T. Ivens, M. Van Ginderen, B. Van Schoubroeck, M. Ehteshami, P. Wigerinck, M. Götte, and K. Hertogs. 2006. Indolopyridones inhibit human immunodeficiency virus reverse transcriptase with a novel mechanism of action. J. Virol. **80:** 12283-12292.

Johnson, A. A., A. S. Ray, J. Hanes, Z. Suo, J. M. Colacino, K. S. Anderson, and K. A. Johnson. 2001. Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. J. Biol. Chem. **276:** 40847-40857.

Joint United Nations Programme on HIV/AIDS. 2008. Global summary of the AIDS epidemic, December 2007.URL: www.unaids.org/en/KnowledgeCentre/HIVData/Epidemiology/epidemiologySlidesAuto.asp. Downloaded April 27, 2009.

Kaminsky, R., C. Schmid, Y. Grether, A. Holý, E. De Clercq, L. Naesens, and R. Brun. 1996. (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S)*-HPMPA]: a purine analogue with trypanocidal activity *in vitro* and *in vivo*. Trop. Med. Int. Health **1:** 255-263.

Kaminsky, R., E. Zweygarth, and E. De Clercq. 1994. Antitrypanosomal activity of phosphonylmethoxyalkylpurines. J. Parasitol. **80:** 1026-1030.

Kern, E. R., C. Hartline, E. Harden, K. Keith, N. Rodriguez, J. R. Beadle, and K. Y. Hostetler. 2002. Enhanced inhibition of orthopoxvirus replication in vitro by alkoxyalkyl esters of cidofovir and cyclic cidofovir. Antimicrob. Agents Chemother. **46:** 991-995.

Kern, E. R., M. N. Prichard, D. C. Quenelle, K. A. Keith, K. N. Tiwari, J. A. Maddry, and J. A. Secrist III. 2009. Activities of certain 5-substituted 4'-thiopyrimidine nucleosides against orthopoxvirus infections. Antimicrob. Agents Chemother. **53:** 572-579.

Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz. 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science **256:** 1783-1790.

Kohlstaedt, L.A., J. Wang, P.A. Rice, J.M. Friedman, T.A. Steitz. 1993. The structure of HIV-1 reverse transcriptase, p. 223-249. *In* A. M. Skalka, S. P. Goff (eds.), Reverse Transcriptase. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Koplan, J. P., K. A. Monsur, S. O. Foster, F. Huq, M. M. Rahaman, S. Huq, R. A. Buchanan, and N. A. Ward. 1975. Treatment of variola major with adenine arabinoside. J. Infect. Dis. **131:** 34-39.

Kornbluth, R. S., D. F. Smee, R. W. Sidwell, V. Snarsky, D. H. Evans, and K. Y. Hostetler. 2006. Mutations in the E9L polymerase gene of cidofovir-resistant vaccinia virus strain WR are associated with the drug resistance phenotype. Antimicrob. Agents Chemother. **50:** 4038-4043.

Koyano, S., T. Suzutani, I. Yoshida, and M. Azuma. 1996. Analysis of phosphorylation pathways of antiherpesvirus nucleosides by varicella-zoster virus-specific enzymes. Antimicrob. Agents Chemother. **40:** 920-923.

Kramata, P., I. Votruba, B. Otová, and A. Holý. 1996. Different inhibitory potencies of acyclic phosphonomethoxyalkyl nucleotide analogs toward DNA polymerases α, δ, and ε. Mol. Pharmacol. **49:** 1005-1011.

Lalezari, J. P., W. L. Drew, E. Glutzer, C. James, D. Miner, J. Flaherty, P. E. Fisher, K. Cundy, J. Hannigan, J. C. Martin, and H. S. Jaffe. 1995. *(S)*-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine (Cidofovir): results of a phase I/II study of a novel antiviral nucleotide analogue. J. Infect. Dis. **171:** 788-796.

Langley, D. R., A. W. Walsh, C. J. Baldick, B. J. Eggers, R. E. Rose, S. M. Levine, A. J. Kapur, R. J. Colonno, and D. J. Tenney. 2007. Inhibition of hepatitis B virus polymerase by entecavir. J. Virol. **81:** 3992-4001.

Laporte, M. G., R. W. Jackson, T. L. Draper, J. A. Gaboury, K. Galie, T. Herbertz, A. R. Hussey, S. R. Rippin, C. A. Benetatos, S. K. Chunduru, J. S. Christensen, G.

A. Coburn, C. J. Rizzo, G. Rhodes, J. O'Connell, A. Y. Howe, T. S. Mansour, M. S. Collett, D. C. Pevear, D. C. Young, T. Gao, D. L. Tyrrell, N. M. Kneteman, C. J. Burns, and S. M. Condon. 2008. The discovery of pyrano[3,4-*b*]indole-based allosteric inhibitors of HCV NS5B polymerase with in vivo activity. ChemMedChem **3:** 1508-1515.

Le Grice, S.F.J. 1993. Human immunodeficiency virus reverse transcriptase, p. 163-191. *In* A. M. Skalka, S. P. Goff (eds.), Reverse Transcriptase. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Lee, G., D. E. Piper, Z. Wang, J. Anzola, J. Powers, N. Walker, and Y. Li. 2006. Novel inhibitors of hepatitis C virus RNA-dependent RNA polymerases. J. Mol. Biol. **357:** 1051-1057.

Levine, S., D. Hernandez, G. Yamanaka, S. Zhang, R. Rose, S. Weinheimer, and R. J. Colonno. 2002. Efficacies of entecavir against lamivudine-resistant hepatitis B virus replication and recombinant polymerases in vitro. Antimicrob. Agents Chemother. **46:** 2525-2532.

Lewis-Jones, S. 2004. Zoonotic poxvirus infections in humans. Curr. Opin. Infect. Dis. **17:** 81-89.

Loeb, L. A., J. M. Essigmann, F. Kazazi, J. Zhang, K. D. Rose, and J. I. Mullins. 1999. Lethal mutagenesis of HIV with mutagenic nucleoside analogs. Proc. Natl. Acad. Sci. USA **96:** 1492-1497.

Love, R. A., H. E. Parge, X. Yu, M. J. Hickey, W. Diehl, J. Gao, H. Wriggers, A. Ekker, L. Wang, J. A. Thomson, P. S. Dragovich, and S. A. Fuhrman. 2003. Crystallographic identification of a noncompetitive inhibitor binding site on the hepatitis C virus NS5B RNA polymerase enzyme. J. Virol. **77:** 7575-7581.

Manns, M. P., G. R. Foster, J. K. Rockstroh, S. Zeuzem, F. Zoulim, and M. Houghton. 2007. The way forward in HCV treatment - finding the right path. Nat. Rev. Drug Discov. **6:** 991-1000.

Martin, J. L., C. E. Brown, N. Matthews-Davis, and J. E. Reardon. 1994. Effects of antiviral nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis. Antimicrob. Agents Chemother. **38:** 2743-2749.

Masur, H., M. A. Michelis, J. B. Greene, I. Onorato, R. A. Stouwe, R. S. Holzman, G. Wormser, L. Brettman, M. Lange, H. W. Murray, and S. Cunningham-Rundles. 1981. An outbreak of community-acquired *Pneumocystis carinii* pneumonia: initial manifestations of cellular immune dysfunction. New Engl. J. Med. **305:** 1431-1438.

McCormick, J. B., I. J. King, P. A. Webb, C. L. Scribmer, R. B. Craven, K. M.

Johnson, L. H. Elliott, and R. Belmont-Williams. 1986. Lassa fever. Effective therapy with ribavirin. New Engl. J. Med. **314:** 20-26.

McDonald, W. F., V. Crozel-Goudot, and P. Traktman. 1992. Transient expression of the vaccinia virus DNA polymerase is an intrinsic feature of the early phase of infection and is unlinked to DNA replication and late gene expression. J. Virol. **66:** 534-547.

McDonald, W. F., and P. Traktman. 1994a. Overexpression and purification of the vaccinia virus DNA polymerase. Protein Expr. Purif. **5:** 409-421.

McDonald, W. F., and P. Traktman. 1994b. Vaccinia virus DNA polymerase: *in vitro* analysis of parameters affecting processivity. J. Biol. Chem. **269:** 31190-31197.

McMahon, M. A., B. L. Jilek, T. P. Brennan, L. Shen, Y. Zhou, M. Wind-Rotolo, S. Xing, S. Bhat, B. Hale , R. Hegarty, C. R. Chong, J. O. Liu, R. F. Siliciano, and C. L. Thio. 2007. The HBV drug entecavir - effects on HIV-1 replication and resistance. New Engl. J. Med. **356:** 2614-2621.

Meadows, K. P., S. K. Tyring, A. T. Pavia, and T. M. Rallis. 1997. Resolution of recalcitrant molluscum contagiosum virus lesions in human immunodeficiency virus-infected patients treated with cidiofovir. Arch. Dermatol. **133:** 987-990.

Merta, A., I. Votruba, J. Jindřich, A. Holý, T. Cihlář, I. Rosenberg, M. Otmar, and T. Y. Herve. 1992. Phosphorylation of 9-(2-phosphonomethoxyethyl)adenine and 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)adenine by AMP(dAMP) kinase from L1210 cells. Biochem. Pharmacol. **44:** 2067-2077.

Merta, A., I. Votruba, I. Rosenberg, M. Otmar, H. Hřebabecký, R. Bernaerts, and A. Holý. 1990. Inhibition of herpes simplex virus DNA polymerase by diphosphates of acyclic phosphonylmethoxyalkyl nucleotide analogues. Antiviral Res. **13:** 209-218.

Meyer, H., M. Perrichot, M. Stemmler , P. Emmerich, H. Schmitz, F. Varaine, R. Shungu, F. Tshioko, and P. Formenty. 2002. Outbreaks of disease suspected of being due to human monkeypox virus infection in the Democratic Republic of Congo in 2001. J. Clin. Microbiol. **40:** 2919-2921.

Migliaccio, G., J. E. Tomassini, S. S. Carroll, L. Tomei, S. Altamura, B. Bhat, L. Bartholomew, M. R. Bosserman, A. Ceccacci, L. F. Colwell, R. Cortese, R. De Francesco, A. B. Eldrup, K. L. Getty, X. S. Hou, R. L. LaFemina, S. W. Ludmerer, M. MacCoss, D. R. McMasters, M. W. Stahlhut, D. B. Olsen, D. J. Hazuda, and O. A. Flores. 2003. Characterization of resistance to non-obligate chain-terminating ribonucleoside analogs that inhibit hepatitis C virus replication *in vitro*. J. Biol. Chem. **278:** 49164-49170.

Mitsuya, H., and S. Broder. 1986. Inhibition of the *in vitro* infectivity and cytopathic

effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. Proc. Natl. Acad. Sci. USA **83:** 1911-1915.

Mitsuya, H., R. F. Jarrett, M. Matsukura, F. Di Marzo Veronese, A. L. DeVico, M. G. Sarngadharan, D. G. Johns, M. S. Reitz, and S. Broder. 1987. Long-term inhibition of human T-lymphotropic virus type III/lymphadenopathy-associated virus (human immunodeficiency virus) DNA synthesis and RNA expression in T cells protected by 2',3'-dideoxynucleosides *in vitro*. Proc. Natl. Acad. Sci. USA **84:** 2033-2037.

Mitsuya, H., K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. N. Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry, and S. Broder. 1985. 3'-Azido-3'-deoxythymidine (BW A509U): An antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. Proc. Natl. Acad. Sci. USA **82:** 7096-7100.

Monsur, K. A., M. S. Hossain, F. Huq, M. M. Rahaman, and M. Q. Haque. 1975. Treatment of variola major with cytosine arabinoside. J. Infect. Dis. **131:** 40-43.

Moss, B. 2007. *Poxviridae:* The viruses and their replication, p. 2905-2945. *In* D. M. Knipe, P. M. Howley (eds.), Fields Virology, fifth edition. Lippincott Williams & Wilkins, a Wolters Kluwer Business, Philadelphia.

Mul, Y. M., R. T. van Miltenburg, E. De Clercq, and P. C. van der Vliet. 1989. Mechanism of inhibition of adenovirus DNA replication by the acyclic nucleoside triphosphate analogue (*S*)-HPMPApp: influence of the adenovirus DNA binding protein. Nucleic Acids Res. **17:** 8917-8929.

Murakami, E., A. Basavapathruni, W. D. Bradley, and K. S. Anderson. 2005. Mechanism of action of a novel viral mutagenic covert nucleotide: molecular interations with HIV-1 reverse transcriptase and host cell DNA polymerases. Antiviral Res. **67:** 10-17.

Neyts, J., and E. De Clercq. 2001. Efficacy of 2-amino-7-(1,3-dihydroxy-2-propoxymethyl)purine for treatment of vaccinia virus (orthopoxvirus) infections in mice. Antimicrob. Agents Chemother. **45:** 84-87.

Neyts, J., E. Verbeken, and E. De Clercq. 2002. Effect of 5-iodo-2'-deoxyuridine on vaccinia virus (orthopoxvirus) infections in mice. Antimicrob. Agents Chemother. **46:** 2842-2847.

Nyanguile, O., F. Pauwels, W. Van den Broeck, C. W. Boutton, L. Quirynen, T. Ivens, L. van der Helm, G. Vandercruyssen, W. Mostmans, F. Delouvroy, P. Dehertogh, M. D. Cummings, J. F. Bonfanti, K. A. Simmen, and P. Raboisson. 2008.

1,5-benzodiazepines, a novel class of hepatitis C virus polymerase nonnucleoside inhibitors. Antimicrob. Agents Chemother. **52:** 4420-4431.

Oien, N. L., R. J. Brideau, T. A. Hopkins, J. L. Wieber, M. L. Knechtel, J. A. Shelly, R. A. Anstadt, P. A. Wells, R. A. Poorman, A. Huang, V. A. Vaillancourt, T. L. Clayton, J. A. Tucker, and M. W. Wathen. 2002. Broad-spectrum antiherpes activities of 4-hydroxyquinoline carboxamides, a novel class of herpesvirus polymerase inhibitors. Antimicrob. Agents Chemother. **46:** 724-730.

Painter, G. R., and K. Y. Hostetler. 2004. Design and development of oral drugs for the prophylaxis and treatment of smallpox infection. Trends Biotechnol. **22:** 423-427.

Palú, G., S. Stefanelli, M. Rassu, C. Parolin, J. Balzarini, and E. De Clercq. 1991. Cellular uptake of phosphonylmethoxyalkylpurine derivatives. Antiviral Res. **16:** 115-119.

Parker, W. B. 2005. Metabolism and antiviral activity of ribavirin. Virus Res. **107:** 165-171.

Parrino, J., and B. S. Graham. 2006. Smallpox vaccines: past, present, and future. J. Allergy Clin. Immunol. **118:** 1320-1326.

Pastor-Anglada, M., M.-A. M. Cano-Solado P. , M. P. Lostao, I. Larráyoz, J. Martínez-Picado, and F. J. Casado. 2005. Cell entry and export of nucleoside analogues. Virus Res. **107:** 151-164.

Pata, J. D., W. G. Stirtan, S. W. Goldstein, and T. A. Steitz. 2004. Structure of HIV-1 reverse transcriptase bound to an inhibitor active against mutant reverse transcriptases resistant to other nonnucleoside inhibitors. Proc. Natl. Acad. Sci. USA **101:** 10548-10553.

Pauwels, R., J. Balzarini, D. Schols, M. Baba, J. Desmyter, I. Rosenberg, A. Holy, and E. De Clercq. 1988. Phosphonomethoxyethyl purine derivatives, a new class of anti-human immunodeficiency virus agents. Antimicrob. Agents Chemother. **32:** 1025-1030.

Prasad, V.R. 1993. Genetic analysis of retroviral reverse transcriptase structure and function, p. 135-162. *In* A. M. Skalka, S. P. Goff (eds.), Reverse Transcriptase. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Prichard, M. N., K. A. Keith, D. C. Quenelle, and E. R. Kern. 2006. Activity and mechanism of action of *N*-methanocarbathymidine against herpesvirus and orthopoxvirus infections. Antimicrob. Agents Chemother. **50:** 1336-1341.

Rao, A. R., J. A. McFadzean, and K. Kamalakshi. 1966. An isothiazole thiosemicarbazone in the treatment of variola major in man. A controlled clinical trial and

laboratory investigations. Lancet **287:** 1068-1072.

Reardon, J. E. 1989. Herpes simplex virus type 1 and human DNA polymerase interactions with 2'-deoxyguanosine 5'-triphosphate analogues: kinetics of incorporation into DNA and induction of inhibition. J. Biol. Chem. **264:** 19039-19044.

Reardon, J. E., and T. Spector. 1989. Herpes simplex virus type 1 DNA polymerase: mechanism of inhibition by acyclovir triphosphate. J. Biol. Chem. **264:** 7405-7411.

Reid, R., E.-C. Mar, E.-S. Huang, and M. D. Topal. 1988. Insertion and extension of acyclic, dideoxy, and ara nucleotides by herpesviridae, human α and human β polymerases. J. Biol. Chem. **263:** 3898-3904.

Rittinger, K., G. Divita, and R. S. Goody. 1995. Human immunodeficiency virus reverse transcriptase substrate-induced conformational changes and the mechanism of inhibition by nonnucleoside inhibitors. Proc. Natl. Acad. Sci. USA **92 :** 8046-8049.

Robbins, B. L., J. Greenhaw, M. C. Connelly, and A. Fridland. 1995. Metabolic pathways for activation of the antiviral agent 9-(2-phosphonylmethoxyethyl)adenine in human lymphoid cells. Antimicrob. Agents Chemother. **39:** 2304-2308.

Sauerbrei, A., C. Meier, A. Meerbach, and P. Wutzler. 2006. Inhibitory efficacy of *cyclo*Sal-nucleoside monophosphates of aciclovir and brivudin on DNA synthesis of orthopoxviruses. Antiviral Chem. Chemother. **17:** 25-31.

Seifer, M., R. K. Hamatake, R. J. Colonno, and D. N. Standring. 1998. In vitro inhibition of hepadnavirus polymerases by the triphosphates of BMS-200475 and lobucavir. Antimicrob. Agents Chemother. **42:** 3200-3208.

Severini, A., X.-Y. Liu, J. S. Wilson, and D. L. J. Tyrrell. 1995. Mechanism of inhibition of duck hepatitis B virus polymerase by (-)-β-L-2',3'-dideoxy-3'-thiacytidine. Antimicrob. Agents Chemother. **39:** 1430-1435.

Sidwell, R. W., J. H. Huffman, G. P. Khare, L. B. Allen, J. T. Witkowski, and R. K. Robins. 1972. Broad-spectrum antiviral activity of Virazole: 1-β–D-ribofuranosyl-1,2,4-triazole-3-carboxamide. Science **177:** 705-706.

Siegal, F. P., C. Lopez, G. S. Hammer, A. E. Brown, S. J. Kornfeld, J. Gold, J. Hassett, S. Z. Hirschman, C. Cunningham-Rundles, B. R. Adelsberg, D. M. Parham, M. Siegal, S. Cunningham-Rundles, and D. Armstrong. 1981. Severe acquired immunodeficiency in male homosexuals, manifested by chronic perianal ulcerative herpes simplex lesions. New Engl. J. Med. **305:** 1439-1444.

Silverman, J. E. Y., M. Ciustea, A. M. D. Shudofsky, F. Bender, R. H. Shoemaker,

and R. P. Ricciardi. 2008. Identification of polymerase and processivity inhibitors of vaccinia DNA synthesis using a stepwise screening approach. Antiviral Res. **80:** 114-123.

Sluis-Cremer, N., N. A. Temiz, and I. Bahar. 2004. Conformational changes in HIV-1 reverse transcriptase induced by nonnucleoside reverse transcriptase inhibitor binding. Curr. HIV Res. **2:** 323-332.

Smee, D. F., R. W. Sidwell, D. Kefauver, M. Bray, and J. W. Huggins. 2002. Characterization of wild-type and cidofovir-resistant strains of camelpox, cowpox, monkeypox, and vaccinia viruses. Antimicrob. Agents Chemother. **46:** 1329-1335.

Smeijsters, L. J. J. W., F. F. J. Franssen, L. Naesens, E. de Vries, A. Holý, J. Balzarini, E. de Clercq, and J. P. Overdulve. 1999. Inhibition of the in vitro growth of *Plasmodium falciparum* by acyclic nucleoside phosphonates. Int. J. Antimicrob. Agents **12:** 53-61.

Smeijsters, L. J. J. W., H. Nieuwenhuijs, R. C. Hermsen, G. M. Dorrestein, F. F. J. Franssen, and J. P. Overdulve. 1996. Antimalarial and toxic effects of the acyclic nucleoside phosphonate *(S)*-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine in *Plasmodium berghei*-infected mice. Antimicrob. Agents Chemother. **40:** 1584-1588.

Smeijsters, L. J. J. W., N. M. Zijlstra, E. de Vries, F. F. J. Franssen, C. J. Janse, and J. P. Overdulve. 1994. The effect of (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine on nuclear and organellar DNA synthesis in erythrocytic schizogony in malaria. Mol. Biochem. Parasitol. **67:** 115-124.

Smeijsters, L. J. J. W., N. M. Zijlstra, J. Veenstra, B. E. Verstrepen, C. Heuvel, J. P. Overdulve, and E. de Vries. 2000. *Plasmodium falciparum* clones resistant to (*S*)-9-(3-hydroxy-2-phosphonylmethoxy-propyl)adenine carry amino acid substitutions in DNA polymerase δ. Mol. Biochem. Parasitol. **106:** 175-180.

Smith, S. K., V. A. Olson, K. L. Karem, R. Jordan, D. E. Hruby, and I. K. Damon. 2009. In vitro efficacy of ST246 against smallpox and monkeypox. Antimicrob. Agents Chemother. **53:** 1007-1012.

Soul-Lawton, J., E. Seaber, N. On, R. Wootton, P. Rolan, and J. Posner. 1995. Absolute bioavailability and metabolic disposition of valaciclovir, the L-valyl ester of acyclovir, following oral administration to humans. Antimicrob. Agents Chemother. **39:** 2759-2764.

Spence, R. A., W. M. Kati, K. S. Anderson, and K. A. Johnson. 1995. Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. Science **267:** 988-993.

Stanitsa, E. S., L. Arps, and P. Traktman. 2006. Vaccinia virus uracil DNA glycosylase interacts with the A20 protein to form a heterodimeric processivity factor for the viral DNA polymerase. J. Biol. Chem. **281:** 3439-3451.

Streeter, D. G., J. T. Witkowski, G. P. Khare, R. W. Sidwell, R. J. Bauer, R. K. Robins, and L. N. Simon. 1973. Mechanism of action of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent. Proc. Natl. Acad. Sci. USA **70:** 1174-1178.

Sudo, K., Y. Miyazaki, N. Kojima, M. Kobayashi, H. Suzuki, M. Shintani , and Y. Shimizu. 2005. YM-53403, a unique anti-respiratory syncytial virus agent with a novel mechanism of action. Antiviral Res. **65:** 125-131.

Sullivan, V., C. L. Talarico, S. C. Stanat, M. Davis, D. M. Coen, and K. K. Biron. 1992. A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature **358:** 162-164.

Summers, J., and S. Litwin. 2006. Examining the theory of error catastrophe. J. Virol. **80:** 20-26.

Tchesnokov, E. P., A. Obikhod, R. F. Schinazi, and M. Götte. 2008. Delayed chain termination protects the anti-hepatitis B virus drug entecavir from excision by HIV-1 reverse transcriptase. J. Biol. Chem. **283:** 34218-34228.

Tomei, L., S. Altamura, L. Bartholomew, A. Biroccio, A. Ceccacci, L. Pacini, F. Narjes, N. Gennari, M. Bisbocci, I. Incitti, L. Orsatti, S. Harper, I. Stansfield, M. Rowley, R. De Francesco, and G. Migliaccio. 2003. Mechanism of action and antiviral activity of benzimidazole-based allosteric inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. J. Virol. **77:** 13225-13231.

Toro, J. R., L. V. Wood, N. K. Patel, and M. L. Turner. 2000. Topical cidofovir: a novel treatment for recalcitrant molluscum contagiosum in children infected with human immunodeficiency virus 1. Arch. Dermatol. **136:** 983-985.

U.S. Food and Drug Administration. 1996. FDA approves cidofovir for AIDS-related retinitis. URL: www.fda.gov/bbs/topics/ANSWERS/AN00743.html. Downloaded April 21, 2009.

Veselý, J., A. Merta, I. Votruba, I. Rosenberg, and A. Holý. 1990. The cytostatic effects and mechanism of action of antiviral acyclic adenine nucleotide analogs in L1210 mouse leukemia cells. Neoplasma **37:** 105-110.

Votruba, I., R. Bernaerts, T. Sakuma, E. De Clercq, A. Merta, I. Rosenberg, and A. Holý. 1987. Intracellular phosphorylation of broad-spectrum anti-DNA virus agent (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine and inhibition of viral DNA synthesis.

Mol. Pharmacol. **32:** 524-529.

Wachsman, M., B. G. Petty, K. C. Cundy, H. S. Jaffe, P. E. Fisher, A. Pastelak, and P. S. Lietman. 1996. Pharmacokinetics, safety and bioavailability of HPMPC (cidofovir) in human immunodeficiency virus-infected subjects. Antiviral Res. **29:** 153-161.

Wang, L. Z., G. L. Kenyon, and K. A. Johnson. 2004. Novel mechanism of inhibition of HIV-1 reverse transcriptase by a new non-nucleoside analog, KM-1. J. Biol. Chem. **279:** 38424-38432.

Wang, M., K. K. S. Ng, M. M. Cherney , L. Chan, C. G. Yannopoulos, J. Bedard, N. Morin, N. Nguyen-Ba, M. H. Alaoui-Ismaili, R. C. Bethell , and M. N. G. James. 2003. Non-nucleoside analogue inhibitors bind to an allosteric site on HCV NS5B polymerase. Crystal structures and mechanism of inhibition. J. Biol. Chem. **278:** 9489-9495.

Weston, B., and G. Storch. 2003. Treatment of orf poxvirus lesion with cidofovir cream. Pediatr. Infect. Dis. J. **22:** 1027-1028.

White, L. K., J.-J. Yoon, J. K. Lee, A. Sun, Y. Du, H. Fu, J. P. Snyder, and R. K. Plemper. 2007. Nonnucleoside inhibitor of measles virus RNA-dependent RNA polymerase complex activity. Antimicrob. Agents Chemother. **51:** 2293-2303.

Whitley, R. J. 2003. Smallpox: a potential agent of bioterrorism. Antiviral Res. **57:** 7-12.

Willer, D. O., M. J. Mann, W. Zhang, and D. H. Evans. 1999. Vaccinia virus DNA polymerase promotes DNA pairing and strand-transfer reactions. Virology **257:** 511-523.

World Health Organization. 1980. Declaration of global eradication of smallpox. Wkly. Epidemiol. Rec. **55:** 148.

Wyles, D. L., K. A. Kaihara, B. E. Korba, R. T. Schooley, J. R. Beadle, and K. Y. Hostetler. 2009. ODE-(S)-HPMPA is a potent and selective inhibitor of hepatitis C virus replication in genotype 1A, 1B and 2A replicons. Antimicrob. Agents Chemother. **53:** 2660-2662.

Xia, Q., J. Radzio, K. S. Anderson, and N. Sluis-Cremer. 2007. Probing nonnucleoside inhibitor-induced active-site distortion in HIV-1 reverse transcriptase by transient kinetic analysis. Protein Sci. **16:** 1728-1737.

Xiong, X., C. Flores, H. Yang, J. J. Toole, and C. S. Gibbs. 1998. Mutations in hepatitis B DNA polymerase associated with resistance to lamivudine do not confer resistance to adefovir *in vitro*. Hepatology **28:** 1669-1673.

Xiong, X., J. L. Smith, and M. S. Chen. 1997. Effect of incorporation of cidofovir into

DNA by human cytomegalovirus DNA polymerase on DNA elongation. Antimicrob. Agents Chemother. **41:** 594-599.

Xiong, X., J. L. Smith, C. Kim, E. Huang, and M. S. Chen. 1996. Kinetic analysis of the interaction of cidofovir diphosphate with human cytomegalovirus DNA polymerase. Biochem. Pharmacol. **51:** 1563-1567.

Yang, G., D. C. Pevear, M. H. Davies, M. S. Collett, T. Bailey, S. Rippen, L. Barone, C. Burns, G. Rhodes, S. Tohan, J. W. Huggins, R. O. Baker, R. L. M. Buller, E. Touchette, K. Waller, J. Schriewer, J. Neyts, E. DeClercq, K. Jones, D. Hruby, and R. Jordan. 2005. An orally bioavailable antipoxvirus compound (ST-246) inhibits extracellular virus formation and protects mice from lethal orthopoxvirus challenge. J. Virol. **79:** 13139-13149.

Yao, X.-D., and D. H. Evans. 2001. Effects of DNA structure and homology length on vaccinia virus recombination. J. Virol. **75:** 6923-6932.

Yokota, T., K. Konno, E. Chonan, S. Mochizuki, K. Kojima, S. Shigeta, and E. De Clercq. 1990a. Comparative activities of several nucleoside analogs against duck hepatitis B virus in vitro. Antimicrob. Agents Chemother. **34:** 1326-1330.

Yokota, T., S. Mochizuki, K. Konno, S. Mori, S. Shigeta, and E. De Clercq. 1990b. Phosphonylmethoxyalkyl derivatives of purine as inhibitors of human hepatitis B virus DNA synthesis. Nucleic Acids Symp. Ser. 17-18.

Yoshizaki, T., N. Wakisaka, S. Kondo , S. Murono, Y. Shimizu, M. Nakashima, A. Tsuji, and M. Furukawa. 2008. Treatment of locally recurrent Epstein-Barr virus-associated nasopharyngeal carcinoma using the anti-viral agent cidofovir. J. Med. Virol. **80:** 879-882.

Zabawski, E. J., and C. J. Cockerell. 1999. Topical cidofovir for molluscum contagiosum in children. Pediatr. Dermatol. **16:** 414-415.

CHAPTER TWO

MECHANISM OF INHIBITION OF VACCINIA VIRUS DNA POLYMERASE BY CIDOFOVIR DIPHOSPHATE[1,2]

Wendy C. Magee, Karl Y. Hostetler and David H. Evans

2.1 INTRODUCTION

Poxviruses are large, double-stranded DNA viruses that replicate in the cytoplasm of infected cells. Members of this virus family can cause severe infections, including human smallpox. Smallpox was declared eradicated in 1980 (World Health Organization, 1980), but concerns over bioterrorism (Henderson *et al.*, 1999; Whitley, 2003) and a recent outbreak of monkeypox in the midwestern United States (Lewis-Jones, 2004), illustrate some need for the continued development of effective new treatment regimens. A number of new treatments are currently the subject of active investigation including immunotherapy. However, antiviral drugs offer a combination of chemical stability and simplicity of delivery that is especially attractive from a public health perspective.

One class of drugs that have been shown to inhibit poxvirus replication are the nucleoside phosphonate analogs of cellular deoxyribonucleotides that were developed by De Clercq *et al.* (1986). These drugs have been shown to be effective against a wide range of DNA viruses and retroviruses (reviewed in De Clercq, 1997) and one of these compounds, *(S)*-1-[3-hydroxy-2-(phosphonylmethoxypropyl]cytosine, also known as

---

[1] A version of this chapter has been published in Antimicrobial Agents and Chemotherapy (2005), 49(8): 3153-3162. *Copyright 2005. American Society for Microbiology.*

[2] This study was supported by operating and equipment grants from the Alberta Heritage Foundation for Medical Research (AHFMR), the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council of Canada (NSERC) to Dr. D. H. Evans. Dr. K. Y. Hostetler was supported by National Eye Institute (NEI) grant EY11834, National Institute of Allergy and Infectious Diseases (NIAID) grant AI29164, and Department of the Army grant DAMD17-01-2-007. The U.S. Army Medical Research Acquisition Activity, Fort Detrick, MD, was the awarding acquisition office. W. C. Magee is the recipient of a University of Alberta Ph.D. scholarship.

The content of this article does not necessarily reflect the position or policy of the U.S. Government, and no official endorsement should be inferred.

cidofovir (CDV), has been granted Food and Drug Administration approval for the treatment of human cytomegalovirus (HCMV)-induced retinitis. CDV has been used off-label in the treatment of orf (Geerinck *et al.*, 2001) and molluscum contagiosum virus infections (Meadows *et al.*, 1997). It has also been shown to block the replication of variola and monkeypox viruses in culture (Baker *et al.*, 2003) and to protect mice from a lethal challenge dose of ectromelia, vaccinia, or cowpox virus (Bray *et al.*, 2000; Buller *et al.*, 2004; Quenelle *et al.*, 2004).

Unfortunately, CDV causes significant problems of nephrotoxicity and can only be administered by intravenous injection. These problems have been addressed by the Hostetler group who have shown that alkoxyglycerol or alkoxypropanediol esters of CDV and cyclic CDV are 50 to 230 times more active than CDV against vaccinia virus *in vitro* (Kern *et al.*, 2002) while also being orally bioavailable (Ciesla *et al.*, 2003). Further studies indicated that this family of CDV analogs also blocked the replication of variola, monkeypox, cowpox and ectromelia viruses *in vitro* (Buller *et al.*, 2004; Huggins *et al.*, 2002; Kern *et al.*, 2002), and when delivered orally protect mice from a lethal challenge with vaccinia, cowpox, or ectromelia viruses (Buller *et al.*, 2004; Quenelle *et al.*, 2004).

CDV is taken up into cells by fluid-phase endocytosis (Connelly *et al.*, 1993) whereas its alkoxy derivatives are more rapidly absorbed through a direct association with the lipid bilayer (Ciesla *et al.*, 2003). Regardless of the uptake route, the alkoxyester link (if present) is then hydrolyzed to yield CDV, which is then is phosphorylated in a two-step process to yield the active intracellular metabolite, CDV diphosphate (CDVpp). The phosphorylation of CDV is carried out by cellular enzymes and in the absence of viral infection (Cihlar and Chen, 1996). This property of CDV results in drug efficacy even against kinase-deficient or mutant viruses. Human DNA polymerases α, β, and γ also exhibit some natural resistance to CDVpp, thus partially explaining the antiviral specificity of the drug (Cherrington *et al.*, 1994; Ho *et al.*, 1992).

Previous studies have examined the effects of CDVpp on the activity of the HCMV DNA polymerase (Xiong *et al.*, 1997; Xiong *et al.*, 1996). This is a member of the B family of DNA polymerases and it possesses both 5′-to-3′ polymerase and 3′-to-5′ exonuclease activities encoded within a single polypeptide. These studies have shown

that CDVpp behaves as a dCTP analog, which HCMV DNA polymerase can incorporate into a growing polynucleotide chain opposite template G's (Xiong *et al.*, 1997; Xiong *et al.*, 1996). Under these circumstances, a single molecule of incorporated CDV somewhat retards further DNA synthesis, while adding two consecutive molecules of CDV opposite a pair of template G's causes a severe reduction or termination of elongation. In addition, a primer bearing a molecule of CDV at the 3′-terminus is refractory to the 3′-to-5′ exonuclease activity of the HCMV enzyme (Xiong *et al.*, 1997). These observations led Xiong *et al.* to suggest that a combination of these two enzymatic effects were responsible for the efficacy of CDV in HCMV-infected cells (Xiong *et al.*, 1997).

These observations also suggest a mechanism by which CDV could inhibit orthopoxvirus DNA replication since poxviruses similarly encode B family DNA polymerases. The best characterized of the poxvirus polymerases is the 116 kDa E9L gene product encoded by vaccinia virus (Earl *et al.*, 1986; McDonald and Traktman, 1994a; Taddie and Traktman, 1991). This enzyme can be produced in a recombinant form (McDonald and Traktman, 1994a) and serves as an excellent surrogate model for its variola virus homolog since the two enzymes differ by only 18 to 20 amino acids over a polypeptide spanning 1,006 amino acids. Vaccinia virus DNA polymerase normally forms part of a larger protein complex (Ishii and Moss, 2002; Klemperer *et al.*, 2001; McCraith *et al.*, 2000; Stanitsa *et al.*, 2006) and its processivity is modified by the A20R and D4R virus gene products (Klemperer *et al.*, 2001; Stanitsa *et al.*, 2006). However, it is nevertheless catalytically active in isolation and this permits the characterization of the substrate specificity. In this communication, we have used steady-state experimental methods and the purified form of vaccinia virus DNA polymerase to show that the effects of CDV on vaccinia DNA polymerase *in vitro* differ in important ways from the effects of the drug on betaherpesvirus DNA polymerases. These effects provide formal biochemical support for the contention that poxviral DNA polymerases are an enzymatic target of these drugs. These studies also provide a starting point for the characterization of viruses exhibiting acquired resistance to CDV (Andrei *et al.*, 2006; Becker *et al.*, 2008; Kornbluth *et al.*, 2006; Smee *et al.*, 2002).

2.2 MATERIALS AND METHODS

2.2.1 Chemicals. Cidofovir diphosphate (CDVpp) was synthesized by Trilink Biotechnologies, San Diego, CA. Radioactive nucleotides were purchased from Amersham Biosciences and unlabeled deoxynucleoside triphosphates (dNTPs) from Fermentas. Oligonucleotides were obtained from Sigma-Genosys.

2.2.2 Vaccinia virus DNA polymerase. Recombinant vaccinia virus DNA polymerase was purified from vaccinia-infected cells according to the protocol of McDonald and Traktman (McDonald and Traktman, 1994a) and appeared homogenous as judged by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) analysis and silver staining (Figure 2.1). The enzyme was stored at –20˚C in glycerol at a concentration of 230 ng/µl. For use in enzyme assays, the enzyme was freshly diluted in 25 mM potassium phosphate (pH 7.4), 5 mM β-mercaptoethanol, 1 mM EDTA, 10% [v/v] glycerol, and 0.1 mg/ml bovine serum albumin (New England Biolabs).

2.2.3 DNA polymerase and exonuclease assays. Different combinations of oligonucleotide primers and templates were used to create different enzymatic substrates (Figure 2.2). The primer strands were first end-labeled using T4 polynucleotide kinase (Fermentas) and [γ-^{32}P]ATP and the unincorporated nucleotides were removed using MicroSpin™ G-25 columns (Amersham Biosciences). Each 10-µl reaction contained one pmol of end-labeled primer (approximately 90,000 cpm) and 3 pmol of template strand in a solution containing 30 mM Tris · HCl (pH 7.9), 5 mM $MgCl_2$, 70 mM NaCl, 1.8 mM dithiothreitol, 80 µg/ml bovine serum albumin, and 0 to 25 ng of purified vaccinia DNA polymerase. Different concentrations of CDVpp and dNTPs were added as indicated. The primer and template strands were mixed, heated to 55°C, and allowed to cool slowly to room temperature prior to adding the remaining reagents. The reactions were incubated at 37°C and stopped by adding 5 µl of gel loading buffer [80% (v/v) formamide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanole FF, 1 mg/ml bromophenol blue]. The reaction products were separated using 10% denaturing polyacrylamide sequencing gels run at 45

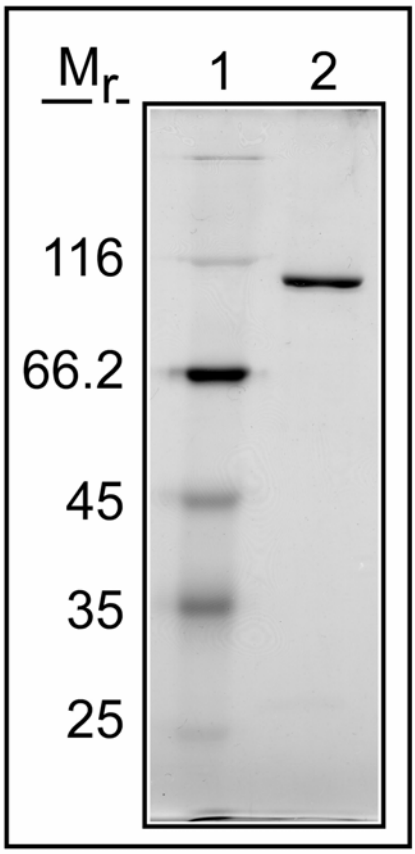

Figure 2.1 The purity of the recombinant vaccinia virus DNA polymerase used in these experiments. About 230 ng of vaccinia virus DNA polymerase was subjected to SDS-PAGE analysis and the gel stained with silver. Unstained protein molecular weight marker (Fermentas) is shown in lane 1.

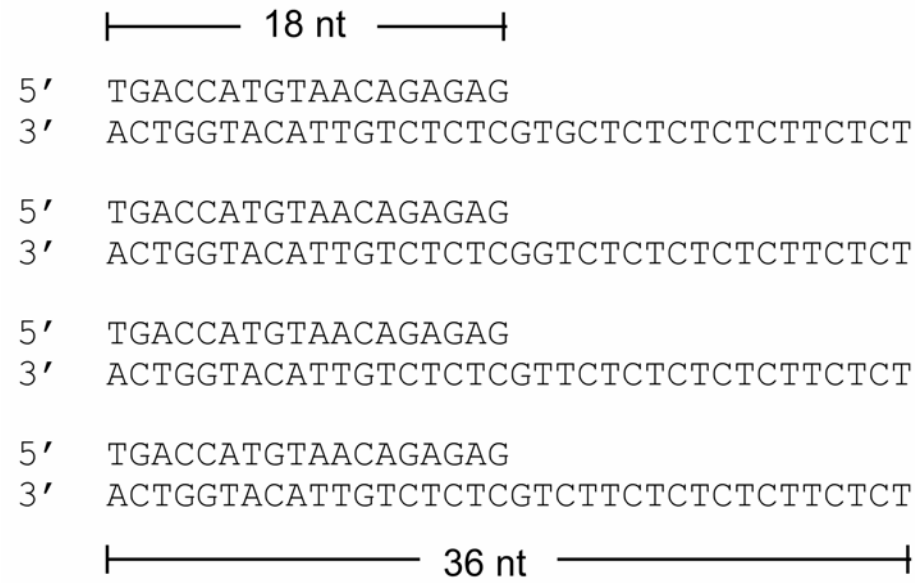

Figure 2.2 Oligonucleotide primer-template pairs used in this study. The P1 primer and templates T1, T2, and T3 were originally described by Xiong *et al.* (1997). Each primer strand was 5′ end-labeled with ^{32}P prior to its annealing with a template strand. The melting point under the salt conditions used in this study (70 mM NaCl, 5 mM $MgCl_2$) is estimated to exceed 70°C.

W for 45 minutes in half-strength Tris-borate-EDTA (Sambrook and Russell, 2001). The gels were fixed in a solution containing 10% [v/v] methanol plus 10% [v/v] acetic acid and dried under vacuum. The dried gels were exposed to phosphorimager screens (Amersham Biosciences) and the images analyzed using a Storm 840 or a Typhoon 8600 Phosphorimager (Amersham Biosciences) with ImageQuant software (v. 5.1).

The Klenow fragment of *Escherichia coli* DNA polymerase I (Fermentas) and dideoxy sequencing reactions were used to generate size standards (Sambrook and Russell, 2001). The relative concentrations of each ddNTP:dNTP pair were adjusted as needed to generate a suitable distribution of marker fragments.

2.2.4 Determination of the K_i value for CDVpp. A filter-based DNA polymerase assay was used to determine the K_i value for CDVpp. Three sets of reactions were prepared, each examining a different CDVpp concentration: 1 μM, 2 μM and 4 μM. Each 100-μl reaction was performed in triplicate and contained 1.67 μg of activated calf thymus DNA (Amersham Biosciences), polymerase buffer [30 mM Tris · HCl (pH 7.9), 5 mM $MgCl_2$, 70 mM NaCl, 1.8 mM dithiothreitol, 80 μg/ml bovine serum albumin], 10 μM (each) dATP, dGTP and dTTP, 0.2 μl of [α^{32}P]dCTP, varying concentrations of dCTP and CDVpp, and 100 ng of vaccinia virus DNA polymerase. The dCTP concentrations in each set of reactions were 0.5 μM, 0.67 μM, 1 μM, 2 μM and 4 μM. The reactions were incubated at 37°C for 10 min and stopped with 200 μl of sodium pyrophosphate, followed by the addition of 200 μg of calf thymus DNA (Sigma) and 600 μl of trichloroacetic acid. After incubation on ice for 5 min, 3 ml of acid-pyro (1 N HCl, 0.1 M sodium pyrophosphate) were added and the solution filtered through glass fiber filters (Schleicher & Schuell), presoaked in 0.1 M sodium pyrophosphate. The filters were washed with acid-pyro and 95% ethanol, allowed to dry, and counted in a Beckman LS 6500 liquid scintillation counter using CytoScint ES™ liquid scintillation fluid (MP Biomedicals). The number of pmol of dCTP incorporated and the velocity of dCTP incorporation for each reaction was calculated by determining the specific activity of dCTP. The data were plotted using a double reciprocal plot and a linear regression fit

using Prism software (v. 4.0b for Macintosh). The results are presented as the mean ± the standard error of the mean.

2.2.5 Determination of K_m and V_{max} values for CDVpp. Standard 10-µl reaction mixtures were prepared as described above (Section 2.2.3) containing ~8 pmol of ^{32}P-labeled primer P1, 32 pmol of template T3, 25 ng of vaccinia DNA polymerase, 10 µM dGTP, and various concentrations of dCTP or CDVpp. After incubation for 0 to 16 min at 37°C, the reactions were stopped and the amount of primer molecules extended by one nucleotide was determined by PAGE and phosphorimager analysis. Initial primer extension rates were calculated for each CDVpp or dCTP concentration and K_m and V_{max} determined from a nonlinear regression fit of these data to the Michaelis-Menten equation. Prism software (v. 4.0b for Macintosh) was used to perform all the curve fits from which the relevant kinetic parameters were obtained. The results are presented as the mean ± the standard error of the mean.

2.3 RESULTS

2.3.1 Adding CDVpp to DNA polymerase reactions promotes chain termination. As a first step in determining the effect of CDVpp on vaccinia virus DNA polymerase, we performed a simple analysis of the rate of primer extension in the absence of any drug. These assays contained 10 µM each dATP, dGTP, and dTTP and 5 µM dCTP. The dNTP concentrations are similar to those estimated to exist *in vivo* in vaccinia virus-infected cells and vary from 2- to 10-fold greater than the calculated K_ms for each of the four nucleotides (Hendricks and Mathews, 1998; McDonald and Traktman, 1994a). Figure 2.3 shows the results of one such assay. Vaccinia virus DNA polymerase has been estimated to incorporate dNTPs at a rate in excess of 30 sec^{-1} under certain conditions (McDonald and Traktman, 1994b), and under our steady state conditions, the majority of the primers were extended out to near the end of the template strand in well under a minute (Figure 2.3, top panel). We noted that the enzyme could never fully extend the primer strand out to the terminus of the duplex and created instead an array of products terminated 1 to 3 nucleotides from the end of the template strand as judged by a

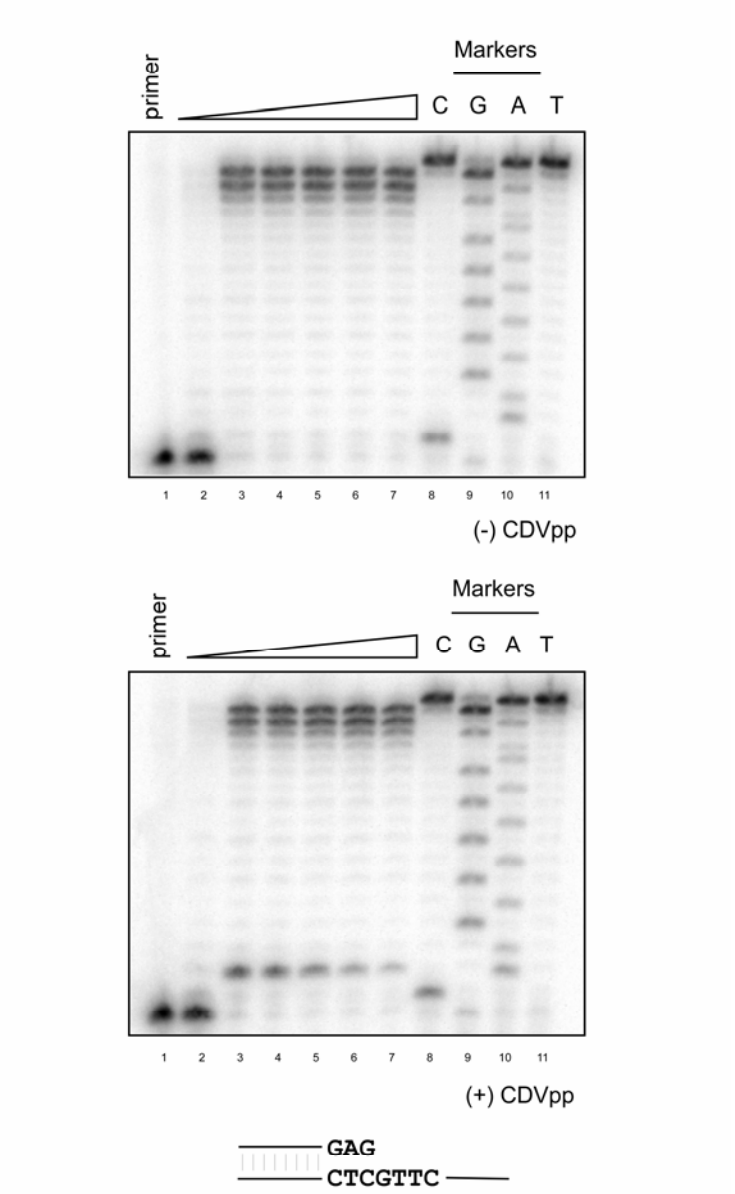

Figure 2.3 CDVpp promotes chain termination in reactions containing a mixture of CDVpp and dCTP. Both reaction mixtures contained ^{32}P-labeled primer P1, template T3, and all four dNTPs (10 μM each dATP, dGTP, and dTTP plus 5 μM dCTP). The reaction mixture analyzed in the lower panel was further supplemented with 10 μM CDVpp. The reactions were started by adding vaccinia virus DNA polymerase, and the mixtures were incubated at 37°C. Sampling was conducted over a time scale ranging from 0 to 15 min, and each reaction was stopped by adding gel loading buffer on ice. The products were subjected to PAGE analysis, and ^{32}P-labeled molecules were detected by phophorimaging. Note the accumulation of premature termination products in the lower panel. Comparison with dideoxy sequencing ladders (at right) shows that these reaction products are terminated 1 nucleotide past the point where CDV would expect to be incorporated.

comparison with the four sequencing lanes (Figure 2.3). Small amounts of other partially extended strands were also sometimes detected at early times in the reaction. None of these effects were artifacts of the templates used in these studies and appear to be characteristic features of the nonprocessive form of vaccinia virus DNA polymerase. For example, a different polymerase (Klenow enzyme) extended essentially all of the primer strands out to the end of the template in reaction mixtures containing ddTTP (Figure 2.3). [Note that the P1-T3 primer-template pair which was used in this reaction mixture contained no template dA's that could direct chain termination in the presence of ddTTP (Figure 2.2).]

In contrast, adding 10 μM CDVpp to these reaction mixtures caused the accumulation of a significant fraction of products (28% at 1 min) terminated two nucleotides beyond the end of the primer strand (Figure 2.3, bottom panel, lanes 3 to 7). When the size of these products is compared with the sequencing ladder it is apparent that these CDV-induced stops are located at position N + 1 where N is the site where a molecule of CDV is expected to be incorporated opposite a template dG (Figure 2.3). In contrast, strands that have avoided suffering this fate seem to have been extended out to the end of the template. It is important to note that this process does not irreversibly block DNA synthesis, because prematurely terminated molecules were slowly chased into full-length extension products over the 15-min time course of these experiments.

All of these studies were conducted using primer P1 annealed to template T3. To test whether these effects might be an idiosyncratic feature of this particular template-primer pair, we repeated the experiment using two additional oligonucleotide duplexes. The results of this experiment are shown in Figure 2.4. Adding CDVpp to reaction mixtures containing a P1-T1 primer-template combination inhibited further chain extension after the primer had been extended by either 2 or 4 nucleotides (Figure 2.4, lanes 2 and 3). Similarly, some termination was seen after primer P1 had been extended either 2 or 3 nucleotides when annealed to template T2 (Figure 2.4, lanes 8 and 9). In all three situations, these termination sites were located at positions N + 1 relative to the site where a template dGMP would direct the incorporation of CDV.

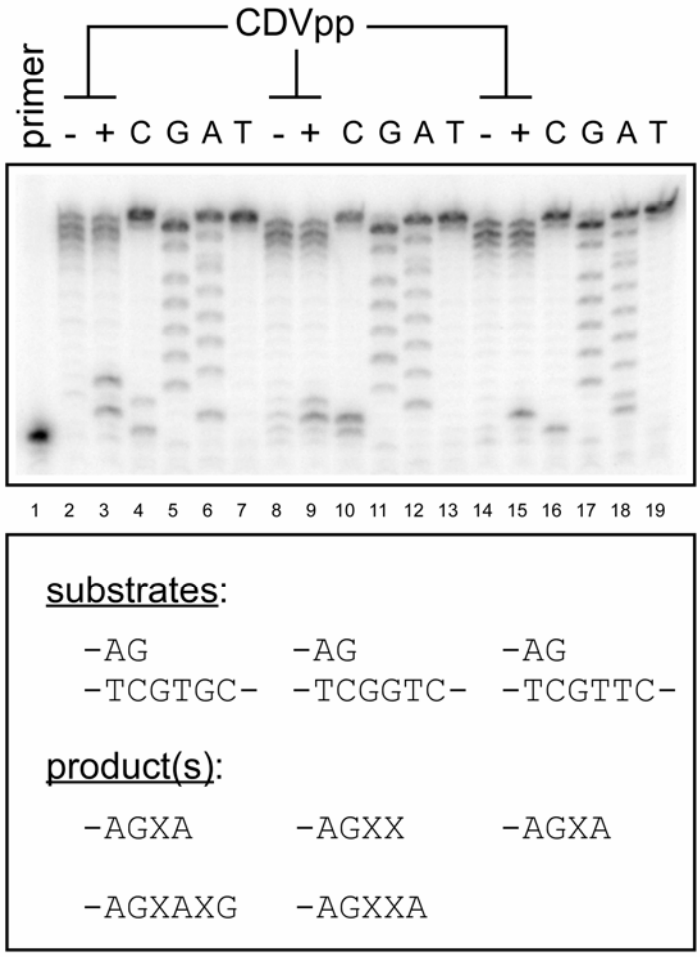

Figure 2.4 CDV promotes the same pattern of chain termination opposite dGMP in a variety of template contexts. Reactions were prepared containing the primer-template combinations shown in the lower panel, all four dNTPs, and ± 10 μM CDVpp as indicated. The six different reactions were then incubated for 10 min at 37°C and subjected to PAGE analysis followed by phosphorimaging. Dideoxy sequencing reactions were performed in parallel for each of the three different primer-template combinations. Comparison with these size standards suggests that all of the prematurely terminated molecules incorporate dC or CDV ("X", lower panel) plus one additional nucleotide residue at the 3′-end.

2.3.2 CDV can be incorporated into DNA. These preliminary studies suggested that CDVpp promotes the termination of DNA synthesis but are complicated by the mixture of dCTP, CDVpp, and other dNTPs used in the reaction mixtures. To test the suitability of CDVpp as a substrate for vaccinia virus DNA polymerase, we investigated the kinetics of incorporation of CDVpp versus dCTP using a combination of primer P1 and template T9. The reaction was supplied with only dCTP or CDVpp, so we expected the primer to be extended at most only 1 nucleotide beyond its 3′ end. The results of this experiment are shown in Figure 2.5. Under these reaction conditions, we could not detect any great differences between CDVpp and dCTP as substrates for vaccinia virus DNA polymerase, with some conversion of the P1 primer into a primer + 1 product occurring in both reaction mixtures even on ice. By the first time point (1 min) the primer was quantitatively converted into the primer + 1 product in both reaction mixtures (Figure 2.5, lanes 3). We also noted that by the 5-min time point, molecules terminated with CDV or dCMP were broken down again into much smaller labeled reaction products (Figure 2.5, lanes 5 to 7). Presumably, the 5′-to-3′ DNA polymerase and 3′-to-5′ proofreading exonuclease activities can consume all of the dNTPs in these reactions and, once this has happened, the proofreading exonuclease can then degrade the substrate down to a limit digest comprised of hexanucleotides and other smaller oligonucleotides. CDVpp is a substrate for the polymerase activity and CDV-terminated DNAs can be attacked by the proofreading exonuclease.

2.3.3 CDV-terminated primers are substrates for the vaccinia DNA polymerase. The pause sites detected in our primer extension assays containing CDVpp are located at positions N + 1 relative to the site of incorporation of CDV. This suggests that vaccinia virus DNA polymerase might encounter some difficulties utilizing CDV-terminated primers. To test this hypothesis, we first added dCMP or CDV residues to primer-template pair P1-T9 as described in Figure 2.5. After sampling the reaction mixture, we added dATP and compared the subsequent rate of chain extension. The results are shown in Figure 2.6. We noted that a dCMP-terminated primer was a good substrate for adding dAMP (Figure 2.6, top panel, lanes 3 to 8), although a dynamic interplay between 3′-to-5′

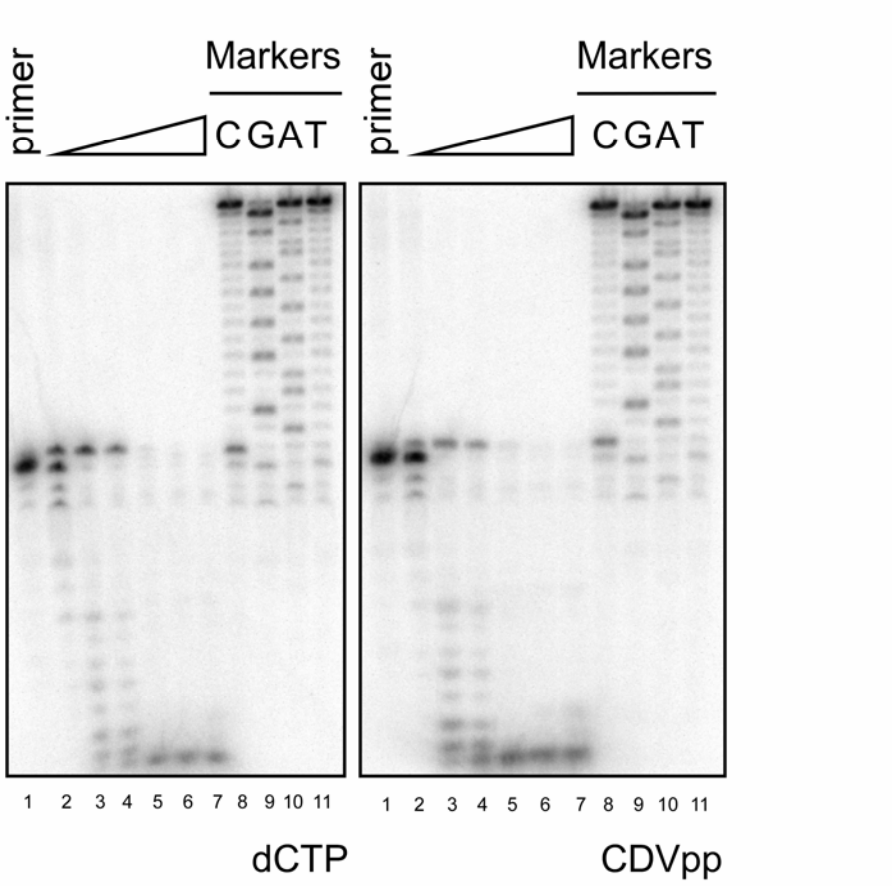

Figure 2.5 Kinetics of dCTP versus CDVpp incorporation. Primer P1 plus template T9 were incubated with vaccinia virus DNA polymerase and either 10 μM dCTP (left panel) or 10 μM CDVpp (right panel). The reaction mixtures were sampled at 0, 1, 2, 5, 10, and 15 min; the reactions were stopped; and then the mixtures were subjected to PAGE analysis. Both compounds are rapidly incorporated into DNA during the initial stages of the reaction; at later time points, the primers are degraded into molecules as small as hexanucleotides.

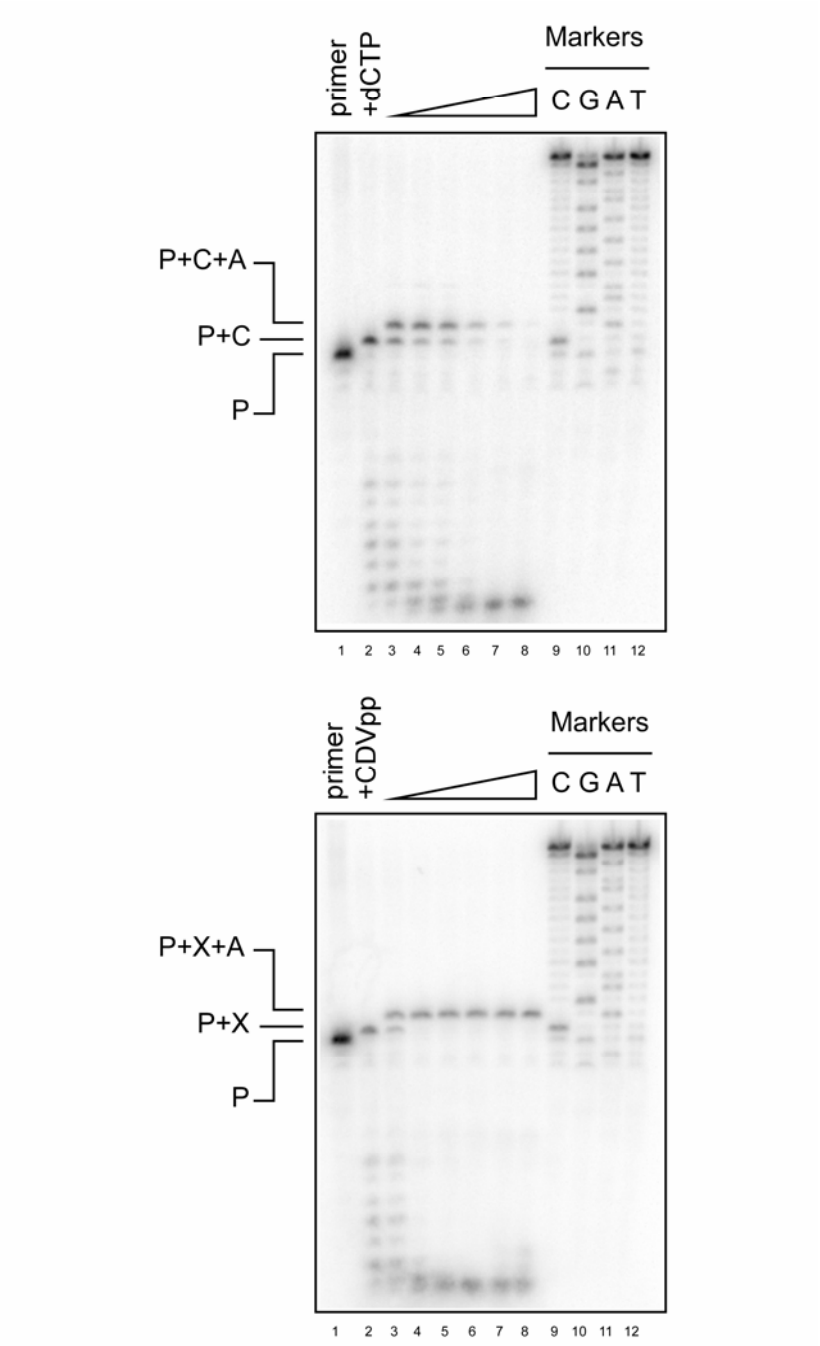

Figure 2.6 Kinetics of dAMP addition to 3′-CDV- or 3′-dCMP-terminated primer strands. Duplex P1-T9 was incubated with vaccinia virus DNA polymerase and either 10 μM dCTP (top panel) or 10 μM CDVpp (bottom panel) for 1 min at 37°C. The concentration of dATP was then adjusted to 10 μM and further samples taken for gel analysis over another 15-min interval. The first samples (lanes 3) were taken on ice.

proofreading exonuclease and polymerase activities was apparent, and the enzyme eventually degraded the reaction products down to oligonucleotides over the course of the reaction. A CDV-terminated primer also directed the addition of dAMP with extension kinetics indistinguishable (using these methods) from a dCMP-terminated primer (Figure 2.6, bottom panel). However, once extended by the addition of CDV plus dAMP, this reaction product was stable over the 15-min course of the experiment.

2.3.4 Primers bearing CDV as the 3′ penultimate base are poor substrates for vaccinia virus DNA polymerase. We next examined what happens when vaccinia virus DNA polymerase encounters a molecule bearing CDV as the penultimate 3′ residue. To do this, we used a P1-T9 primer-template pair and a series of sequential assembly reaction mixtures to incorporate CDV (10 μM CDVpp) followed by residue N + 1 (10 μM dATP) into DNA. We then tested the substrate properties of this primed structure after adding the remaining three dNTPs (no dCTP). As a control, dCTP was substituted for CDVpp. The results of these experiments are shown in Figure 2.7. As noted in previous experiments (Figure 2.6), vaccinia virus DNA polymerase produced a mix of extension products terminated in dCMP or dCMP-dAMP (Figure 2.7, top panel, lane 3). These two control reaction products were nearly instantaneously extended out to the ends of the template strand in the presence of dGTP, dATP, and dTTP (Figure 2.7, top panel, lanes 4 to 9). Primers terminated in CDV-dAMP were synthesized in high yield (Figure 2.7, bottom panel, lane 3), but the rate of extension of this product by vaccinia virus DNA polymerase clearly lagged well behind the rate of extension of control primers. Based upon the calculated distribution of label in substrates and products (Figure 2.8) we estimated that the rate of primer extension, measured as a first-order rate constant, is at least threefold slower when CDV replaces dCMP as the 3′ penultimate nucleotide (K = 3 min^{-1} versus 0.9 min^{-1} for extension from dCMP- versus CDV-bearing primers, respectively). However, there is a large standard error in the estimate of the control rate (95% confidence intervals for K_{dCMP} = 0 to 24 min^{-1}) and thus this calculation will probably underestimate the rate difference by at least a factor of ten.

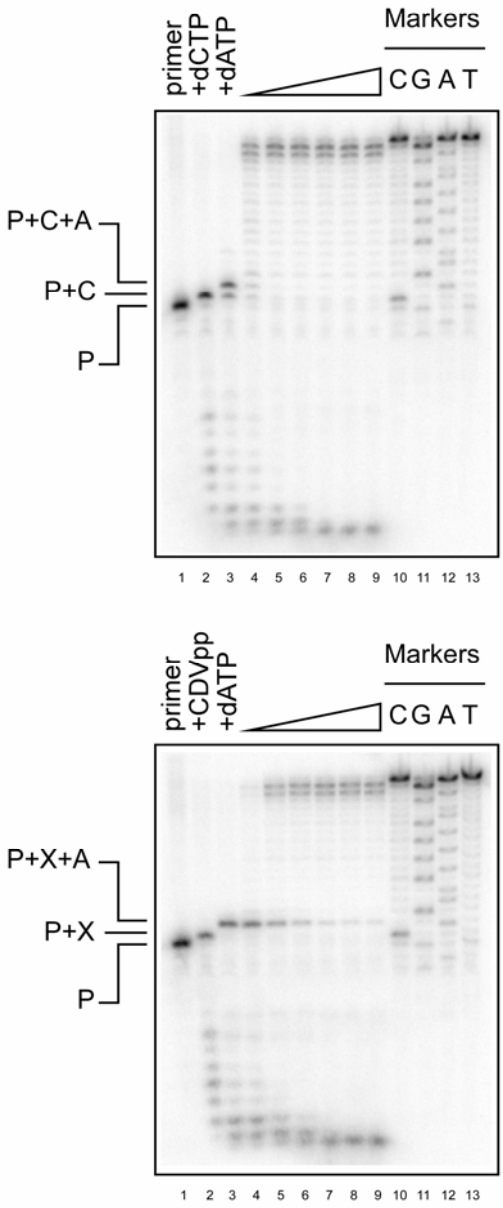

Figure 2.7 Kinetics of chain extension using a 3′-CDV-dAMP-terminated primer. Substrates were prepared as described in the legend to Figure 2.6, consisting of duplex P1-T9 that had been extended two nucleotides using dCTP-dATP (top) or CDVpp-dATP (bottom) as illustrated by Figures 2.5 and 2.6. (Note that vaccinia DNA polymerase always generates a mixture of dCMP- and dCMP-dAMP-terminated products, whereas only a single CDV-dAMP product is formed.) A mixture of dATP, dGTP, and dTTP was then added and further samples taken for gel analysis over another 15-min interval. The first samples (lanes 4) were taken on ice.

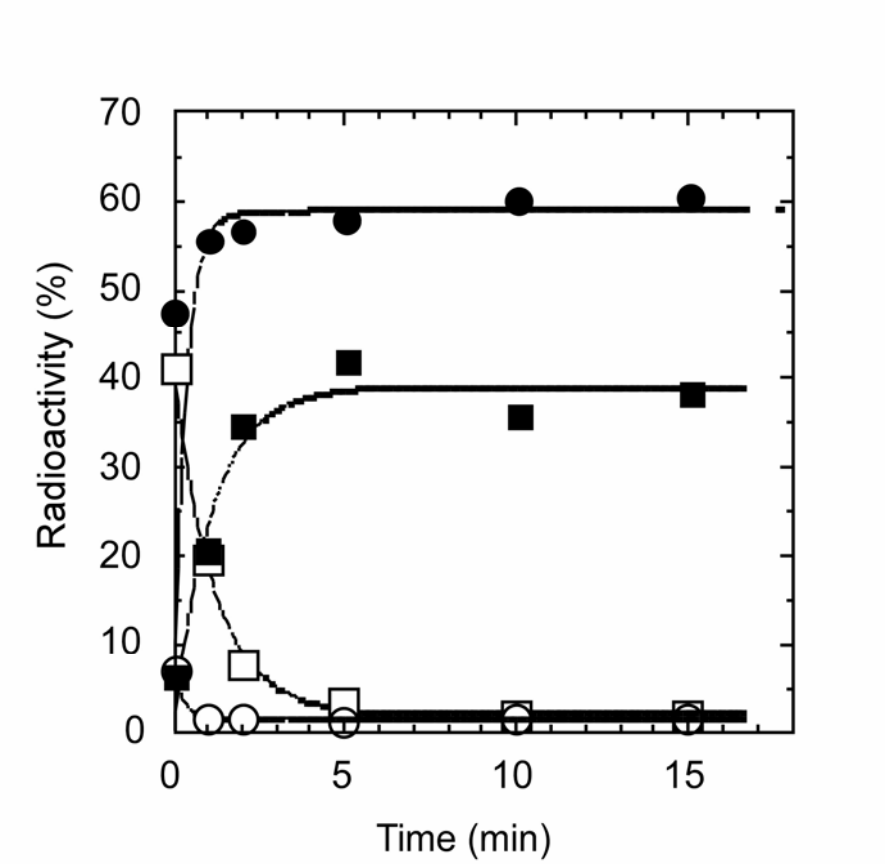

Figure 2.8 Quantitative analysis of the kinetics of chain extension. The amounts of label present in substrate and product bands, as a proportion of the total label in each lane (Figure 2.7) was determined using ImageQuant software and curve fits calculated using the program Prism 4.0b. The substrates were defined as the sum of label in bands labeled "P + C" (or X) and "P + C + A" (or X) in Figure 2.7. The products are defined as all labeled species larger than these substrate bands. The points are: dCTP substrates (-○-) and products (-●-), CDV substrates (-□-) and products (-■-).

We also looked to see if the enzyme could extend a primer incorporating two CDV molecules. Previous experiments with CMV DNA polymerase indicated that the presence of two consecutive CDV molecules dramatically reduced or terminated DNA synthesis (Xiong *et al.*, 1997). To compare the behavior of the two DNA polymerases, an assay was performed using primer P1 and template T2. The primer-template pair was first incubated with 10 μM CDVpp or dCTP (as a control), and then 10 μM dATP was added. Samples were taken for analysis after a brief incubation period, followed by the addition of dATP, dGTP and dTTP (10 μM each) to the remainder of the mixture. This reaction mixture was then sampled over the next 80 min. These results are shown in Figure 2.9. Molecules terminating with 5′-CC-OH 3′ and 5′-CCA-OH 3′ were again very rapidly extended out to the ends of the template within less than a minute (Figure 2.9, top panel, lanes 4 to 9). In contrast, the rate of elongation of a primer incorporating two CDV molecules was drastically reduced, as judged by the very slow incorporation of the next (dAMP) residue and the subsequent extension of the primer out to the end of the template strand. However, it was also apparent that two CDV molecules did not completely block DNA synthesis catalyzed by vaccinia virus DNA polymerase. We saw the accumulation of primers terminated with 5′-XXA-OH 3′ and 5′-XXAG-OH 3′ (where X = CDV), but molecules extended immediately beyond this point were not seen and appeared to be chased into full-length products over the 80-min incubation time.

2.3.5 A single CDV molecule is a substrate for vaccinia virus DNA polymerase 3′-to-5′ exonuclease. Vaccinia virus DNA polymerase encodes a 3′-to-5′ proofreading exonuclease activity and we also tested whether molecules terminated with CDV were substrates for this activity. To address this question, we first added a single dCMP or CDV residue to primer-template pair P1-T9 as described in Figure 2.5. We purified these reaction products free of any unincorporated dCTP or CDVpp and then incubated the two extension products with fresh enzyme in the absence of any additional dNTPs or CDVpp. The results of this experiment are shown in Figure 2.10. Molecules terminated with CDV (Figure 2.10, bottom panel) were degraded as quickly as were dCMP-terminated primers

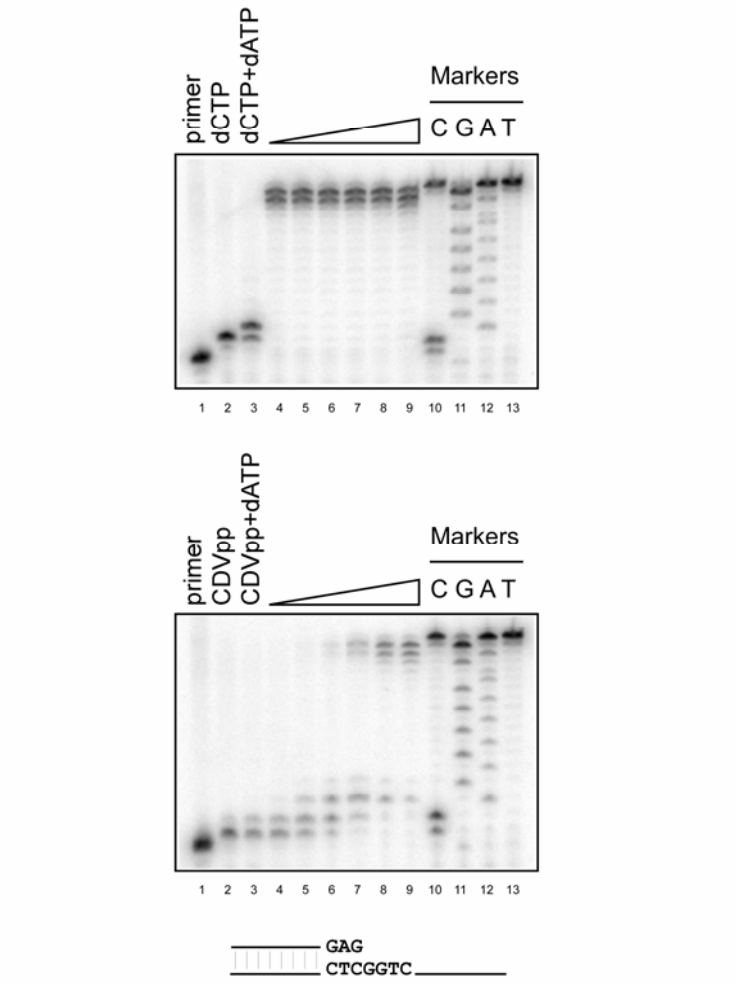

Figure 2.9 The presence of two consecutive template dG's slows, but does not completely block, DNA synthesis. A series of reaction mixtures were used to incorporate dCMP-dAMP (top panel) or CDV-dAMP (bottom panel) onto the 3′ end of primer P1. This was accomplished by incubating P1-T2 with DNA polymerase plus 10 μM dCTP (top) or 10 μM CDVpp (bottom) for 1 min at 37°C. After sampling the two reaction mixtures, dATP was added to a final concentration of 10 μM in each reaction mixture and the incubation continued for another 1 min at 37°C. Further samples were taken, and then a dNTP mixture was added to both reactions to produce final concentrations of 10 μM each of dATP, dGTP, and dTTP with no additional dCTP. These solutions were incubated at 37°C with sampling at 1, 5, 10, 20, 40, and 80 min. A mixture of primers terminating in 5′-CC-OH 3′ or 5′-CCA-OH 3′ (top panel, lane 3) is fully extended to the end of the template strand in less than a minute by vaccinia virus DNA polymerase (lanes 4 to 9). However, the enzyme only reluctantly synthesizes molecules encoding two CDV resides (bottom panel, lanes 2 and 3) and takes much longer to assemble a primer terminating in 5′-XXA-OH 3′ (where X = CDV, lanes 4 to 9). These molecules do eventually chase into various amounts of full-length products over an 80-min period (lanes 7 to 9).

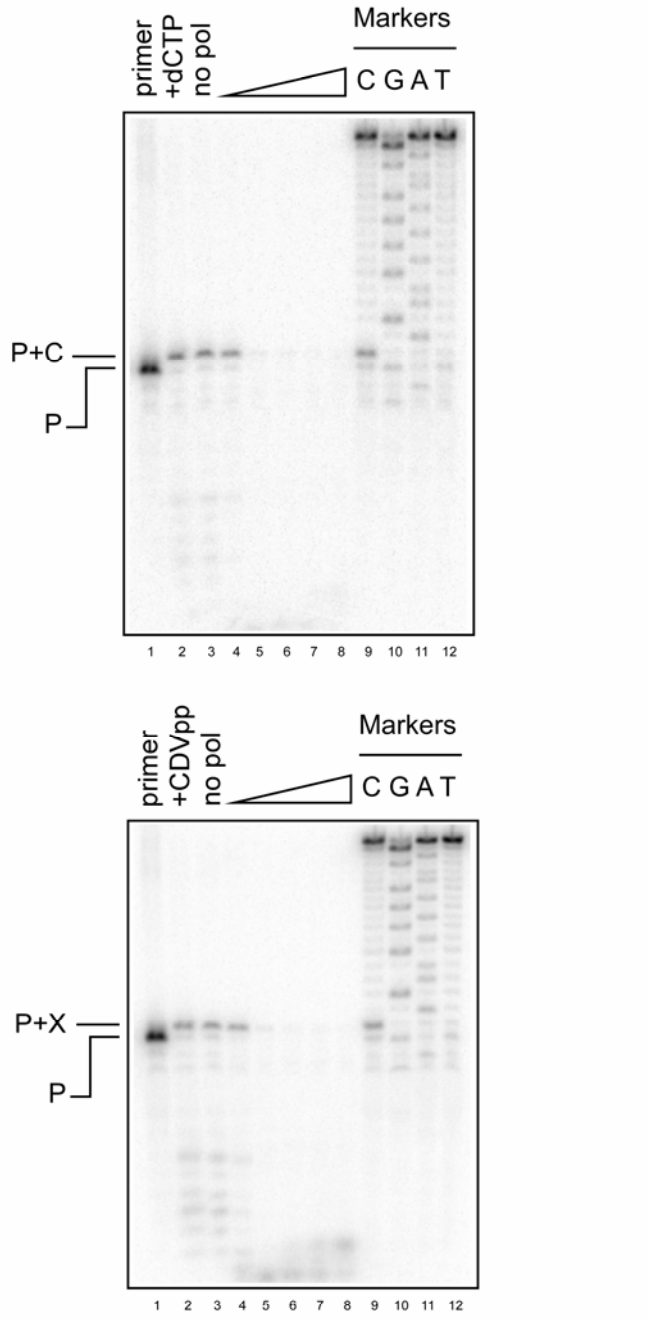

Figure 2.10 Excision of CDV from a 3′-CDV-terminated strand. Duplex P1-T9 was extended 1 nucleotide using either dCTP (top) or CDVpp (bottom) and then purified free of reaction materials by gel filtration. The purified product (lanes 2) was then incubated with vaccinia virus DNA polymerase at 37°C, in the absence of dNTPs, with sampling at 0, 1, 5, 10, and 20 min (lanes 4 to 8). Lanes 3 show a reaction incubated for 20 min in the absence of polymerase. Both types of extension products are substrates for the 3′-to-5′ proofreading exonuclease.

(Figure 2.10, top panel). This shows that a single CDV molecule is a substrate for the 3′-to-5′ proofreading exonuclease activity if it is located at the 3′ end of the primer strand.

2.3.6 The CDV + 1 product is not a substrate for the 3′-to-5′ exonuclease. In contrast to primers bearing a 3′-terminal CDV molecule, the CDV + 1 reaction product was completely resistant to attack by the proofreading exonuclease. For this assay, primer P1 and template T9 were again used. The primer-template pair was incubated together with 10 μM each CDVpp (or dCTP) and dATP, and vaccinia virus DNA polymerase, to create primers terminated with either 5′-CA-OH 3′, or 5′-XA-OH 3′ residues. The unincorporated dCTP, CDVpp, and/or dATP were removed by gel filtration, and then a new reaction was prepared containing DNA polymerase but lacking any dNTPs (as in Section 2.3.5). Figure 2.11 shows the results of this experiment. Molecules terminated with a 5′-CA-OH 3′ sequence were rapidly attacked by the exonuclease, generating a series of degradation products of which the smallest detectable by these methods were 6 to 7 nucleotides in length (Figure 2.11, left panel, lanes 4 to 10). In contrast, the 5′-XA-OH 3′ terminated primer was stable over 80 minutes of incubation. A second molecule 13 ± 1 nucleotides long was also resistant to this activity (Figure 2.11, right panel). These likely correspond to primer molecules that during the initial extension step (with CDVpp and dATP, but without dGTP) were excised back to the next nearest 5′-CA-3′ residue. If this sequence motif had been replaced by 5′-XA-OH 3′ it would create a 13-mer molecule (Figure 2.2) that, like the 20-mer, would also be resistant to nuclease attack. We concluded that the CDV + 1 extension product is resistant to attack by the vaccinia virus DNA polymerase 3′-to-5′ proofreading exonuclease.

2.3.7 Determination of the K_i of CDVpp. We attempted to determine the K_i value for CDVpp by using a filter-based DNA polymerase assay. Because CDVpp is an analog of dCTP and can be used by vaccinia virus DNA polymerase in place of this natural substrate, we predicted that this drug would act as a competitive inhibitor. Reactions were prepared in which the CDVpp and dCTP concentrations were varied, and the incorporation of dCTP into an activated calf thymus DNA template was determined by

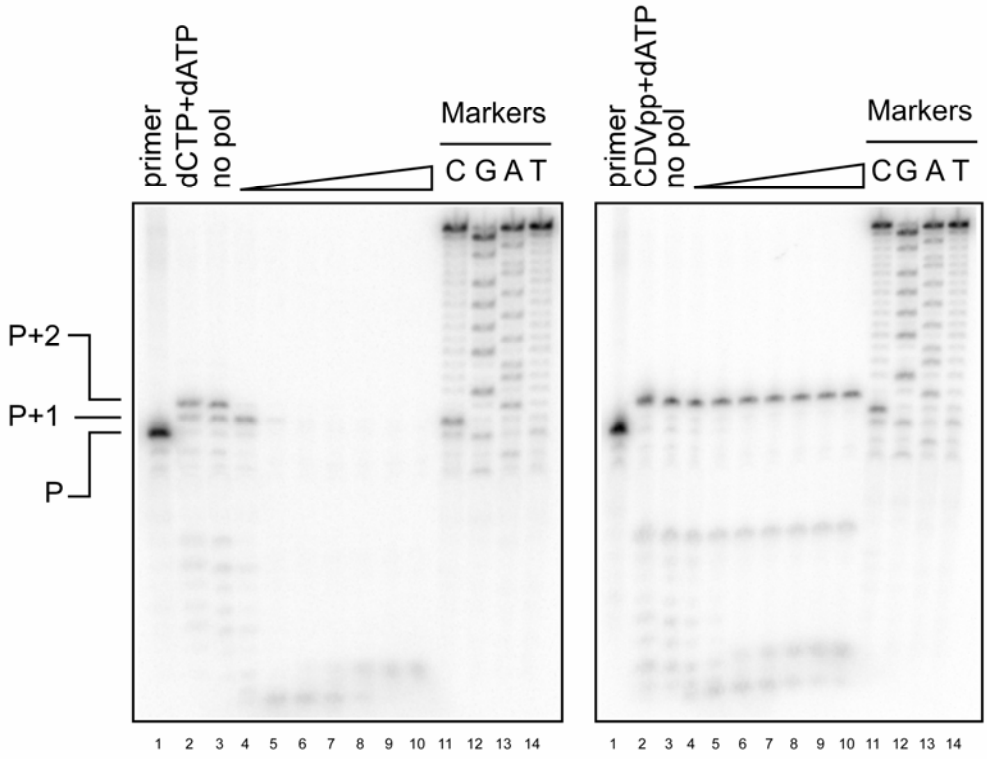

Figure 2.11 CDV inhibits the 3′-to-5′ exonuclease activity of vaccinia virus DNA polymerase when located in the penultimate position of the primer strand. Primer-template pair P1-T9 was incubated with vaccinia virus DNA polymerase at 37°C for 1 min in the presence of 10 μM dCTP and 10 μM dATP (left panel) or 10 μM CDVpp and 10 μM dATP (right panel). The unincorporated nucleotides and CDVpp were removed by gel filtration. The purified product (lanes 2) was then incubated with vaccinia virus DNA polymerase at 37°C, in the absence of dNTPs, with sampling at 0, 1, 5, 10, 20, 40, and 80 min. Lanes 3 show reactions incubated for 80 min in the absence of polymerase. Molecules terminated with 5′-C-OH 3′ or 5′-CA-OH 3′ are rapidly degraded (left), whereas molecules terminated with 5′-XA-OH 3′ are completely resistant to exonucleolytic attack (right).

labeling with [α^{32}P]dCTP. The double reciprocal plot of the data is shown in Figure 2.12. Increasing the concentration of a competitive inhibitor will result in a group of lines that intercept the 1/V axis at the same point but have increasing slopes (Nelson and Cox, 2005; Spector and Cleland, 1981). However, the results generated from our data do not intercept the Y-axis at a common point [0.54 ± 0.32 min · pmol^{-1} (1 μM CDVpp), 1.6 ± 0.5 min · pmol^{-1} (2μM CDVpp) and 1.3 ± 0.4 min · pmol^{-1} (4 μM CDVpp)], and the slopes of the lines [2.0 ± 0.2 (1 μM CDVpp), 2.0 ± 0.4 (2 μM CDVpp), 1.8 ± 0.3 (4 μM CDVpp)] are not significantly different. Based on these results, the inhibition constant for CDVpp could not be determined.

2.3.8 Determination of the K_m of CDVpp. Since the K_i of CDVpp could not be calculated using a standard filter-based DNA polymerase assay, we decided to determine the K_m of this substrate so that it could be compared to that of the natural substrate, dCTP. Our primer extension assays provided a method for estimating this value. Reaction mixtures were prepared containing a P1-T3 primer-template pair, 25 ng of vaccinia virus DNA polymerase, and 10 μM dGTP. The dGTP was added to maximize the stability of the dGMP-terminated primer in the presence of various concentrations of CDVpp. The kinetics of CDV incorporation were then determined in the presence of 0.3 to 30 μM CDVpp, monitoring the amount of labeled primer plus CDV extension product using polyacrylamide gel electrophoresis and phosphorimaging (Figure 2.13). The various initial rates of CDV incorporation were then used to estimate that the K_m and V_{max} for CDVpp, under these reaction conditions, are 23 ± 6 μM and 3.0 ± 0.4 pmol · min^{-1}, respectively. Each reaction contained 25 ng (0.22 pmol) of DNA polymerase and, assuming that the enzyme is fully active, the turnover number (k_{cat}) for CDVpp can be estimated as ~0.2 sec^{-1}. The same methods were used to determine K_m (3.8 ± 0.7 μM), V_{max} (2.4 ± 0.2 pmol · min^{-1}), and k_{cat} (~0.2 sec^{-1}) for dCTP (Figure 2.14).

2.4 DISCUSSION

The effect of CDVpp on the activity of vaccinia virus DNA polymerase was investigated using an *in vitro* primer extension assay. Adding CDVpp to a pool of

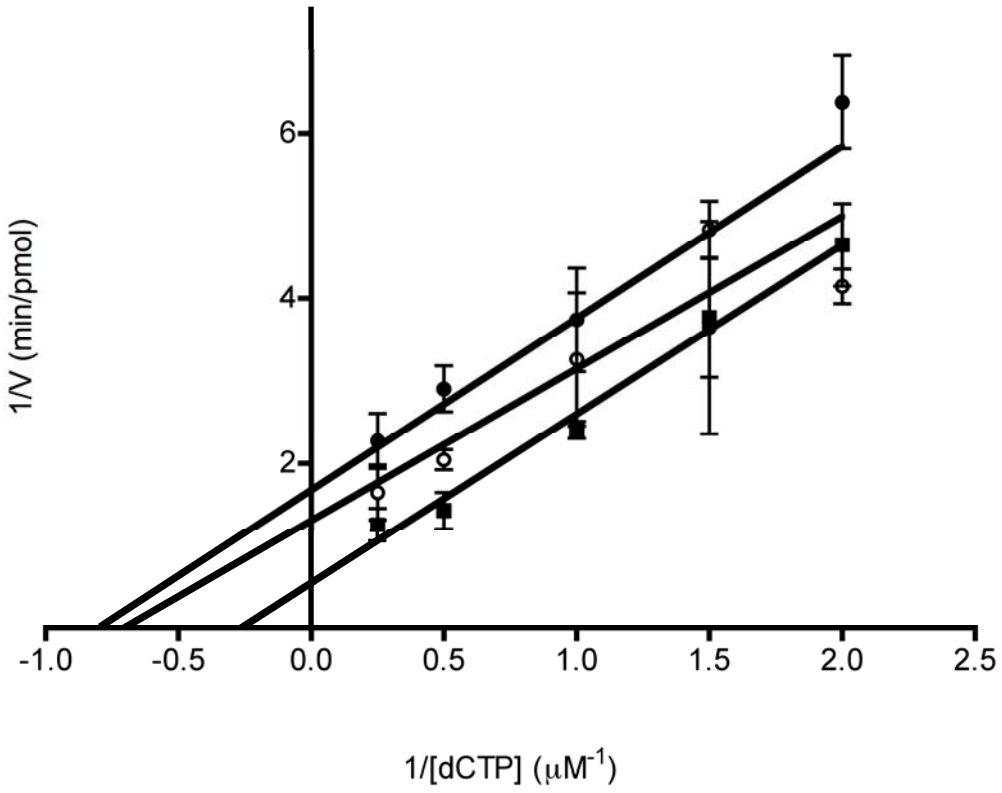

Figure 2.12 Double reciprocal plot of the rate of dCTP incorporation in the presence of increasing concentrations of CDVpp. The rate of incorporation of dCTP into an acid-precipitatible material was measured in the presence of 1 μM CDVpp (-■-), 2 μM CDVpp (-●-) or 4 μM CDVpp (-○-). The reciprocal of these rates were plotted against the reciprocal of the dCTP concentration using a linear regression analysis and Prism 4.0 software (GraphPad). The Y-intercepts were determined to be 0.54 ± 0.32 min · $pmol^{-1}$, 1.6 ± 0.5 min · $pmol^{-1}$ and 1.3 ± 0.4 min · $pmol^{-1}$ and the slopes of the lines calculated to be 2.0 ± 0.2, 2.0 ± 0.4, and 1.8 ± 0.3 for 1 μM CDVpp, 2μM CDVpp and 4 μM CDVpp, respectively.

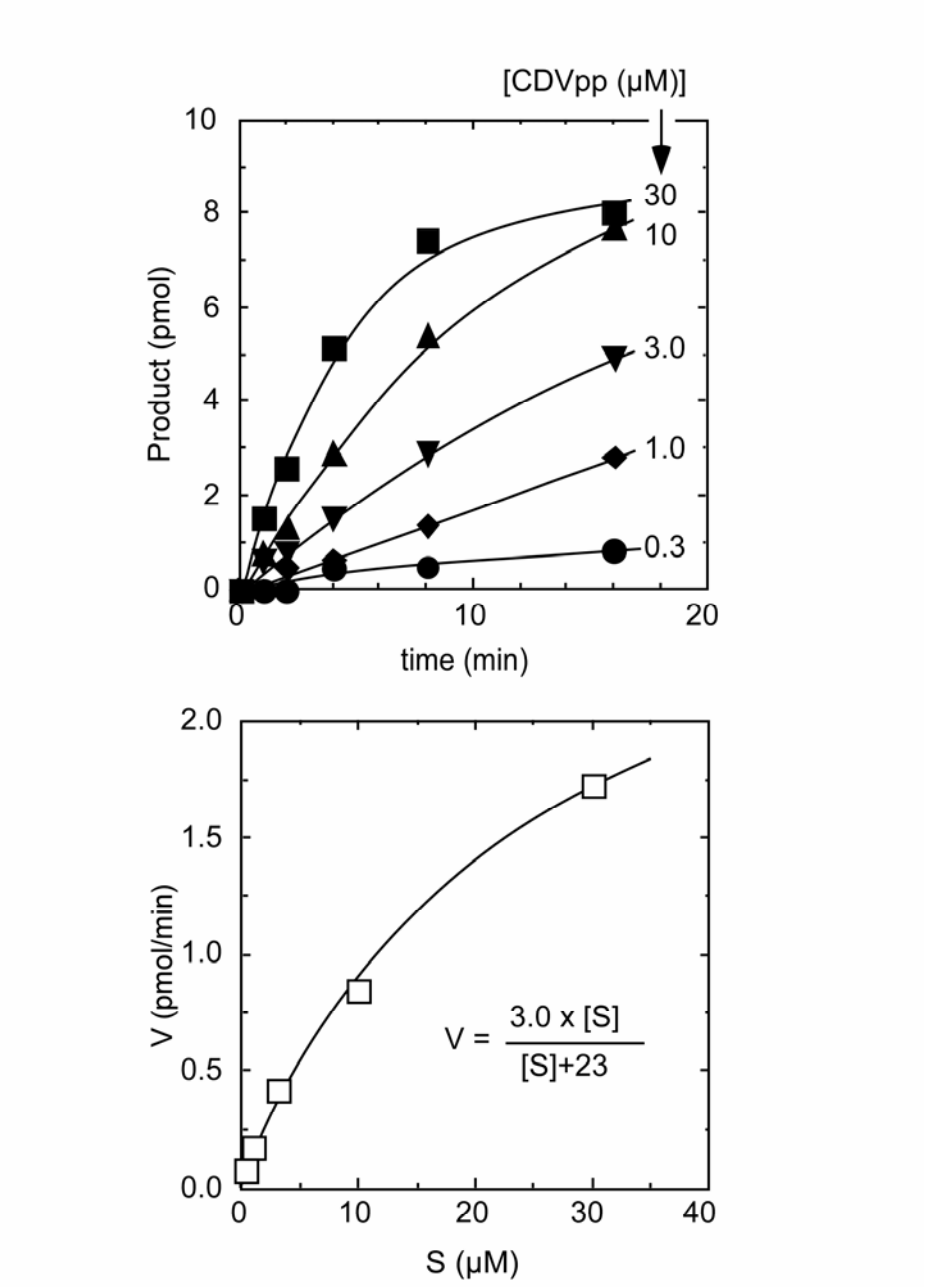

Figure 2.13 Kinetics of CDVpp incorporation. The rate of CDV incorporation was determined using the primer-template pair P1-T3 and electrophoretic methods shown in Figure 2.5 and initial rates calculated from the data plotted as shown in the upper panel using nonlinear regression analysis and Prism 4.0 software (GraphPad). These data were replotted as shown in the bottom panel and the same nonlinear regression method used to calculate the Michaelis-Menten constants K_m (23 ± 6 μM) and V_{max} (3.0 ± 0.4 pmol · min^{-1}).

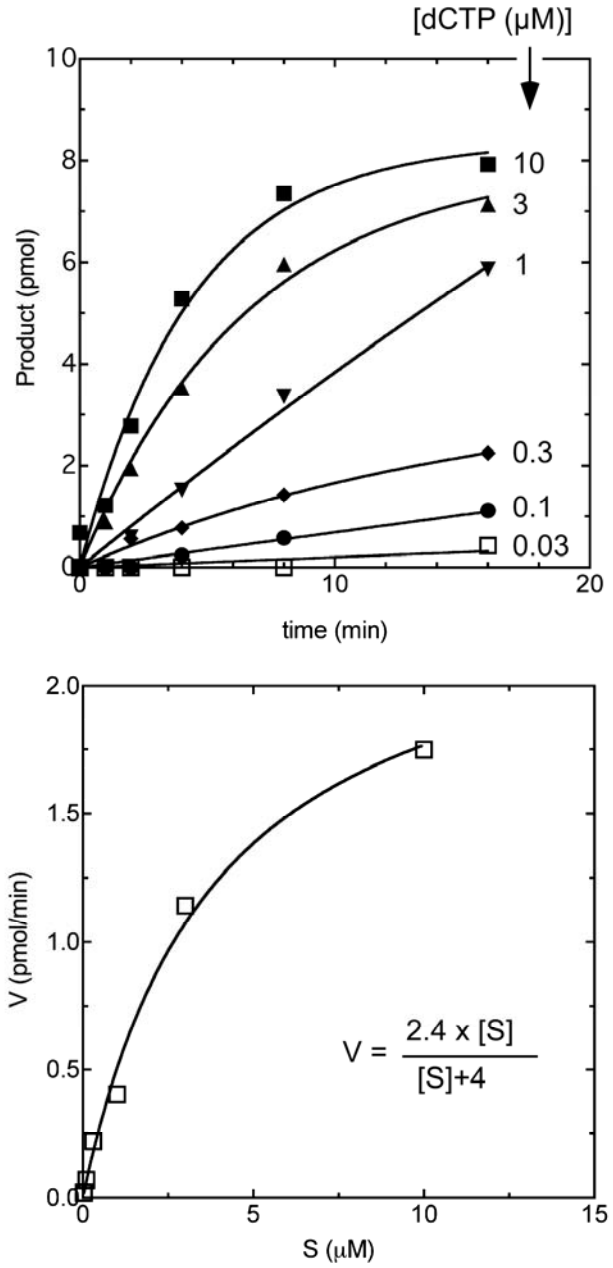

Figure 2.14 Kinetics of dCTP incorporation. The initial rates of dCMP incorporation were determined as described in the legend to Figure 2.13 and are shown in the upper panel. The lower panel shows a replotting of the data using a nonlinear regression method. These data were used to determine the Michaelis-Menten constants K_m (3.8 ± 0.7 μM) and V_{max} (2.4 ± 0.2 pmol · min^{-1}) for dCTP.

ordinary dNTPs caused the DNA polymerase to pause during chain extension at a point always one nucleotide past the site where CDV would be incorporated opposite a template dG. These observations provide a straightforward rationale for the antiviral activity against orthopoxviruses that has been reported both *in vitro* and *in vivo*. They also explain why CDV-resistant viruses encode mutations in the E9L gene (DNA polymerase), mutations that can be shown to be responsible for the drug resistant phenotype by marker rescue methods (Andrei *et al.*, 2006; Becker *et al.*, 2008; Kornbluth *et al.*, 2006).

Vaccinia virus DNA polymerase can use CDVpp as a substrate and can extend a CDV-terminated primer by one more nucleotide (Figures 2.5, 2.6 and 2.13). The differences between the substrate properties of dCTP and CDVpp are not readily apparent from the steady-state data seen in Figure 2.5, but a more detailed kinetic analysis clearly shows that CDVpp is a less favored substrate than dCTP. This is illustrated by the fact that the catalytic efficiency is about fivefold higher for dCTP as a substrate versus CDVpp, with the difference primarily accounted for by differences in the K_m. The effects of CDV on the relative rate of addition of the next templated nucleotide (dATP) is more difficult to judge by these methods given the effects of the product on the proofreading exonuclease activity (see below) and the consequential biased accumulation of the CDV + 1 product. Nevertheless, the presence of a CDV molecule linked to the 3′ terminus of the primer strand still permitted the addition of the next nucleotide with kinetics superficially comparable to dATP addition to a dCMP-terminated primer (Figure 2.6). CDV is thus not a chain-terminating drug in the classical sense, since it can be incorporated into DNA and still prime chain extension after incorporation into the nascent strand. From a structural perspective, CDVpp must be capable of occupying the nucleotide-binding site and serving as a substrate for the nucleophilic attack on the α-phosphonate. After translocation of the newly incorporated CDV molecule to the primer-terminus binding site, vaccinia virus DNA polymerase must also be able to reposition the hydroxyl group with sufficient accuracy to permit further polymerization. The crystal structure of tenofovir [*R*-9-(2-(phosphonomethoxypropyl)adenine] complexed with human immunodeficiency virus type 1 reverse transcriptase illustrates the conformational

flexibility of nucleoside phosphonates (Tuske *et al.*, 2004) that presumably accounts for this enzymatic behavior.

Although these substrate properties can partially explain the anti-poxvirus activity of CDVpp, more dramatic effects of the drug are seen at the next step in the polymerization cycle. Molecules bearing a CDV residue at the penultimate 3′ primer position are only very slowly extended into full-length chains (Figure 2.7). The rate-limiting factor appears to be the reaction steps catalyzing the addition of the next nucleotide to the CDV + 1 product (Figure 2.7). Although these molecules are poor substrates, they are still slowly converted into full-length products with a half-life of ~0.8 min under our conditions (Figure 2.8). Generally, the same effect was seen when two molecules of CDV were incorporated into the primer strand. However, the transient accumulation of a CDV_2 + 2 product and the much-delayed appearance of full-length product (Figure 2.9) suggested that the deleterious effects of CDV are propagated further and with greater effect under such circumstances. Base-paired deoxynucleoside monophosphates are relatively inflexible molecules and this property, combined with structural distortions imposed by a nucleoside phosphonate linkage, could result in the misalignment of 3′-hydroxyl terminus with deleterious effects on the next nucleotide addition step. Alternatively, the enzyme might have difficulty repositioning the CDV + 1 reaction product within the polymerase reaction site, which would also inhibit further chain extension.

The same effects are seen when one examines the susceptibility of CDV-containing molecules to attack by the 3′-to-5′ proofreading exonuclease. CDV is excised from the 3′ end of the primer at a rate comparable to dCMP (Figure 2.10) and shows that vaccinia virus DNA polymerase has little difficulty hydrolyzing a phosphonate ester linkage. This is not true of the CDV + 1 substrate, where the 3′-terminal (-1) phosphodiester linkage is completely resistant to hydrolysis (Figure 2.11). These observations can be rationalized using the same structural arguments outlined above. CDV-terminated molecules might exhibit sufficient conformational flexibility to still serve as exonuclease substrates, while the constraints imposed by a phosphonate linkage at the –2 bond may render the phosphodiester bond at –1 resistant to hydrolysis.

We had attempted to determine a K_i value for CDVpp using vaccinia virus DNA polymerase in order to compare the value to those previously reported for cowpox virus DNA polymerase, HCMV DNA polymerase, and human DNA polymerases α, β, and γ (Cherrington *et al.*, 1994; Ho *et al.*, 1992; Smee *et al.*, 2002; Xiong *et al.*, 1996). Unfortunately, our data did not fit the expected competitive inhibition model with respect to dCTP (Nelson and Cox, 2005; Spector and Cleland, 1981). As an alternative substrate for the polymerase, CDVpp is expected to be only a competitive inhibitor of vaccinia virus DNA polymerase and not an uncompetitive inhibitor that would bind to a site distinct from the active site of the enzyme (Nelson and Cox, 2005). The reasons why a K_i value for CDVpp could not be determined are unclear. One possibility is that the different effects of CDVpp and CDV on vaccinia virus DNA polymerase, depending on the context in which the enzyme encounters the drug (as a substrate, at the 3′ end of the primer, at the 3′ penultimate position of the primer, or as two consecutive molecules at the 3′ end of the primer), complicates the analysis with an undefined template like activated calf thymus DNA. That Smee *et al.* (2002) were able to calculate a K_i value for cowpox virus DNA polymerase (26 ± 4 μM) would tend to discount this possibility. Primer extension analyses like those described here have not been performed with the cowpox enzyme; however, the high percent identity between these two proteins (>98%) suggests that CDVpp will have a similar effect on this enzyme. Similarly, although CDV shows differential effects on the HCMV DNA polymerase [a single drug molecule slows elongation by HCMV DNA polymerase and two consecutively incorporated molecules cause chain termination (Xiong *et al.*, 1997)], a K_i value for CDVpp of 6.6 ± 0.8 μM was calculated for this enzyme (Xiong *et al.*, 1996). A direct comparison between the results obtained using these three enzymes are difficult to make, as the experimental designs differ or are unknown. Smee *et al.* used only a single competitive concentration (0.25 μM) of [^{3}H]dCTP in their assay, a value 10-fold lower than the K_m of this substrate (2.3 ± 0.7 μM) (Smee *et al.*, 2002). Further, these authors calculated the K_i value by determining the concentration of CDVpp that reduced the rate of the reaction by 50% (IC_{50}) and using the formula $IC_{50} = K_i (1 + S/K_m)$, where S is the concentration of dCTP (Cheng and Prusoff, 1973). The method used by Xiong *et al.* is unclear, as the assay used

is not described (Xiong *et al.*, 1996). The DNA polymerase assays used to calculate K_i values for CDVpp and human DNA polymerases α, β, and γ (51.0 μM, 520 μM and 299 μM, respectively) are similar to those that we performed except that no cold dCTP was used in the reactions and the [^{3}H]dCTP concentration was varied (Cherrington *et al.*, 1994; Ho *et al.*, 1992). As the effect of CDVpp on these DNA polymerases using primer extension assays has not been determined, it is unknown whether this change in experimental design would account for the inability to obtain a K_i value for CDVpp and vaccinia virus DNA polymerase.

As described above, HCMV DNA polymerase exhibits some of the same enzymatic responses to CDVpp (Xiong *et al.*, 1997; Xiong *et al.*, 1996). In particular, the two different virus enzymes utilize CDVpp less efficiently than dCTP (Xiong *et al.*, 1996). Substituting CDVpp for dCTP also causes the transient accumulation of the CDV + 1 reaction product. However, poxvirus polymerases appear capable of slowly copying templates encoding a dGpdG motif in the presence of CDVpp (Figure 2.9), while these residues completely block readthrough catalyzed by HCMV polymerase. A much more notable difference is that HCMV polymerase cannot excise CDV from a CDV-terminated primer (Xiong *et al.*, 1997), whereas it is the CDV + 1 reaction product that creates the greatest difficulties for vaccinia virus DNA polymerase. These differences suggest that the two enzymes interact with CDV and CDV-bearing DNAs in sometimes different ways, and caution must be observed extrapolating from the molecular genetic properties of CDV-resistant herpesviruses to poxvirus systems.

In conclusion, these data provide insights into the anti-poxvirus activity of nucleoside phosphonate drugs. These compounds inhibit chain extension, and since DNA synthesis is a key regulator of the virus life cycle, they are expected to compromise a diverse array of other processes including intermediate and late gene transcription and virus assembly. The incorporation of CDV into DNA also completely inhibits the associated 3′-to-5′ exonuclease. The proofreading exonuclease serves a critical role in minimizing replication errors and probably also catalyzes virus genetic recombination (Hamilton *et al.*, 2007; Willer *et al.*, 2000; Yao and Evans, 2003). Future studies will use

CDV and CDV-resistant viruses as a tool for investigating these two critical viral systems.

2.5 AUTHOR CONTRIBUTION TO DATA

Wendy Magee performed all of the experiments described in this study. The graphs in Figures 2.8, 2.13 and 2.14 were prepared by Dr. David Evans using data generated by Wendy Magee.

2.6 REFERENCES

Andrei, G., D. B. Gammon, P. Fiten, E. De Clercq, G. Opdenakker, R. Snoeck, and D. H. Evans. 2006. Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice. J. Virol. **80:** 9391-9401.

Baker, R. O., M. Bray, and J. W. Huggins. 2003. Potential antiviral therapeutics for smallpox, monkeypox and other orthopoxvirus infections. Antiviral Res. **57:** 13-23.

Becker, M. N., M. Obraztsova, E. R. Kern, D. C. Quenelle, K. A. Keith, M. N. Prichard, M. Luo, and R. W. Moyer. 2008. Isolation and characterization of cidofovir resistant vaccinia viruses. Virology J. **5:** 58.

Bray, M., M. Martinez, D. F. Smee, D. Kefauver, E. Thompson, and J. W. Huggins. 2000. Cidofovir protects mice against lethal aerosol or intranasal cowpox virus challenge. J. Infect. Dis. **181:** 10-19.

Buller, R. M., G. Owens, J. Schriewer, L. Melman, J. R. Beadle, and K. Y. Hostetler. 2004. Efficacy of oral active ether lipid analogs of cidofovir in a lethal mousepox model. Virology **318:** 474-481.

Cheng, Y.-C., and W. H. Prusoff. 1973. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibtion (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. **22:** 3099-3108.

Cherrington, J. M., S. J. Allen, B. H. McKee, and M. S. Chen. 1994. Kinetic analysis of the interaction between the diphosphate of (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine, ddCTP, AZTTP, and FIAUTP with human DNA polymerases β and γ. Biochem. Pharmacol. **48:** 1986-1988.

Ciesla, S. L., J. Trahan, W. B. Wan, J. R. Beadle, K. A. Aldern, G. R. Painter, and K. Y. Hostetler. 2003. Esterification of cidofovir with alkoxyalkanols increases oral

bioavailability and dimishes drug accumulation in the kidney. Antiviral Res. **59:** 163-171.

Cihlar, T., and M. S. Chen. 1996. Identification of enzymes catalyzing two-step phosphorylation of cidofovir and the effect of cytomegalovirus infection on their activities in host cells. Mol. Pharmacol. **50:** 1502-1510.

Connelly, M. C., B. L. Robbins, and A. Fridland. 1993. Mechanism of uptake of the phosphonate analog (*S*)-1-(3-hydroyx-2-phosphonylmethoxypropyl)cytosine (HPMPC) in Vero cells. Biochem. Pharmacol. **46:** 1053-1057.

De Clercq, E. 1997. Acyclic nucleoside phosphonates in the chemotherapy of DNA virus and retrovirus infections. Intervirology **40:** 295-303.

De Clercq, E., A. Holý, I. Rosenberg, T. Sakuma, J. Balzarini, and P. C. Maudgal. 1986. A novel selective broad-spectrum anti-DNA virus agent. Nature **323:** 464-467.

Earl, P. L., E. V. Jones, and B. Moss. 1986. Homology between DNA polymerases of poxviruses, herpesviruses, and adenoviruses: Nucleotide sequence of the vaccinia virus DNA polymerase gene. Proc. Natl. Acad. Sci. USA **83:** 3659-3663.

Geerinck, K., G. Lukito, R. Snoeck, R. De Vos, E. De Clercq, Y. Vanrenterghem, H. Degreef, and B. Maes. 2001. A case of human orf in an immunocompromised patient treated successfully with cidofovir cream. J. Med. Virol. **64:** 543-549.

Hamilton, M. D., A. A. Nuara, D. B. Gammon, R. M. Buller, and D. H. Evans. 2007. Duplex strand joining reactions catalyzed by vaccinia DNA polymerase. Nucleic Acids Res. **35:** 143-151.

Henderson, D. A., T. V. Inglesby, J. G. Bartlett, M. S. Ascher, E. Eitzen, P. B. Jahrling, J. Hauer, M. Layton, J. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. Perl, P. K. Russell, and K. Tonat. 1999. Smallpox as a biological weapon: medical and public health management. JAMA **281:** 2127-2137.

Hendricks, S. P., and C. K. Mathews. 1998. Allosteric regulation of vaccinia virus ribonucleotide reductase, analyzed by simultaneous monitoring of its four activities. J. Biol. Chem. **273:** 29512-29518.

Ho, H.-T., K. L. Woods, J. J. Bronson, H. De Boeck, J. C. Martin, and M. J. M. Hitchcock. 1992. Intracellular metabolism of the antiherpes agent (S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine. Mol. Pharmacol. **41:** 197-202.

Huggins, J. W., R. O. Baker, J. R. Beadle, and K. Y. Hostetler. 2002. Orally active ether lipid prodrugs of cidofovir for the treatment of smallpox. Antiviral Res. **53:** A66.

Ishii, K., and B. Moss. 2002. Mapping interaction sites of the A20R protein component of the vaccinia virus DNA replication complex. Virology **303:** 232-239.

Kern, E. R., C. Hartline, E. Harden, K. Keith, N. Rodriguez, J. R. Beadle, and K. Y. Hostetler. 2002. Enhanced inhibition of orthopoxvirus replication in vitro by alkoxyalkyl esters of cidofovir and cyclic cidofovir. Antimicrob. Agents Chemother. **46:** 991-995.

Klemperer, N., W. McDonald, K. Boyle, B. Unger, and P. Traktman. 2001. The A20R protein is a stoichiometric component of the processive form of vaccinia virus DNA polymerase. J. Virol. **75:** 12298-12307.

Kornbluth, R. S., D. F. Smee, R. W. Sidwell, V. Snarsky, D. H. Evans, and K. Y. Hostetler. 2006. Mutations in the E9L polymerase gene of cidofovir-resistant vaccinia virus strain WR are associated with the drug resistance phenotype. Antimicrob. Agents Chemother. **50:** 4038-4043.

Lewis-Jones, S. 2004. Zoonotic poxvirus infections in humans. Curr. Opin. Infect. Dis. **17:** 81-89.

McCraith, S., T. Holtzman, B. Moss, and S. Fields. 2000. Genome-wide analysis of vaccinia virus protein-protein interactions. Proc. Natl. Acad. Sci. USA **97:** 4879-4884.

McDonald, W. F., and P. Traktman. 1994a. Overexpression and purification of the vaccinia virus DNA polymerase. Protein Expr. Purif. **5:** 409-421.

McDonald, W. F., and P. Traktman. 1994b. Vaccinia virus DNA polymerase: *in vitro* analysis of parameters affecting processivity. J. Biol. Chem. **269:** 31190-31197.

Meadows, K. P., S. K. Tyring, A. T. Pavia, and T. M. Rallis. 1997. Resolution of recalcitrant molluscum contagiosum virus lesions in human immunodeficiency virus-infected patients treated with cidiofovir. Arch. Dermatol. **133:** 987-990.

Nelson, D. L., and M. M. Cox. 2005. Lehninger Principles of Biochemistry, 4th ed. W. H. Freeman and Company, New York.

Quenelle, D. C., D. J. Collins, W. B. Wan, J. R. Beadle, K. Y. Hostetler, and E. R. Kern. 2004. Oral treatment of cowpox and vaccinia virus infections in mice with ether lipid esters of cidofovir. Antimicrob. Agents Chemother. **48:** 404-412.

Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Smee, D. F., R. W. Sidwell, D. Kefauver, M. Bray, and J. W. Huggins. 2002. Characterization of wild-type and cidofovir-resistant strains of camelpox, cowpox, monkeypox, and vaccinia viruses. Antimicrob. Agents Chemother. **46:** 1329-1335.

Spector, T., and W. W. Cleland. 1981. Meanings of K_i for conventional and alternate-substrate inhibitors. Biochem. Pharmacol. **30:** 1-7.

Stanitsa, E. S., L. Arps, and P. Traktman. 2006. Vaccinia virus uracil DNA glycosylase interacts with the A20 protein to form a heterodimeric processivity factor for the viral DNA polymerase. J. Biol. Chem. **281:** 3439-3451.

Taddie, J. A., and P. Traktman. 1991. Genetic characterization of the vaccinia virus DNA polymerase: identification of point mutations conferring altered drug sensitivities and reduced fidelities. J. Virol. **65:** 869-879.

Tuske, S., S. G. Sarafianos, A. D. Clark Jr., J. Ding, L. K. Naeger, K. L. White, M. D. Miller, C. S. Gibbs, P. L. Boyer, P. Clark, G. Wang, B. L. Gaffney, R. A. Jones, D. M. Jerina, S. H. Hughes, and E. Arnold. 2004. Structures of HIV-1 RT-DNA complexes before and after incorporation of the anti-AIDS drug tenofovir. Nat. Struct. Mol. Biol. **11:** 469-474.

Whitley, R. J. 2003. Smallpox: a potential agent of bioterrorism. Antiviral Res. **57:** 7-12.

Willer, D. O., X.-D. Yao, M. J. Mann, and D. H. Evans. 2000. *In vitro* concatemer formation catalyzed by vaccinia virus DNA polymerase. Virology **278:** 562-569.

World Health Organization. 1980. Declaration of global eradication of smallpox. Wkly. Epidemiol. Rec. **55:** 148.

Xiong, X., J. L. Smith, and M. S. Chen. 1997. Effect of incorporation of cidofovir into DNA by human cytomegalovirus DNA polymerase on DNA elongation. Antimicrob. Agents Chemother. **41:** 594-599.

Xiong, X., J. L. Smith, C. Kim, E. Huang, and M. S. Chen. 1996. Kinetic analysis of the interaction of cidofovir diphosphate with human cytomegalovirus DNA polymerase. Biochem. Pharmacol. **51:** 1563-1567.

Yao, X.-D., and D. H. Evans. 2003. Characterization of the recombinant joints formed by single-strand annealing reactions in vaccinia virus-infected cells. Virology **308:** 147-156.

CHAPTER THREE

CIDOFOVIR AND *(S)*-9-[3-HYDROXY-(2-PHOSPHONOMETHOXY)PROPYL]ADENINE ARE HIGHLY EFFECTIVE INHIBITORS OF VACCINIA VIRUS DNA POLYMERASE WHEN INCORPORATED INTO THE TEMPLATE STRAND[1,2]

Wendy C. Magee, Kathy A. Aldern, Karl Y. Hostetler, and David H. Evans

3.1 INTRODUCTION

(S)-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine [*(S)*-HPMPA] is an acyclic analog of dAMP and was the first of the nucleoside phosphonate drugs described in the research literature (De Clercq *et al.*, 1986). *(S)*-HPMPA was first shown to exhibit activity against a range of DNA viruses, including herpesviruses, vaccinia virus, and adenovirus, as well as Moloney murine sarcoma retrovirus (De Clercq *et al.*, 1986). Later work has shown that it is also effective against hepatitis B viruses (Yokota *et al.*, 1990a; Yokota *et al.*, 1990b). Although the drug did not show any activity against human immunodeficiency virus in its original formulation (Pauwels *et al.*, 1988), Hostetler *et al.* have recently shown that the alkoxyalkyl ester derivatives are active against this virus, as well as against mutant viruses resistant to azidothymidine, lamivudine, tenofovir, and nevirapine (Hostetler *et al.*, 2006). The antimicrobial range of *(S)*-HPMPA also includes parasites such as trypanosomes (Kaminsky *et al.*, 1996; Kaminsky *et al.*, 1994), *Schistosoma mansoni* (Botros *et al.*, 2003), *Plasmodium falciparum,* and *Plasmodium berghei* (de Vries *et al.*, 1991; Smeijsters *et al.*, 1999).

(S)-HPMPA is taken up into cells by endocytosis (Palú *et al.*, 1991) and converted

---

[1] A version of this chapter has been published in Antimicrobial Agents and Chemotherapy (2008), 52(2): 586-597. *Copyright 2008. American Society for Microbiology.*

[2] This study was supported by Canadian Institutes of Health Research (CIHR) and Natural Sciences and Engineering Research Council of Canada (NSERC) grants to Dr. D. H. Evans and National Institutes of Health (NIH) grants AI-066499 and AI-064615 to Dr. K. Y. Hostetler.

into the active metabolite *(S)*-HPMPA diphosphate [*(S)*-HPMPApp] by cellular kinases (Merta *et al.*, 1992). *(S)*-HPMPApp is an analog of dATP, and different studies have described a variety of cytotoxic effects at higher drug doses, which are most likely caused by the inhibition of cellular DNA replication (Bronson *et al.*, 1989; Veselý *et al.*, 1990; Votruba *et al.*, 1987). Rat cell DNA polymerases α, δ, and ε can use *(S)*-HPMPApp as a substrate and incorporate two to four consecutive *(S)*-HPMPA molecules into a growing DNA strand (Birkus *et al.*, 2004; Kramata *et al.*, 1996). More specifically, *(S)*-HPMPApp is a strong inhibitor of DNA polymerase ε (K_i/K_m = 0.07), a moderate inhibitor of DNA polymerase δ (K_i/K_m = 0.25) and a weak inhibitor of DNA polymerase α (K_i/K_m = 2.29) (Kramata *et al.*, 1996), but not an inhibitor of DNA polymerase β (Merta *et al.*, 1990). Interestingly, both DNA polymerases δ and ε can still excise *(S)*-HPMPA from a primer terminus, with polymerase ε showing more effective removal of the drug (Birkus *et al.*, 2004).

Similar studies have examined the effects of *(S)*-HPMPApp on parasitic and viral DNA synthesis. *(S)*-HPMPA inhibits the replication of human and duck hepatitis B viruses (Yokota *et al.*, 1990a; Yokota *et al.*, 1990b) and herpes simplex virus type 1 (De Clercq *et al.*, 1987). Herpes simplex virus DNA polymerase can use *(S)*-HPMPApp as a substitute for dATP, but curiously *(S)*-HPMPApp is a poor inhibitor of the enzyme itself (Merta *et al.*, 1990). Adenovirus DNA polymerases are inhibited by *(S)*-HPMPApp, which causes a block in replication at the level of DNA elongation (Mul *et al.*, 1989). Trypanosomal DNA replication is also inhibited by *(S)*-HPMPA (Kaminsky *et al.*, 1998), but although *P. falciparum* DNA polymerases α and γ are inhibited by *(S)*-HPMPApp, the *in vivo* target of the drug appears to be polymerase δ (de Vries *et al.*, 1991; Smeijsters *et al.*, 2000).

These studies suggest that *(S)*-HPMPApp affects different polymerases in different ways, but the mechanism linking the effects on DNA synthesis to a cytotoxic, antiviral, or antiparasitic effect is not well understood. To address this question, we have chosen to examine the antiviral effects of nucleoside phosphonate drugs using vaccinia virus and vaccinia DNA polymerase as a model system. Orthopoxviruses are acutely sensitive to nucleoside phosphonate drugs, and it has been suggested they might prove

useful for treating renascent smallpox (Baker *et al.*, 2003; Keith *et al.*, 2003). Vaccinia polymerase is a B family DNA polymerase (Ito and Braithwaite, 1991) and possesses both 5′-to-3′ polymerase and 3′-to-5′ exonuclease activities (Challberg and Englund, 1979). We have previously shown that when a related compound, cidofovir [*(S)*-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine] [*(S)*-HPMPC)] diphosphate (CDVpp), is incorporated into DNA it inhibits both primer extension and drug excision by vaccinia DNA polymerase (Chapter 2; Magee *et al.*, 2005). *(S)*-HPMPA and CDV differ only in the structure of the base moiety, so we hypothesized that the two compounds would have a similar mechanism of inhibition. *(S)*-HPMPA has been shown to be more toxic in mice than CDV (Bronson *et al.*, 1989), but subsequent work yielded conflicting results on the relative cytotoxicity and selectivity indices between the two drugs (Baker *et al.*, 2003; Beadle *et al.*, 2006; Keith *et al.*, 2003; Lebeau *et al.*, 2006; Snoeck *et al.*, 2002). The latter results are most likely due to differences in the sensitivity of the various cells lines used to *(S)*-HPMPA and CDV. *(S)*-HPMPA shows greater efficacy against orthopoxviruses in culture (De Clercq *et al.*, 1987; Keith *et al.*, 2003; Lebeau *et al.*, 2006; Snoeck *et al.*, 2002) and the hexadecyloxypropyl (HDP) ester of *(S)*-HPMPA is 80-fold more active than the HDP-ester of CDV (Beadle *et al.*, 2006; Kern *et al.*, 2002). Therefore, we predicted that *(S)*-HPMPA would have a more profound effect on these aspects of enzyme activity than does CDV. Oddly, *(S)*-HPMPApp is not as good an inhibitor of primer extension as is CDVpp. We demonstrate that both drugs, when incorporated into the template strand, cause a profound block in DNA elongation. These results show that CDV and *(S)*-HPMPA are more complex drugs than has been previously recognized, affecting DNA elongation when incorporated in both the primer and template strands and blocking 3′-to-5′ exonuclease activity when located in the primer strand.

3.2 MATERIAL AND METHODS

3.2.1 Chemicals. *(S)*-HPMPApp and CDVpp were prepared by custom synthesis by TriLink Biotechnologies. *(S)*-HPMPA was a gift of Dr. G. Andrei, Rega Institute for Medical Research, Leuven, Belgium. CDV was obtained from Moravek Biochemicals.

Radiolabeled cordycepin triphosphate ([α-^{32}P]3′-deoxyATP) was purchased from PerkinElmer, [γ-^{32}P]ATP and [α-^{32}P]dATP were obtained from GE Healthcare, and the unlabeled deoxynucleoside triphosphates (dNTPs) were from Fermentas. Oligonucleotides were purchased from Sigma-Genosys or Integrated DNA Technologies. [2-^{14}C]CDV (56 mCi/mmol), *(S)*-[8-^{14}C]HPMPA (57 mCi/mmol) and their alkoxyester derivatives HDP-[2-^{14}C]CDV (50 mCi/mmol) and HDP-*(S)*-[8-^{14}C]HPMPA (50 mCi/mmol) were synthesized by Moravek Biochemicals using unlabeled intermediates and methods provided by Dr. J. Beadle, as previously described (Beadle *et al.*, 2006).

3.2.2 Enzymes. Vaccinia virus DNA polymerase was purified from cells coinfected with recombinant vaccinia vTMPOL and vTF7.5 viruses as described previously (McDonald and Traktman, 1994). The enzyme was freshly diluted in polymerase dilution buffer [25 mM potassium phosphate (pH 7.4), 5 mM β-mercaptoethanol, 1 mM EDTA, 10% (v/v) glycerol, 0.1 mg/ml bovine serum albumin] prior to use. T4 polynucleotide kinase, the Klenow fragment of DNA polymerase I, uracil-DNA glycosylase (UDG), and terminal deoxynucleotidyl transferase (TdT) were purchased from Fermentas. Moloney murine leukemia virus (MMLV) reverse transcriptase was obtained from Invitrogen.

3.2.3 Cells and virus. All cells and virus were obtained from the American Type Culture Collection. MRC-5 human lung fibroblasts were grown in minimal essential medium with Earle's salts containing 2% (v/v) fetal bovine serum. BSC40 African green monkey kidney epithelial cells and vaccinia virus [Western Reserve (WR) strain] were cultured in minimal media containing 5% (v/v) fetal bovine serum, 1% (v/v) amino acids, 1% (v/v) L-glutamine, and 1% (v/v) antibiotic/antimycotic. Cells were maintained at 37°C in a 5% CO_2 atmosphere.

3.2.4 Plaque reduction assays. Plaque reduction assays were performed in triplicate using 200 plaque-forming units (pfu) of virus per 60-mm dish. Virus-infected BSC40 cells were cultured for 48 h and then stained with crystal violet. The 50% effective concentration (EC_{50}) was calculated from a nonlinear curve fit using Prism 4.0b software.

3.2.5 Slot blot hybridization. BSC40 cells were infected with vaccinia virus at a multiplicity of infection of 10 in 60-mm dishes. The cells were then incubated at 37°C and harvested at the times indicated by scraping and centrifugation. [To examine the effects of drugs on virus replication, cells were preincubated with *(S)*-HPMPA or CDV for 24 h prior to infection, and then more drug added after the virus was added.] The cell pellets were washed and resuspended in 1.5 ml of 10X saline sodium citrate containing 1 M ammonium acetate and stored at -80°C. The samples were frozen and thawed three times and clarified by centrifugation, and then 25-µl aliquots were mixed with an equal volume of 0.8 M NaOH plus 20 mM EDTA, boiled for 10 min, cooled on ice, and diluted with 125 µl of 0.4 M NaOH and 10 mM EDTA. The samples were applied in duplicate to a Zeta-Probe membrane (Bio-Rad) by using a vacuum manifold, washed, and immobilized with UV light. A 3.1 kb probe spanning the DNA polymerase gene was prepared using the PCR (Andrei *et al.*, 2006; Yao and Evans, 2004), purified, and labeled with [α-^{32}P]dATP using a random priming labeling kit (Roche). The membrane was processed using a Southern blot hybridization procedure (Sambrook and Russell, 2001) and label detected by using a Typhoon phosphorimager.

3.2.6 DNA polymerase and exonuclease assays. Oligonucleotide primer-template pairs (Figure 3.1) were used as substrates for DNA polymerase and exonuclease assays as previously described (Chapter 2; Magee *et al.*, 2005). The primers were first end-labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. Reaction products were resolved on 10 to 15% polyacrylamide gels and also analyzed as described previously (Chapter 2; Magee *et al.*, 2005). To determine the K_m and V_{max} values for *(S)*-HPMPApp, we prepared 10-µl reaction mixtures containing ~9 pmol of ^{32}P-labeled primer P1, 35 pmol of template T11, 25 ng of vaccinia polymerase, 10 µM dGTP, various concentrations of dATP [or *(S)*-HPMPApp], and polymerase buffer [30 mM Tris · HCl (pH 7.9), 5 mM $MgCl_2$, 70 mM NaCl, 1.8 mM dithiothreitol, 80 µg/ml bovine serum albumin]. The reaction products were separated on 15% polyacrylamide gels and analyzed using phosphorimaging. To determine the fidelity of drug incorporation, 10-µl reactions were prepared containing 1 pmol of 5′-^{32}P end-labeled primer P1, 3 pmol of template DNA

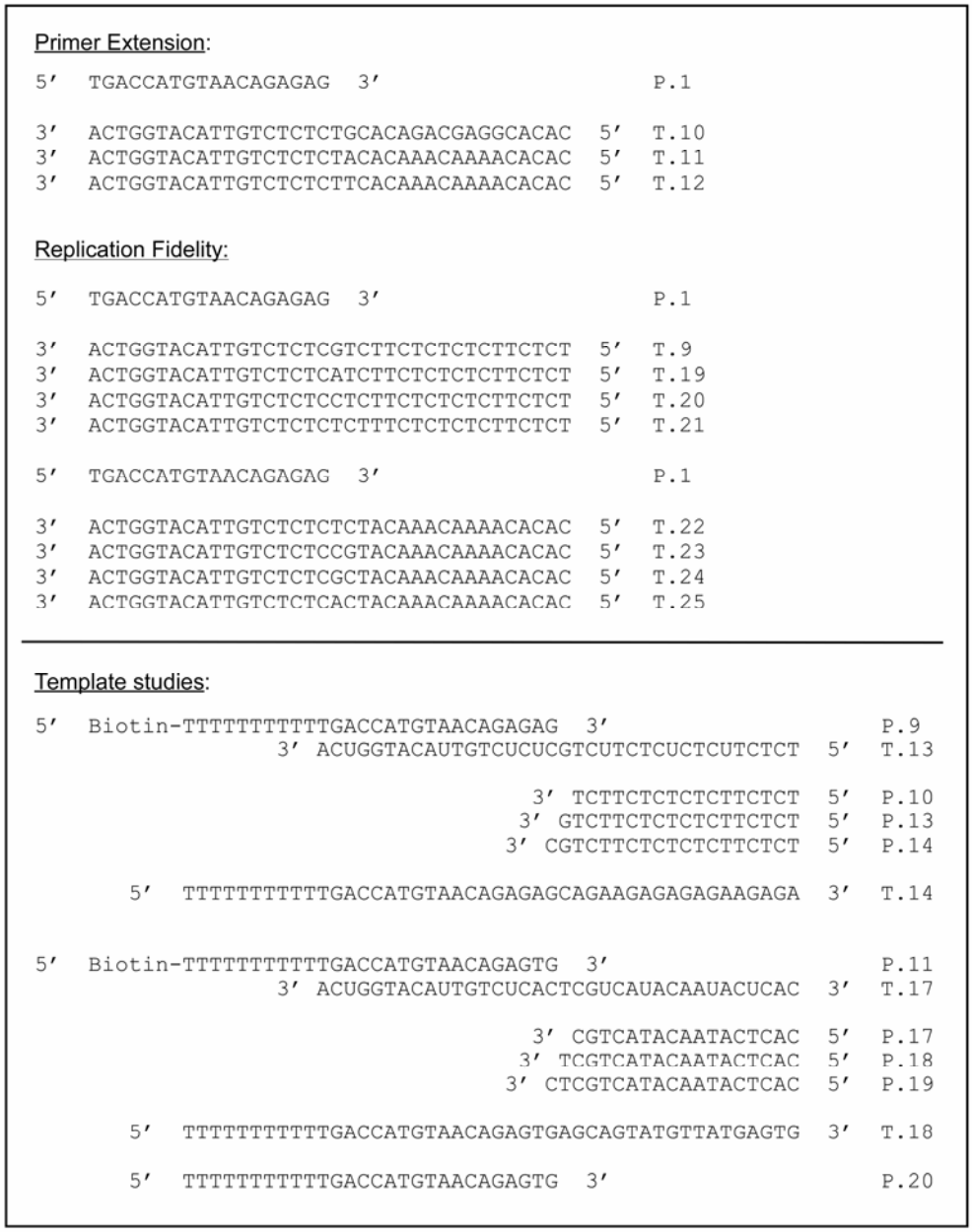

Figure 3.1 Oligonucleotide primer-template pairs used in the present study. Primer P1 was originally described by Xiong *et al.* (1997). The primer DNA was 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase prior to annealing with the template DNA.

(templates T9 or T19 to 25, Figure 3.1), 10μM CDVpp or *(S)*-HPMPApp, buffer, and 25 ng of vaccinia virus DNA polymerase. In controls, dCTP and dATP were substituted for CDVpp and *(S)*-HPMPApp, respectively. Reactions were incubated for 1 min at 37°C for CDVpp and dCTP reactions and at 25°C for *(S)*-HPMPApp and dATP reactions and stopped by adding 5 μl of gel loading buffer.

3.2.7 Preparation of CDV- and *(S)*-HPMPA-containing templates. CDV-containing templates were prepared using a two-step procedure. First, ~25 pmol of primer P9 was annealed to a threefold excess of the deoxyuridine-containing template T13 (Figure 3.1), followed by incubation at 37°C for 5 min in a reaction containing 25 μM (each) CDVpp (or dCTP), dATP, and dGTP and 2.5 ng vaccinia virus DNA polymerase/μl in polymerase buffer. The reactions were stopped by adding EDTA (to 45 μM), and the DNA was purified by using chloroform extraction and G-25 MicroSpin columns (GE Healthcare). The DNA was incubated at 37°C for 15 min with 1 U of UDG and then heated at 95°C for 10 min to cleave apyrimidinic sites. The reactions were extracted with phenol and chloroform, and the DNA precipitated with ethanol. An aliquot of this DNA was also labeled using TdT and [α-^{32}P]3′-deoxyATP to assess the extent of primer extension.

(S)-HPMPA-containing templates were prepared in a similar way except that *(S)*-HPMPA was incorporated using a different primer-template pair plus one additional enzymatic step. About 30 pmol of primer P11 was mixed with a threefold excess of template T17 (Figure 3.1), and the primer then extended by two residues at 37°C for 1 h in a reaction containing 10 μM each *(S)*-HPMPApp and dGTP, 0.01 M dithiothreitol, first-strand buffer, and 8 U of MMLV reverse transcriptase/μl. The DNA was purified using chloroform extraction and G-25 MicroSpin columns and precipitated with ethanol. This *(S)*-HPMPA-containing primer was then converted to a full-length extension product using vaccinia polymerase and all four dNTPs, purified, treated with UDG, purified again, and characterized as described above. A template containing dAMP (instead of *(S)*-HPMPA) was prepared the same way, except that the reverse transcriptase step was omitted.

3.2.8 DNA polymerase assays using CDV- or *(S)*-HPMPA-containing templates. Enzyme substrates were prepared by annealing 5′-end-labeled primers to drug-containing templates in a ~3:1 (template/primer) molar ratio. These DNAs were added to reactions containing the indicated dNTPs (50 μM each), polymerase buffer, and 2.5 ng vaccinia DNA polymerase/μl and incubated at 37°C. The reactions were stopped by adding EDTA and the biotinylated strands (and hybridized DNAs) recovered by using M-280 streptavidin Dynabeads as directed by the manufacturer (Invitrogen). The products were then size fractioned and detected by phosphorimaging. Size standards were generated by using dideoxy-sequencing reactions and Klenow DNA polymerase plus the same 5′ end-labeled primers used in the vaccinia DNA polymerase assays annealed to templates T14 and T18 (Figure 3.1) (Sambrook and Russell, 2001).

3.2.9 Cell uptake and HPLC analysis of *(S)*-HPMPA and CDV metabolites. *(S)*-[8-^{14}C]HPMPA and HDP-*(S)*-[8-^{14}C]HPMPA (3 μM) were added to 24-well plates containing MRC-5 fibroblasts, and after 24 h at 37°C the media was removed, the cell monolayer was washed twice with cold phosphate-buffered saline, and cell uptake of drug was assessed by liquid scintillation counting in quadruplicate as previously described (Aldern *et al.*, 2003). For measurement of *(S)*-HPMPA and HDP-*(S)*-HPMPA conversion to their mono- and diphosphates, radioactive drugs were added to 25-cm^2 flasks of near-confluent MRC-5 cells (10 μM), followed by incubation for 24 h. The medium was removed, and the monolayer washed twice with cold phosphate-buffered saline, followed by the addition of 0.6 ml of distilled water. The flasks were twice frozen and thawed and sonicated for 5 min in a cold sonicator bath, and the flask contents scraped into a glass tube. Cold trichloroacetic acid was added to a final concentration of 8% and the contents vortex mixed and centrifuged for 10 min at 4°C. The supernatant was removed, an aliquot was subjected to liquid scintillation counting, and another aliquot (10,000 dpm) was subjected to high-pressure liquid chromatography analysis as previously described (Aldern *et al.*, 2003). The method used a Partisil 10 SAX column (4.6 x 15 cm), with SAX guard column, equilibrated with 20 mM potassium phosphate buffer (pH 5.8) and operating at a flow rate of 1 ml/min. The sample was applied to the

column and, after 9 min of isocratic operation, eluted with a 20 to 700 mM potassium phosphate buffer gradient, over 20 min, followed by a 5-min terminal hold. Each 1-ml fraction was collected and analyzed by liquid scintillation counting using FloScint IV fluid. The retention times of [2-^{14}C]CDVpp (25 to 27 min) (Aldern *et al.*, 2003) and *(S)*-[8-^{14}C]HPMPApp (32 to 33 min) were identical to that of pure reference standards. The data shown are the average of two separate determinations.

3.3 RESULTS

3.3.1 Effect of *(S)*-HPMPA on vaccinia DNA replication. *(S)*-HPMPA inhibits viral DNA replication *in vitro,* but at lower concentrations than seen using CDV. A plaque reduction assay was first performed to determine the EC_{50} values for each drug against vaccinia virus strain WR in BSC40 cells (Figure 3.2). These values were determined to be 18 μM for *(S)*-HPMPA and 46 μM for CDV, confirming the greater efficacy of *(S)*-HPMPA against vaccinia virus (De Clercq *et al.*, 1987; Keith *et al.*, 2003; Lebeau *et al.*, 2006; Snoeck *et al.*, 2002) and similar to values determined previously using vaccinia virus strain Copenhagen in MRC-5 cells (Table 3.1). To determine the effect of *(S)*-HPMPA and CDV on virus DNA synthesis we then tested two concentrations of each drug corresponding to the EC_{50} and twice the EC_{50}. BSC40 cells were pretreated (or mock treated) with drug for 24 h prior to infection and then infected with virus while maintaining the drug selection pressure. The cells were harvested, and the amount of viral DNA accumulated was determined at different time points by slot blot hybridization using a ^{32}P-labeled vaccinia gene probe. Figure 3.3 shows the results of this experiment. No viral DNA was detected in any of the mock-infected samples or in any of the infected cell samples at 2 h postinfection (Figure 3.3, top panel). In drug-free media, viral DNA began to be detected at 4 h postinfection, followed by a large accumulation at 6 h postinfection. As predicted, CDV and *(S)*-HPMPA caused a substantial reduction in DNA synthesis relative to what was detected in cells infected with virus in the absence of drug. Moreover, DNA replication was inhibited to a similar extent when cells were treated with concentrations of drug corresponding to the respective EC_{50}s or to twice their respective EC_{50}s (Figure 3.3, bottom panel). Thus, both drugs cause comparable effects

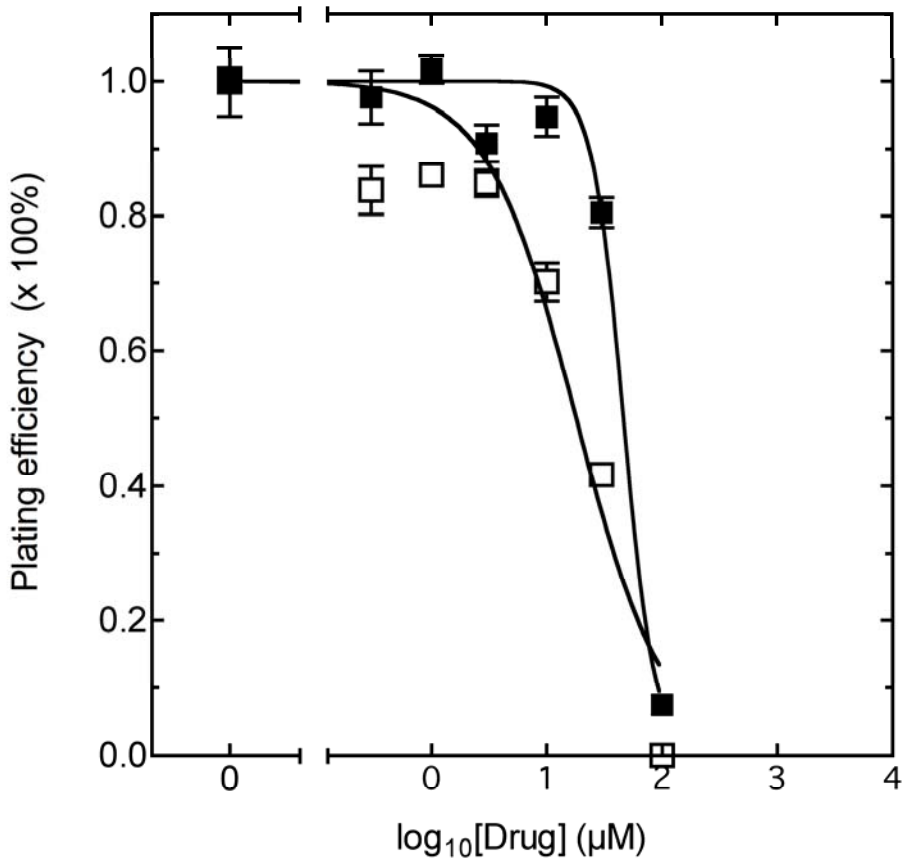

Figure 3.2 Plaque reduction assay. BSC40 cells were infected with vaccinia WR in the presence of increasing concentrations of CDV (-■-) or *(S)*-HPMPA (-□-), or in the absence of drug. Each sample was assayed in triplicate. After culturing for 48 h, the cells were stained with crystal violet and the number of plaques counted. The plating efficiency at each drug concentration was determined relative to the plating efficiency in the absence of drug (100%). The 50% effective concentrations (EC_{50}) were calculated from a sigmoidal dose-response curve fit using Prism 4.0b software.

Table 3.1 Metabolic properties of CDV, *(S)*-HPMPA and their hexadecyloxypropyl esters and antiviral activity against vaccinia virus strain Copenhagen *in vitro*[1,2]

	Cell uptake			Anabolic phosphorylation					Antiviral activity	
Drug	**Drug concn (μM)**	**Mean uptake (pmol/well) ± SD**	**Source or ref.**	**Drug concn (μM)**	**N**	**Np**	**Npp**	**Source or ref.**	**Mean EC_{50}, (μM) ± SD**	**Source or ref.**
CDV	3.0	3.60 ± 0.29 (3)	Aldern *et al.*, 2003	10	274	1.0	1.3	Aldern *et al.*, 2003	46 ± 12 (2)	Kern *et al.*, 2002
HDP-CDV	3.0	187 ± 12.0 (2)	Aldern *et al.*, 2003	10	697	63	132	Aldern *et al.*, 2003	0.80 ± 0.40 (2)	Kern *et al.*, 2002
(S)-HPMPA	3.0	2.77 ± 0.20 (4)	This study	10	29	6.0	21	This study	2.70 ± 2.40 (2)	Beadle *et al.*, 2006
HDP-*(S)*-HPMPA	3.0	155 ± 16 (4)	This study	10	93	68	451	This study	0.01 ± 0.004 (2)	Beadle *et al.*, 2006

[1] Drug uptake was measured in MRC-5 fibroblasts in 24-well plates 24 h after each compound was added to the culture media. Anabolic phosphorylation was assessed in MRC-5 cells grown in T-75 flasks, 24 h after the addition of ^{14}C-labeled versions of each compound. Abbreviations: N, nucleotide; Np, nucleotide monophosphate; Npp, nucleotide diphosphate.

[2] Values in parentheses are the numbers of independent replicates.

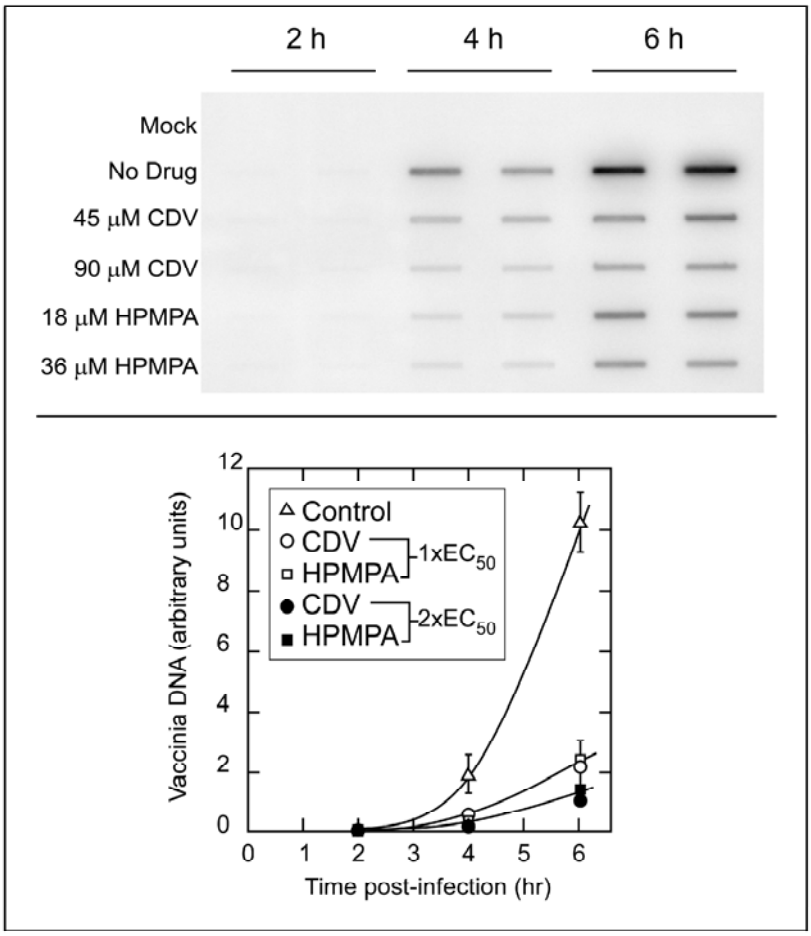

Figure 3.3 Effect of CDV and *(S)*-HPMPA on vaccinia virus DNA synthesis *in vitro.* BSC40 cells were cultured for 24 h, with or without the indicated concentrations of drugs, and then infected with vaccinia WR at a multiplicity of infection of 10. Fresh culture media was added, also containing CDV or *(S)*-HPMPA where indicated, and then all of the nucleic acids recovered at times 2, 4, or 6 h postinfection. A slot blotting protocol followed by phosphorimager analysis (top panel), was used to quantify the amount of accumulated viral DNA [in arbitrary units] using a ^{32}P-labeled vaccinia DNA polymerase gene as a probe (bottom panel). The concentrations of each drug used correspond to the EC_{50} (18 and 45 μM) and twice the EC_{50} (36 and 90 μM) for *(S)*-HPMPA and CDV, respectively.

on viral replication, but less *(S)*-HPMPA is required than CDV in the culture media.

3.3.2 Cellular uptake and metabolism of *(S)*-HPMPA and CDV and their hexadecyloxypropyl esters. To further evaluate the reasons for the much greater inhibitory effect of *(S)*-HPMPA and HDP-*(S)*-HPMPA, versus CDV and HDP-CDV, we measured the uptake and conversion of these compounds to their diphosphates (the triphosphate equivalent and the active metabolite) as shown in Table 3.1. The cell uptake of CDV and *(S)*-HPMPA was comparable (4.0 versus 2.8 pmol/well), but the conversion of *(S)*-HPMPA to the diphosphate in 24 h was 16-fold greater than that of CDV (21 versus 1.3 pmol/plate). Esterification with hexadecyloxypropyl increased the cell uptake of both CDV and *(S)*-HPMPA by 47- and 55-fold, respectively. However, the amount of *(S)*-HPMPApp formed was still greater than CDVpp, (451 versus 132 pmol/flask) (Aldern *et al.*, 2003). Thus, the intracellular level of CDVpp and *(S)*-HPMPApp correlate roughly with antiviral efficacy of the compounds shown in Table 3.1.

3.3.3 *(S)*-HPMPApp is a weak chain terminator. These observations suggested that the greater bioactivity of *(S)*-HPMPA, relative to CDV, is at least partially explained by differences in the uptake and conversion of the two drugs to their diphosphoryl derivatives. Nevertheless, we decided to test the effect of *(S)*-HPMPApp on vaccinia DNA polymerase, to confirm that *(S)*-HPMPApp and CDVpp have comparable effects on this enzyme. Curiously they do not.

We first examined what happens when *(S)*-HPMPApp is added to a primer extension assay in a reaction containing an 18-nucleotide ^{32}P-labeled primer annealed to a 36-nucleotide template (primer P1 and template T11, Figure 3.1). The reactions also contained all four dNTPs at concentrations approximating those observed *in vivo* (5 μM dATP, 10μM dCTP, 10 μM dGTP and 10 μM dTTP) and were incubated at 37°C. When 10 μM *(S)*-HPMPApp was added to the reactions, it caused a very weak stop at the N + 1 position, where N is the expected site of incorporation of *(S)*-HPMPA opposite a dTMP residue in the template (compare Figure 3.4A and 3.4B). We also noted that adding *(S)*-HPMPApp caused a slight reduction in the production of molecules terminated at the N,

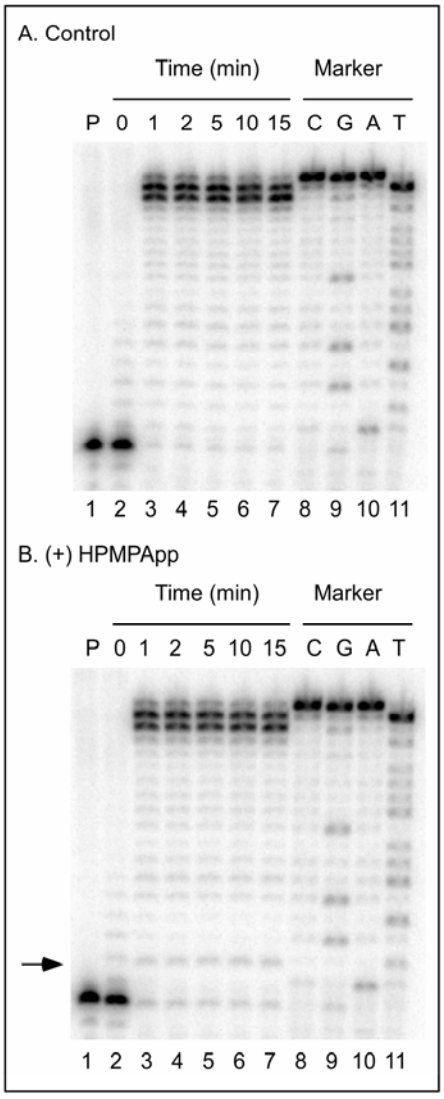

Figure 3.4 *(S)*-HPMPApp is a weak inhibitor of primer extension assays. The reactions contained ^{32}P-labeled primer P1 annealed to template T11, four dNTPs (5 μM dATP, 10μM dCTP, 10 μM dGTP and 10 μM dTTP), 2.5 ng of vaccinia DNA polymerase/μl, and 0 μM (A) or 10 μM (B) *(S)*-HPMPApp. The mixture was incubated at 37°C, and samples removed at the indicated time points and mixed with a formamide-containing stop/loading buffer. The reaction products were then separated on 10% denaturing polyacrylamide gels and the radioactivity detected by using a phosphorimager. Lanes numbered 1 show the position of the labeled primer. Size markers were prepared by using dideoxy sequencing reactions and Klenow DNA polymerase (lanes 8 to 11). The band corresponding to the *(S)*-HPMPA + 1 extension product was seen (arrowed, panel B) but comprised ~1.4% of the total label in each of lanes 3 to 7. The same band comprised ~0.3% of the extension products in panel A.

N + 2, and N + 3 positions compared with the ladder of incomplete extension products seen in the control reaction. Since adding *(S)*-HPMPApp to the primer extension reaction produced only a weak stop, whereas adding CDVpp to a similar reaction mixture resulted in a strong stop one nucleotide after a template dG (Chapter 2; Magee *et al.*, 2005), we investigated whether the yield of premature termination products was affected by the *(S)*-HPMPApp concentration. No substantial differences in the intensity of the N + 1 (or other) termination products were detected using *(S)*-HPMPApp concentrations varying from 0.1 μM to 100 μM (Figure 3.5).

3.3.4 Vaccinia DNA polymerase can use *(S)*-HPMPApp as a substrate and extend a primer containing *(S)*-HPMPA. We next wanted to compare the substrate properties of *(S)*-HPMPApp versus dATP using the P1-T11 primer-template combination. These experiments were complicated by the fact that, in the absence of other dNTPs, both compounds were incorporated into DNA and then sometimes excised so rapidly as to cause the complete degradation of the labeled primer strand in less than a minute at 37°C (Figure 3.6). After some experimentation with reaction temperatures ranging from 0 to 25°C (Figure 3.7), we found that the problem can be avoided by incubating the reactions at 25°C (Figure 3.8). This sufficiently slows the reaction to permit ready detection of both polymerase and exonuclease activities under steady-state conditions, and most of the subsequent experiments were performed at 25°C unless otherwise noted.

These assays were next used to determine the K_m and V_{max} for *(S)*-HPMPApp and for dATP. The ^{32}P-labeled primer P1 was annealed to template T11, mixed with various concentrations of *(S)*-HPMPApp or dATP, followed by incubation at 37°C for 0, 1, 2, or 4 min with vaccinia DNA polymerase. We also added the nucleotide found at the 3′-end of the P1 strand to each reaction (dGTP, 10 μM) to minimize attack on that end by the 3′-to-5′ exonuclease and permit use of a 37°C reaction temperature. The reaction products were separated by using a 15% polyacrylamide gel and detected by using a phosphorimager. The amount of primer extended by one nucleotide in each reaction was determined by using ImageQuant software, and the results were analyzed using one-phase exponential association, to determine an initial velocity, and the Michaelis-Menten

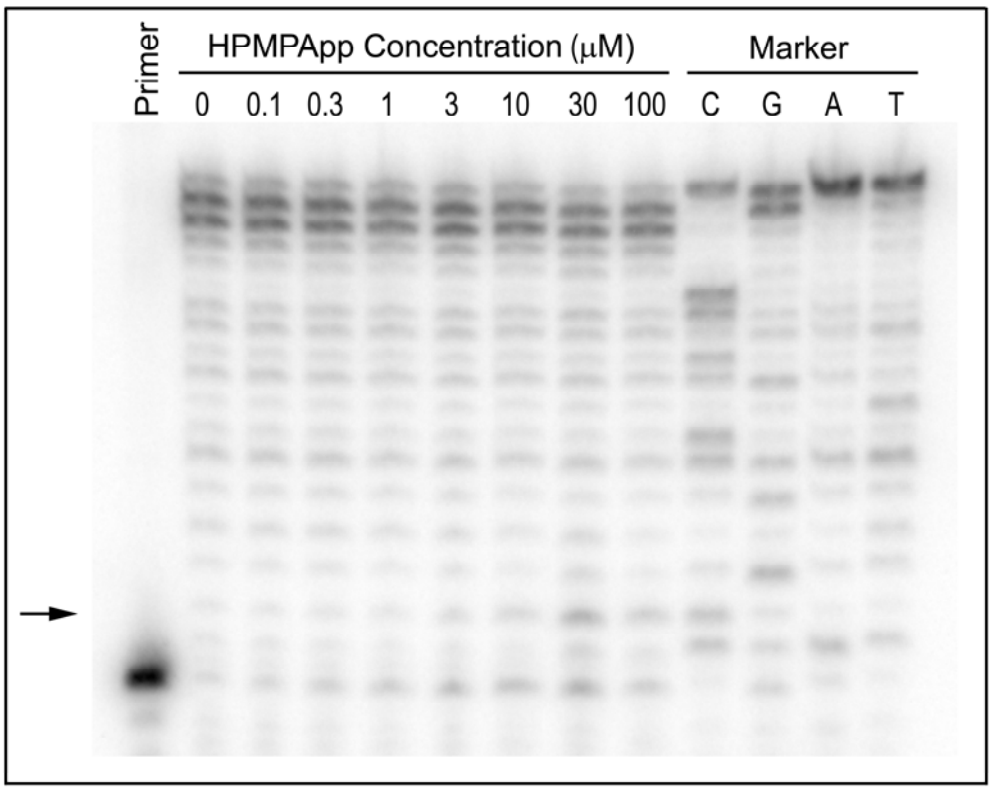

Figure 3.5 The effect of *(S)*-HPMPApp concentration on the yield of premature termination products. Primer P1 was labeled with ^{32}P, annealed to template T10, and incubated at 37°C in the presence of four dNTPs (5 μM dATP, 10μM dCTP, 10 μM dGTP and 10 μM dTTP) and 2.5 ng of vaccinia DNA polymerase/μl. Reactions were further supplemented with *(S)*-HPMPApp concentrations ranging from 0 to 100 μM as indicated. After incubation for 10 min, the reactions were stopped by the addition of gel loading buffer and the reaction products were size fractionated on a 10% denaturing polyacrylamide gel. A DNA sequencing ladder shown on the right was prepared for use as a size marker. The *(S)*-HPMPA + 1 product is indicated by the arrow.

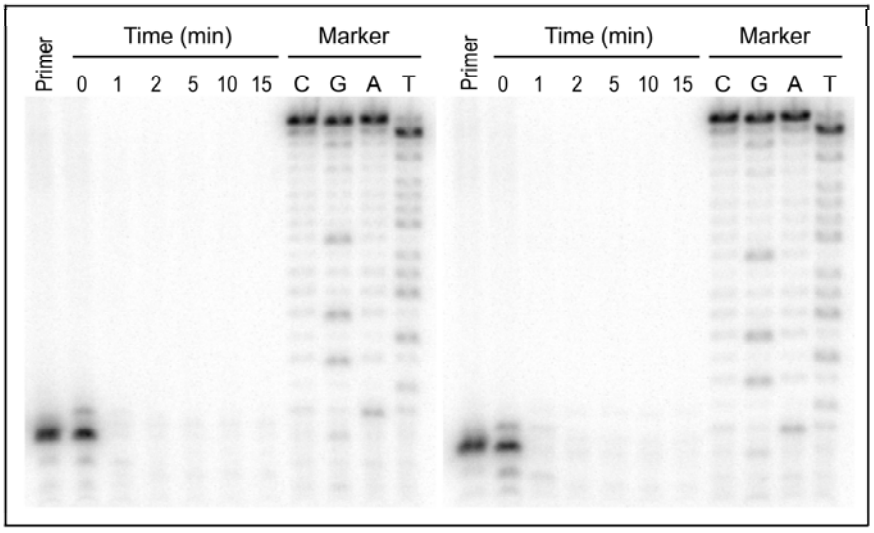

Figure 3.6 Kinetics of dATP versus *(S)*-HPMPApp incorporation at 37°C. Primer template pair P1-T11 was incubated with vaccinia virus DNA polymerase and either 10 μM dATP (left panel) or 10 μM *(S)*-HPMPApp (right panel). The reaction mixtures were sampled at 0, 1, 2, 5, 10, and 15 min; the reactions were stopped; and then the mixtures were subjected to denaturing polyacrylamide gel analysis. Both compounds are rapidly incorporated into DNA and then rapidly degraded in less than 1 min.

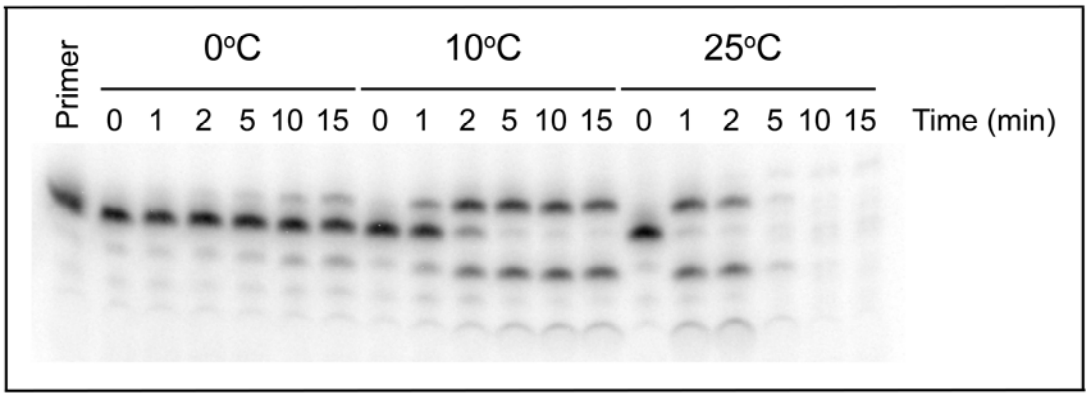

Figure 3.7 The effect of reaction temperature on the incorporation of *(S)*-HPMPApp. The incorporation of *(S)*-HPMPApp into DNA was examined at 0°C, 10°C, and at 25°C. Primer-template pair P1-T11 was incubated at the indicated temperatures with 10 μM of *(S)*-HPMPApp and 2.5 ng of vaccinia DNA polymerase /μl for reaction times ranging from 0 to 15 min. After stopping each reaction with gel loading buffer, the reaction products were subjected to denaturing polyacrylamide gel electrophoresis and phosphorimager analysis.

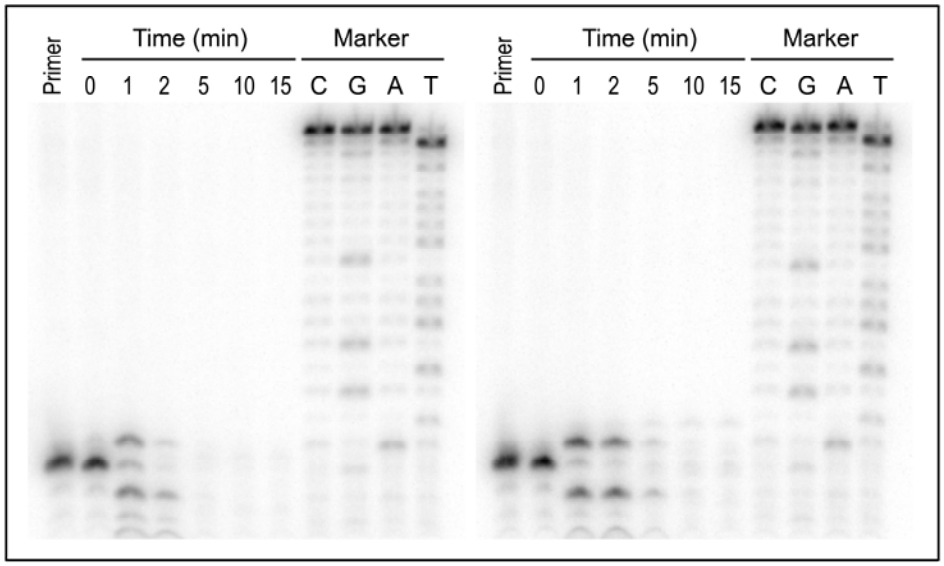

Figure 3.8 Kinetics of dATP versus *(S)*-HPMPApp incorporation at 25°C. Reaction mixtures were prepared as described in the legend to Figure 3.6 except that the incubation temperature was 25°C instead of 37°C. The incorporation of both compounds and their subsequent degradation are slowed sufficiently to allow detection of both processes.

equation. The K_m of *(S)*-HPMPApp was calculated to be 3.8 ± 0.8 μM and the V_{max} was calculated at 2.1 ± 0.1 pmol/min. The K_m and V_{max} of dATP were determined to be 4.6 ± 0.5 μM and 2.0 ± 0.07 pmol/min, respectively. These data indicate that *(S)*-HPMPApp is as good a substrate for vaccinia DNA polymerase as is dATP.

Having shown that *(S)*-HPMPApp is a good substrate for vaccinia virus DNA polymerase, we next examined what effect the presence of the drug near the primer terminus would have on chain extension. Of particular interest was the effect of *(S)*-HPMPA when incorporated into the penultimate position of the primer strand ("*(S)*-HPMPA + 1"), since it is the CDV + 1 structure that is less well used by vaccinia and herpes DNA polymerases. Figure 3.9A shows such a stepwise comparison of the substrate properties of dAMP versus *(S)*-HPMPA at 25°C. As noted above, *(S)*-HPMPA can be incorporated into DNA with kinetics resembling dATP. The next nucleotide (dTTP), was then added to the *(S)*-HPMPA-terminated primer in a manner also similar to that seen in the dAMP-terminated control reaction. Finally, when an equimolar mixture of dCTP, dGTP, and dTTP was added to each of these reactions (to 30 μM total concentration) the primer was rapidly extended out to the end of the template strand. We noted that there might be a slight lag in the production to the full-length products from a primer terminating in *(S)*-HPMPA plus dTMP compared with extension from a primer terminating in dAMP plus dTMP (Figure 3.9A). Nevertheless, nearly all of the *(S)*-HPMPA-containing labeled primer was rapidly chased into higher-molecular-weight products within moments of starting the reactions.

3.3.5 Vaccinia DNA polymerase can use primers containing two consecutive *(S)*-HPMPA molecules. CDV has been shown to greatly reduce the rate of primer extension by vaccinia virus and human cytomegalovirus (HCMV) DNA polymerases whenever the template contains two consecutive dG residues (Chapter 2; Magee *et al.*, 2005; Xiong *et al.*, 1997). These data have led to the suggestion that CDV might be especially prone to causing chain termination whenever it is incorporated into adjacent sites in the primer (De Clercq, 2004) and led us to test whether *(S)*-HPMPA might have similar effects on vaccinia DNA polymerase. For this experiment, a ^{32}P-labeled primer P1 was annealed to

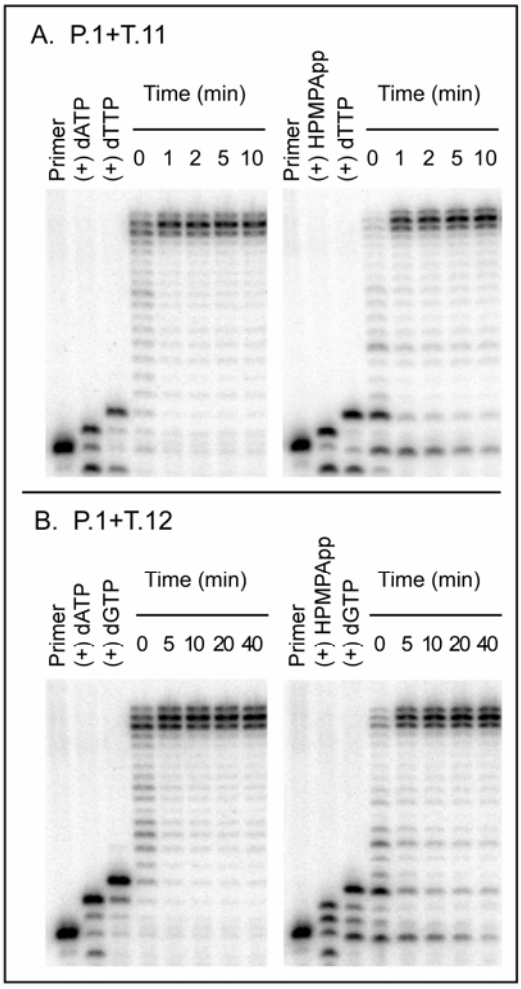

Figure 3.9 *(S)*-HPMPA can be incorporated into DNA and extended by vaccinia DNA polymerase. (A) Primer P1 was labeled with ^{32}P, annealed to template T11, and incubated for 1 min with 2.5 ng of vaccinia DNA polymerase/µl plus 10 µM dATP or *(S)*-HPMPApp at 25°C. A sample of the product was removed from each reaction and added to formamide stop buffer. dTTP was then added to each of the remaining mixtures, to a final concentration of 10 µM, and the incubation continued for another minute. A second sample was removed, followed by the addition of dCTP, dGTP and dTTP (all to 10 µM final concentration), and the incubation was continued with periodic sampling. The reaction products were then size fractionated and detected by phosphorimaging. A slight lag may be seen in the extension of molecules terminated by *(S)*-HPMPA + dGMP, but the majority of the primer chases into a series of extension products in less than a minute at 25°C. (B) Experiment similar to that in panel A except that template T12 directs the incorporation of two consecutive molecules of dAMP or *(S)*-HPMPA.

template T12 (Figure 3.1) and used as an alternative substrate. The P1-T12 primer-template was incubated with vaccinia DNA polymerase and with either 10 μM dATP or 10 μM *(S)*-HPMPApp (Figure 3.9B) for one minute. A sample was taken of each reaction, and the next nucleotide (dGTP) was added to the remainder to a final concentration of 10 μM. After another minute of incubation, a sample was taken, and the remaining reaction mixture was adjusted to include 10 μM each for dTTP and dCTP. The reaction was then sampled at different time points. We observed that vaccinia DNA polymerase can readily incorporate two consecutive molecules of dATP into DNA, extend this primer further by one nucleotide, and then rapidly extend this primer in the presence of all four dNTPs. Replacing dATP with *(S)*-HPMPApp produced similar results. Although there is again a slight lag on the initial production of fully extended primer products, as seen by the decreased intensity of the full-length bands in the first minute, the incorporation of two consecutive *(S)*-HPMPA molecules into the growing DNA strand did not cause a dramatic reduction in the rate of DNA elongation compared with two molecules of CDV (Chapter 2; Magee *et al.*, 2005).

3.3.6 Effect of (S)-HPMPA on the 3′-to-5′ proofreading exonuclease activity. To determine what effect *(S)*-HPMPA has on the 3′-to-5′ exonuclease activity of vaccinia DNA polymerase, the 5′-to-3′ polymerase activity was first used to incorporate *(S)*-HPMPA (or dAMP) into the terminus of ^{32}P-labeled primer P1 annealed to template T11. We also added dTTP to some reactions to position the *(S)*-HPMPA/dAMP moiety at the penultimate location in the primer strand. Unincorporated nucleotides and *(S)*-HPMPApp were removed by gel filtration. The substrates were then incubated with fresh DNA polymerase, in the absence of dNTPs and at 25°C, to examine exonuclease activity in the absence of any polymerase activity. Figure 3.10 (left panel) shows the results obtained when the enzyme is presented with substrates incorporating dAMP or *(S)*-HPMPA at the 3′-primer terminus. A primer terminated with dAMP was completely converted to an array of smaller products by vaccinia DNA polymerase in less than a minute. A primer terminated with *(S)*-HPMPA was also rapidly degraded although we noted that there was still a trace of the *(S)*-HPMPA-terminated primer band visible after 1 min of incubation

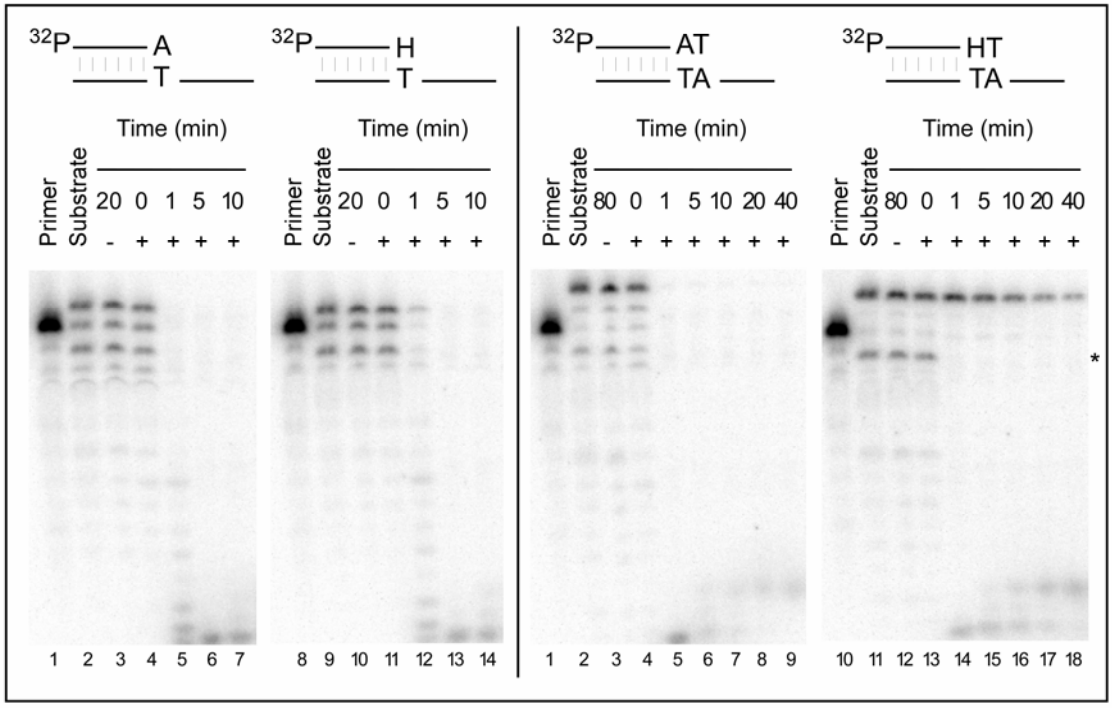

Figure 3.10 Vaccinia DNA polymerase can excise *(S)*-HPMPA from the primer terminus but not if *(S)*-HPMPA is the penultimate 3′-nucleotide. Primer P1 was labeled with ^{32}P, annealed to template T11, and incubated for 1 min with 2.5 ng of vaccinia DNA polymerase/µl at 25°C in the presence of 10 µM dATP, 10 µM *(S)*-HPMPApp, 10 µM (each) dATP and dTTP or 10 µM (each) *(S)*-HPMPApp and dTTP. This produced molecules bearing the 3′ structures indicated in the Figure ("H" = *(S)*-HPMPA). The unincorporated nucleotides and *(S)*-HPMPApp were removed by gel filtration, and the purified substrates incubated with fresh enzyme at 25°C. The reactions were sampled at the times indicated, mixed with formamide stop buffer, and size fractionated on 10% polyacrylamide gels, and the radioactivity was detected by phosphorimaging. Water was substituted for DNA polymerase in the no polymerase controls (indicated with "-" symbols). Primers terminated with *(S)*-HPMPA + dTMP are highly resistant to exonuclease attack (lanes 14 to 18, at right), but did not inhibit exonuclease attack on a trace of contaminating molecules terminated with dAMP or *(S)*-HPMPA (asterisk).

(Figure 3.10, left, lane 12). A primer terminating in dAMP plus dTMP was also rapidly degraded by the vaccinia DNA polymerase (Figure 3.10, right panel) with nearly complete conversion of this primer to smaller products seen after a minute of incubation. In contrast, a primer terminating in *(S)*-HPMPA plus dTMP was refractory to exonuclease activity. We estimate that the half-life of this product at 25°C is about 7 min (Figure 3.11), and although the exonuclease is more active if the reaction is incubated at 37°C (Figure 3.12), the half-life is still at least ~4 min (Figure 3.11). It should also be noted that these *(S)*-HPMPA-containing molecules are not irreversible inhibitors of the exonuclease activity. During the preparation of these substrates a small amount of a contaminating product is left behind that is one nucleotide smaller than the original primer P1 (Figure 3.10, asterisk). This DNA should bear either a 3′-terminal dAMP or *(S)*-HPMPA residue, and even in the presence of the molecules terminated with *(S)*-HPMPA + dTMP, it is completely degraded within a minute of adding the enzyme.

3.3.7 CDV and *(S)*-HPMPA are faithfully incorporated into DNA by vaccinia DNA polymerase. One mechanism that might account for the greater activity of *(S)*-HPMPA relative to CDV could be that *(S)*-HPMPA is a substrate that is especially prone to misincorporation by vaccinia polymerase. This would cause mutations and deleterious effects on replication. To examine whether *(S)*-HPMPA is misincorporated into DNA by this enzyme, a simple primer extension analysis was performed. For these reactions, ^{32}P-labeled primer P1 was annealed to different templates containing each of the four nucleotides located immediately after the primer terminus (Figure 3.1). These primer-template pairs were incubated with vaccinia DNA polymerase and 10 μM CDVpp (or dCTP) or 10 μM *(S)*-HPMPApp (or dATP) for 1 min. The results are presented in Figure 3.13, where it can be seen that CDV and dCMP are incorporated opposite only dG and *(S)*-HPMPA and dAMP are incorporated opposite only dT. Thus, under the conditions of this experiment (which we estimate could detect 1 to 2% misincorporation), both CDV and *(S)*-HPMPA are faithfully incorporated into DNA by vaccinia polymerase.

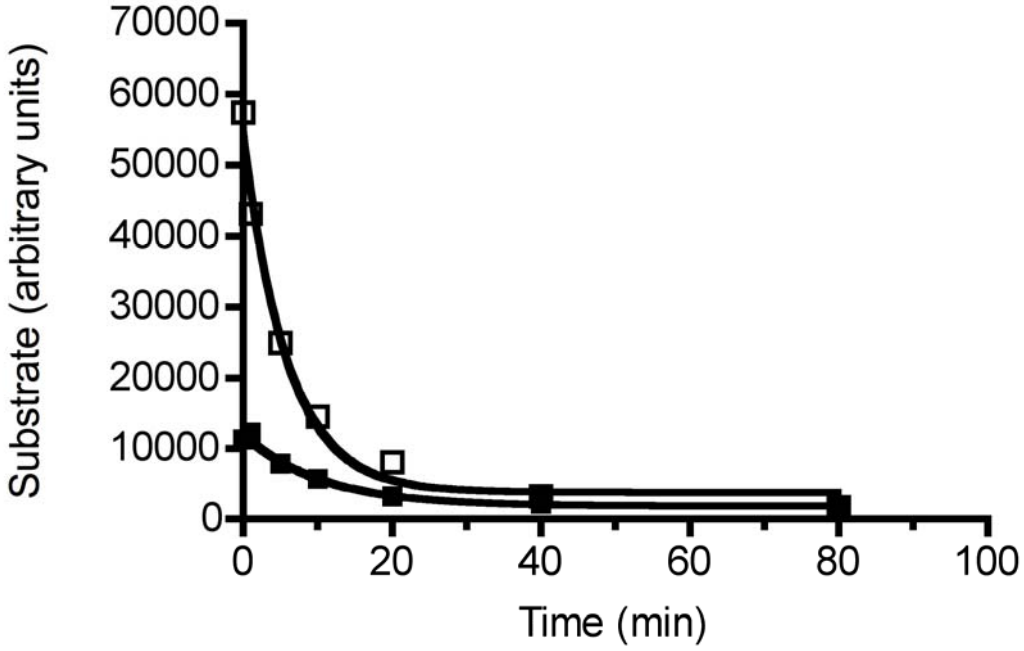

Figure 3.11 Degradation of the *(S)*-HPMPA + 1 product by the vaccinia DNA polymerase 3′-to-5′ proofreading exonuclease. The amount of the *(S)*-HPMPA + 1 product present at various times after incubation with vaccinia DNA polymerase at 25°C (-■-) or 37°C (-□-) was determined by phosphorimager analysis using ImageQuant software. The half-life of this product at each temperature was determined from a nonlinear fit of the data using Prism 4.0b software. These values were determined to be approximately 7 min and 4 min, respectively.

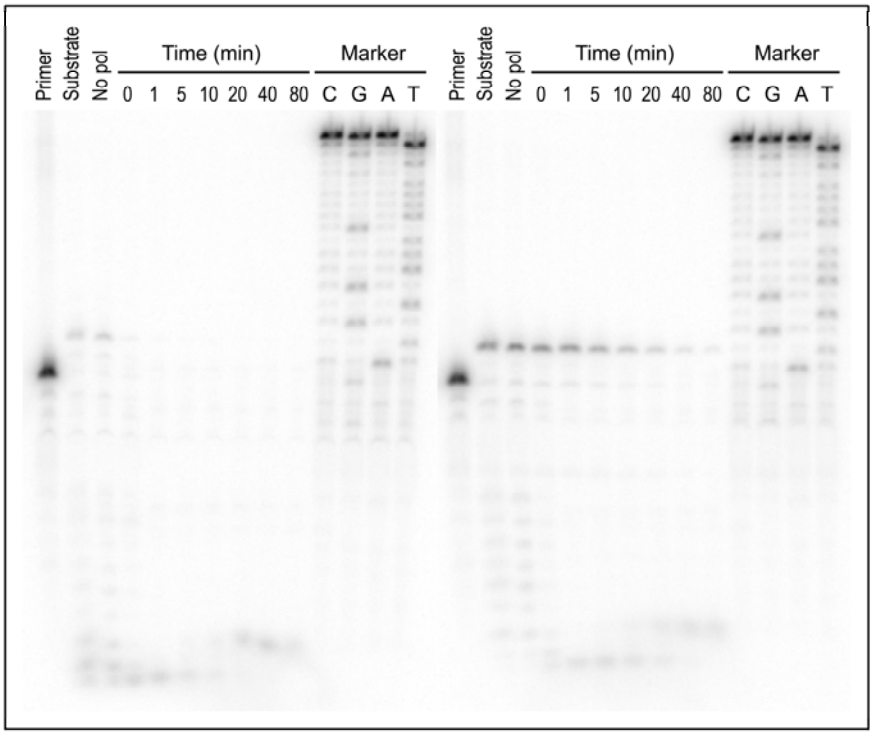

Figure 3.12 Excision of molecules terminating with dATP + 1 or *(S)*-HPMPA + 1 by vaccinia DNA polymerase at 37°C. Duplex P1-T11 was extended 2 nucleotides using either dATP and dTTP (left) or *(S)*-HPMPApp and dTTP (right) at 37°C and then purified free of reaction materials by gel filtration. The purified product (lanes marked "Substrate") was then incubated with vaccinia virus DNA polymerase at 37°C, in the absence of dNTPs, with sampling at 0, 1, 5, 10, 20, 40 and 80 min. Lanes marked "No pol" show 80 min reactions in which water was substituted for polymerase.

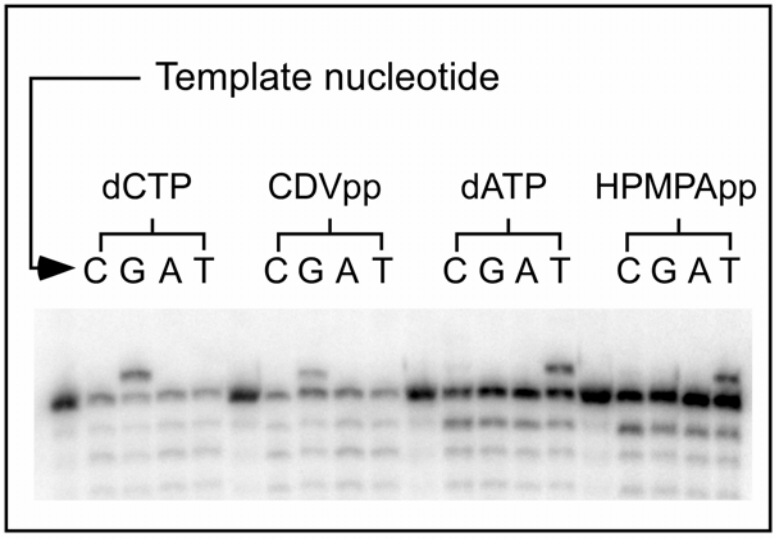

Figure 3.13 CDV and *(S)*-HPMPA are faithfully incorporated into DNA by vaccinia DNA polymerase. The reaction mixtures contained ^{32}P end-labeled primer P1 annealed to different template strands and were incubated with the indicated nucleotides (each at 10 μM) and 2.5 ng of vaccinia DNA polymerase/μl at 37°C (dCTP and CDVpp) or 25°C (dATP and *(S)*-HPMPApp). The nucleotide encoded by the template strand at the position immediately after the primer terminus is indicated on the figure. Each reaction was stopped after 1 min, and the products separated by gel electrophoresis and detected using a phosphorimager.

3.3.8 Preparation of oligonucleotide templates containing CDV or *(S)*-HPMPA. When one considers the data outlined above, one factor that differentiates *(S)*-HPMPA from CDV is that *(S)*-HPMPA could be more readily incorporated into DNA than CDV. This raises the interesting question of what effects the two drugs have on DNA synthesis when located in the template strand. Since there is currently no chemical method to synthesize these drug-containing DNAs, we used the enzymatic approach illustrated in Figure 3.14 to produce these templates.

In order to synthesize a CDV-containing template, the 5′ biotinylated primer, P9, was annealed to template T13 (Figure 3.1) and extended using vaccinia DNA polymerase and CDVpp (or dCTP for control purposes) plus dATP and dGTP. The T13 template strand contains seven uracil residues that permitted its degradation using uracil-DNA glycosylase and heat. The UDG-resistant, CDV-containing (or dCMP-containing) DNAs were then purified for use as template strands. Aliquots of the UDG-resistant template DNA was also end labeled with ^{32}P using TdT and cordycepin triphosphate in order to assess the extent of P9 extension.

The preparation of an *(S)*-HPMPA-containing template followed a similar procedure, using 5′ biotinylated primer P11 annealed to template T17. However, this approach was complicated by the fact that the uracil in the template can promote the incorporation of many *(S)*-HPMPA molecules, and attempts to add the drug in a stepwise manner, using vaccinia polymerase, were frustrated by exonuclease attack on the P11 primer. Therefore, we used MMLV reverse transcriptase to incorporate *(S)*-HPMPA plus the next nucleotide (dGMP) into the terminus of primer P11, purified the product, and generated a full-length copy using vaccinia polymerase and four dNTPs. The product was then processed as described above. We tried using the same method to prepare a dAMP-containing control template, but the extensive misincorporation of dAMP and/or dGMP by MMLV reverse transcriptase prevented us from doing so (Figure 3.15, lanes 4 and 9). This problem can be avoided by limiting the time of incubation of the reverse transcriptase with *(S)*-HPMPApp and dGTP (Figure 3.15, lane 10), but we found no good method to avoid it using dATP plus dGTP. As a result, this template was prepared like the CDV- and dCMP-containing templates, namely by incubating the primer-template

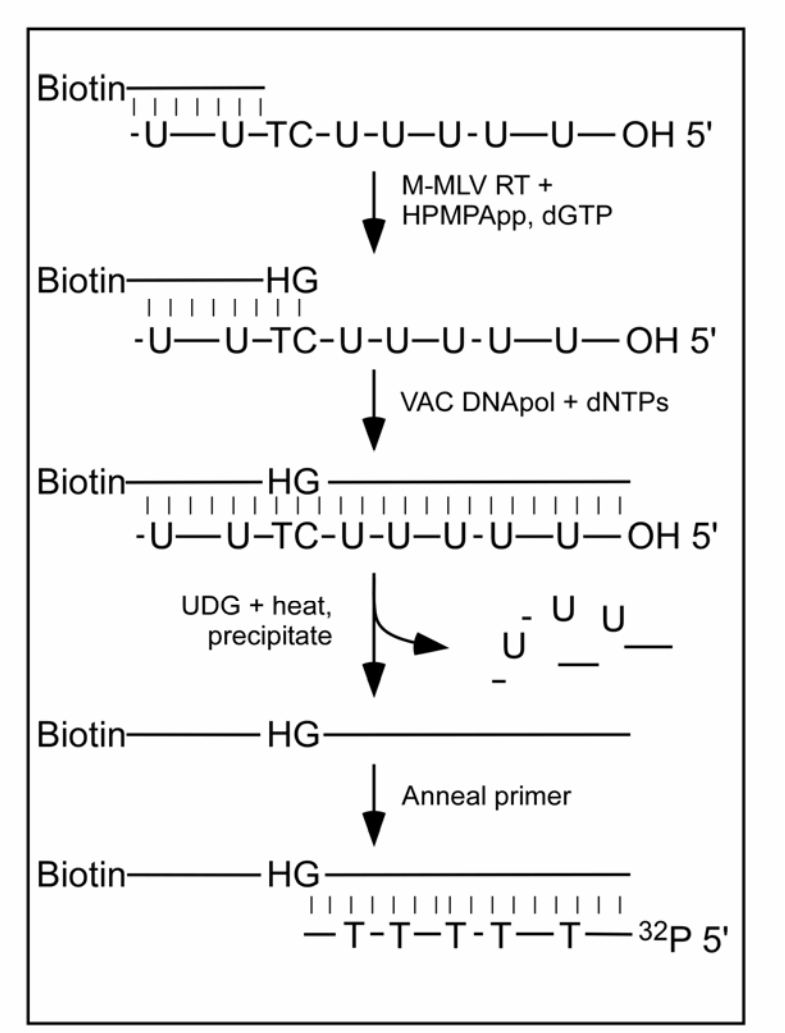

Figure 3.14 Scheme used to incorporate CDV and *(S)*-HPMPA into a template strand. The figure shows the method used to incorporate *(S)*-HPMPA into DNA. MMLV reverse transcriptase was used to add *(S)*-HPMPA ("H") and dGMP to a DNA comprising primer P11 annealed to template T17 (Figure 3.1). The products were purified and further extended using vaccinia DNA polymerase and four dNTPs. The T17 strand was then degraded with uracil glycosylase, and the *(S)*-HPMPA-containing strand was purified and annealed to ^{32}P-labeled primers P17, P18, or P19 (Figure 3.1). Molecules containing CDV, and control DNAs, were prepared the same way, except we omitted the MMLV reverse transcriptase step. We also used different primers and templates to direct the incorporation of CDV and dCMP (Figure 3.1).

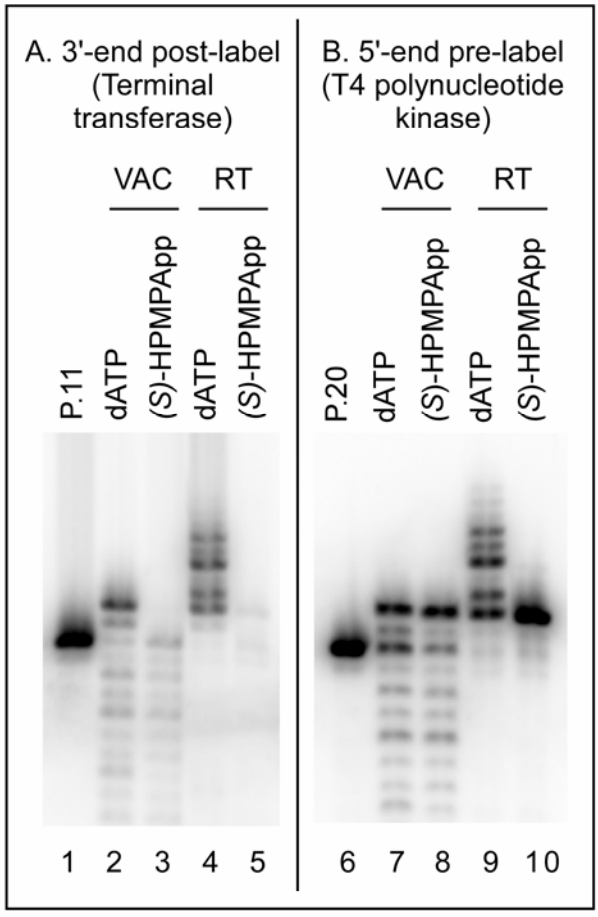

Figure 3.15 *(S)*-HPMPA inhibits labeling with TdT. (A) Primer P11 was annealed to strand T17 and incubated at 37°C with vaccinia DNA polymerase (1 min) or MMLV reverse transcriptase (1 h), in the presence of 10 μM dGTP and either 10 μM dATP or 10 μM *(S)*-HPMPApp. The T17 strand was degraded using UDG and heat, and the biotinylated P11 strand was recovered by using magnetic beads. The DNAs were labeled using terminal transferase and [α^{32}P]3′-deoxyATP, subjected to electrophoresis, and the radioactivity detected by using a phosphorimager. Primer P11 was purified and labeled the same way but not incubated with either polymerase (lane 1). (B) Primer P20 was 5′ end labeled and annealed to template T17 and then incubated with vaccinia polymerase or MMLV reverse transcriptase as described above. The reaction products were size fractionated by electrophoresis and detected using phosphorimaging. Both polymerases can incorporate *(S)*-HPMPA (and dGMP) into DNA, as judged using pre-labeled primers (lanes 8 and 10), but these N + 1 products are not postlabeled with terminal transferase (lanes 3 and 5). The TdT is still active, as shown by the capacity to label any of the molecules encoding dAMP (lanes 2 and 4). VAC, vaccinia DNA polymerase; RT, reverse transcriptase.

pair P11-T17 with vaccinia polymerase plus all four dNTPs, followed by UDG and heat treatment and ethanol precipitation.

During the preparation of these substrates, aliquots were taken of the reaction intermediates and labeled with ^{32}P using terminal transferase to assess the extent of elongation of primer P11. We observed little or no labeling of the biotinylated-P11 products if they were expected to contain *(S)*-HPMPA (Figure 3.15 lanes 3 and 5). We hypothesized that these might be poor substrates for TdT and confirmed this by performing control reactions using the nonbiotinylated primer P20 instead of P11 (Figure 3.1). This permitted 5′ end labeling of the primer using T4 polynucleotide kinase and showed that the "absence" of an extension product (Figure 3.15, lanes 3 and 5) was an artifact of the TdT not being able to extend *(S)*-HPMPA-containing primers.

3.3.9 CDV or *(S)*-HPMPA are faithfully copied by vaccinia DNA polymerase. To test the coding properties of these drug lesions, the newly prepared templates were annealed to 5′-^{32}P end-labeled primers and incubated with vaccinia DNA polymerase plus one each of the four dNTPs at 50 μM concentration. After a 1-min incubation, the products that had annealed to the template were retrieved using Dynabeads, size fractionated, and visualized by phosphorimaging. As shown in Figure 3.16, only dGMP is incorporated opposite dCMP and CDV (top panel) and only dTMP is incorporated opposite dAMP and *(S)*-HPMPA (bottom panel). These results show that CDV and *(S)*-HPMPA are both faithfully incorporated into DNA and then copied by vaccinia DNA polymerase.

3.3.10 The presence of CDV or *(S)*-HPMPA in the template strand blocks primer extension. We next examined what effect these drug lesions had on primer extension. We synthesized three kinds of primers for the present study, designed so that the 3′ terminus was located one nucleotide preceding, next to, or one nucleotide past the site of incorporation of the drug residue (Figure 3.1, primers P10 to P14 and P17 to P19). These strands were ^{32}P end labeled, annealed to the prepared templates, and incubated at 37°C with vaccinia DNA polymerase and all four dNTPs at 50 μM each. The results of this

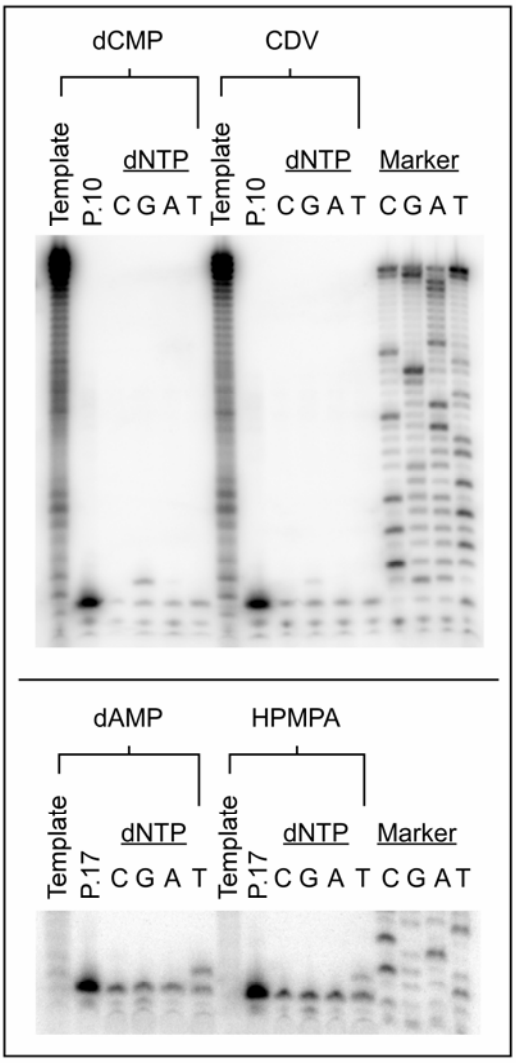

Figure 3.16 CDV and *(S)*-HPMPA are faithfully copied by vaccinia DNA polymerase. The four template strands were prepared containing dCMP/CDV (top panel) or dAMP/*(S)*-HPMPA (bottom panel) and annealed to ^{32}P-labeled primers P10 or P17 as indicated. These primer-template pairs were incubated with 2.5 ng of vaccinia DNA polymerase/µl at 37°C for 1 min in the presence of each of the indicated single dNTPs (50 µM), and the products were recovered using magnetic beads. The reaction products were then separated on a 10% polyacrylamide gel and the radioactivity was visualized by phosphorimaging. CDV and *(S)*-HPMPA direct the incorporation of dGMP and dTMP, respectively. Each of the enzymatically prepared template strands was also separately labeled with terminal transferase to measure the length of the original extension products (template). Sequencing ladders were prepared using primer P10 annealed to template T14 (top panel) or primer P17 annealed to template T18 (bottom panel).

study are shown in Figure 3.17. In all of these experiments, the primers annealed to control (i.e. drug-free) strands were all rapidly extended the full length of the enzymatically-copied strand, regardless of where we positioned the 3′ end relative to the dAMP or dCMP template nucleotide. This demonstrated the integrity of templates produced in this manner. In contrast, the presence of CDV or *(S)*-HPMPA strongly inhibited primer extension.

With CDV-containing templates (Figure 3.17, top panel), primer P10 was first extended by just one nucleotide and then attacked and degraded by the 3′-to-5′ exonuclease. At no point did we ever see the complete conversion of the primer to the primer plus one nucleotide (N + 1); instead a mix of molecules corresponding to N - 1, N, and N + 1 products was formed and then degraded (Figure 3.17, top left image). No net DNA synthesis was seen with primers P13 and P14; instead these DNAs were progressively degraded to a series of shorter molecules with the most prominent products being the N - 1 and N - 2 bands (for P13) and N - 2 and N - 3 bands (for P14) (Figure 3.17, top middle and top right images). A similar effect was seen using templates containing *(S)*-HPMPA. Primer P17 was converted to a mix of N and N + 1 products, P18 to a mix of N and N - 1 products, and P19 to a mix of N - 1 and N - 2 products (Figure 3.17, bottom images). However, although both drugs had similar inhibitory effects on DNA extension, a dramatic difference was seen in the stability of the DNAs formed in these reactions. All of the substrates and products detected in reactions using the CDV-containing template were completely degraded in less than 15 min, whereas those products that result from using the *(S)*-HPMPA-containing templates were stable throughout the entire reaction. This exonucleolytic attack on these strands occurs despite using high (200 μM) concentrations of dNTPs and suggests that the two different drugs, and resulting DNA structures, may have dramatically different effects on the rate of nucleotide turnover by vaccinia DNA polymerase.

3.4 DISCUSSION

The nucleoside phosphonate family of drugs exhibits a varying degree of activity against orthopoxviruses with the purine analog, *(S)*-HPMPA, being more active than the

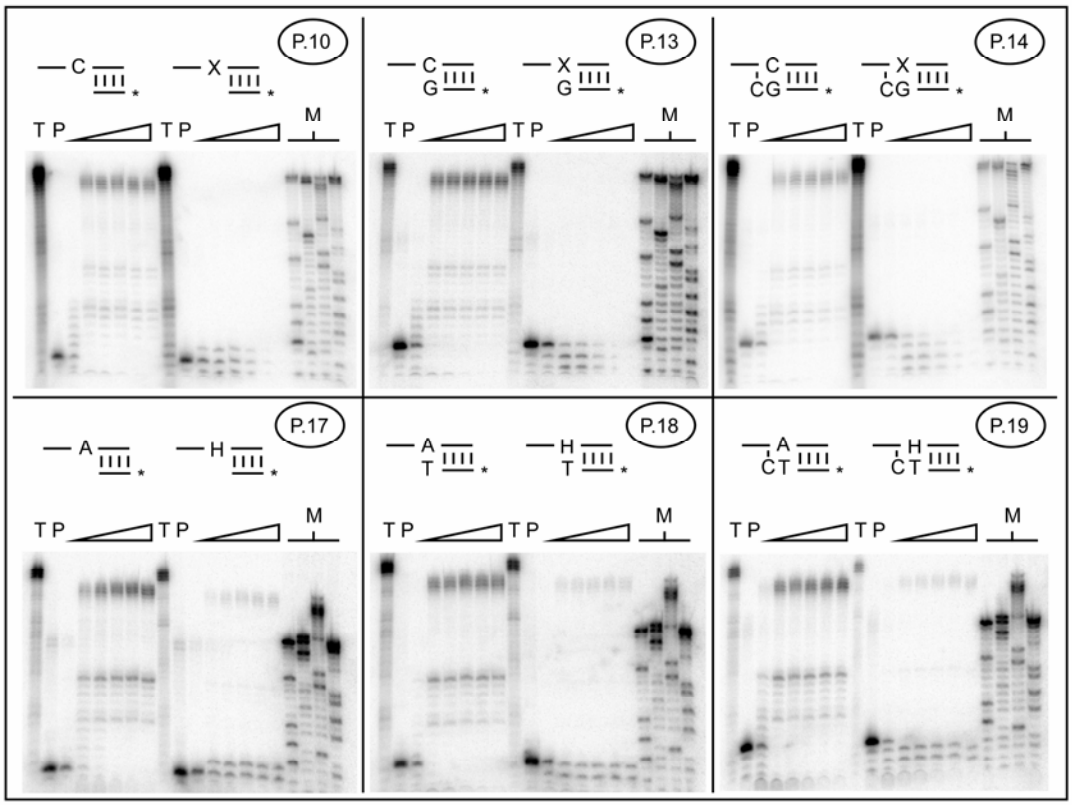

Figure 3.17 Effects of templates bearing nucleoside phosphonate drugs on trans-lesion DNA synthesis. Templates were prepared bearing dCMP/CDV (top panels) or dAMP/*(S)*-HPMPA (bottom panels) and annealed to the six indicated ^{32}P-labeled primer strands. These primers terminate at positions N + 1 (P10 and P17), N (P13 and P18), and N - 1 (P14 and P19), where N is site of drug incorporation ["X" = CDV, "H" = *(S)*-HPMPA]. Each of these primer-template pairs was incubated with 2.5 ng of vaccinia DNA polymerase/µl at 37°C for 0, 1, 2, 5, 10 or 15 min (triangles) in the presence of all four dNTPs (50 µM each), and the products recovered by using magnetic beads. The reaction products were then separated on a 10% polyacrylamide gel and the radioactivity visualized by phosphorimaging. All of the six primers were rapidly extended across control molecules bearing dCMP or dAMP residues. In contrast P10 and P17 were extended only one nucleotide (left-hand column), and the drugs blocked net DNA synthesis from the other four primers. Each of the enzymatically prepared template strands were also separately labeled with terminal transferase and [α^{32}P]3′-deoxyATP to measure the length of the original extension products ("T"). The electrophoretic properties of unmodified primer strands are illustrated in lanes marked "P".

pyrimidine analog, CDV, as judged by a 2.5- to 17-fold difference in EC_{50} *in vivo* (Figure 3.2 and Table 3.1). Metabolic studies provide some insight into the reasons for this difference since cells exposed to these drugs also appear to more rapidly convert *(S)*-HPMPA into *(S)*-HPMPApp and provide intracellular concentrations of *(S)*-HPMPApp that are 16-fold higher than that of CDVpp with the unmodified nucleotides and 3.4 fold higher with HDP-*(S)*-HPMPA versus HDP-CDV (Table 3.1). A variety of data also clearly implicates the replication machinery, and DNA polymerase in particular, as being the primary target of both drugs. In particular one selects for spontaneous mutations in the E9L (DNA polymerase) gene by continued passage of vaccinia virus in the presence of CDV, and such CDV-resistant mutations create cross-resistance to most other nucleoside phosphonate drugs including *(S)*-HPMPA (Andrei *et al.*, 2006). When one exposes vaccinia virus-infected cells to biologically equivalent concentrations of both drugs (i.e., concentrations equal to the EC_{50}), one sees nearly equal levels of inhibition of virus DNA replication, as judged by the amount of virus DNA synthesized (Figure 3.3). However, it is not clear what might be "wrong" with the substrate properties of *(S)*-HPMPApp as judged by the primer extension assays used in this study.

We have previously shown that the diphosphoryl derivative of CDV, CDVpp, can be used and incorporated into DNA by vaccinia DNA polymerase (Chapter 2; Magee *et al.*, 2005). CDVpp is a poor substrate for the vaccinia enzyme relative to the natural substrate, dCTP [$K_{m,\ CDVpp} = 23 \pm 6$ μM, $K_{m,\ dCTP} = 3.8 \pm 0.7$ μM (Chapter 2; Magee *et al.*, 2005)]. Its incorporation results in only a slight decrease in the rate of chain extension after adding one CDV molecule, but causes premature chain termination, as evidenced by the appearance of "pause sites", after the addition of the next nucleotide (Chapter 2; Magee *et al.*, 2005). DNA synthesis is also profoundly inhibited when two consecutive molecules of CDV are incorporated into the 3′ end of the primer strand. These effects of CDVpp on vaccinia DNA polymerase are similar to the effects on HCMV DNA polymerase (Xiong *et al.*, 1997). Given that *(S)*-HPMPA is more biologically active than CDV but shares a similar chemical structure within the nucleoside phosphonate moiety, we hypothesized *(S)*-HPMPApp would interact with vaccinia DNA polymerase in a manner similar to CDVpp, but that the effects of the drug would simply be more

exaggerated and perhaps detectable at lower concentrations. This is not what is observed.

The first experiment we performed was a simple primer extension assay containing all four dNTPs. When *(S)*-HPMPApp was added to this reaction, a weak stop at a position corresponding to 2 nucleotides longer than the original primer strand was detected (Figure 3.4B). This is in contrast to that seen with CDVpp, where the addition of this compound at an equal concentration (10 μM) resulted in the formation of a strong stop at this N + 1 position (Chapter 2; Magee *et al.*, 2005). We next examined the kinetics of incorporation of *(S)*-HPMPApp relative to dATP. These studies showed that *(S)*-HPMPApp is as good if not a better substrate for the polymerase than is dATP ($K_{m,\ (S)\text{-}HPMPApp}$ = 3.8 ± 0.8 μM versus $K_{m,\ dATP}$ = 4.6 ± 0.5 μM, with comparable V_{max}), and it is also very rapidly excised with kinetics comparable to dAMP from the 3′ end of the primer strand (Figure 3.10). Our experiments also showed that when *(S)*-HPMPA is incorporated into DNA, it can then serve as a primer for further extension with kinetics similar to that seen in a control reaction, where dATP replaced *(S)*-HPMPApp (Figure 3.9A). Finally, whereas molecules encoding CDV + 1 nucleotide are poor primers, and molecules bearing two consecutive molecules of CDV are nearly inert in elongation reactions (Chapter 2; Magee *et al.*, 2005), the analogous *(S)*-HPMPA containing structures have not nearly as marked effects on the elongation rate (Figure 3.9). The only striking difference between molecules bearing *(S)*-HPMPA and dAMP, as the penultimate 3′-nucleotide, is that the presence of *(S)*-HPMPA clearly inhibits the activity of the 3′-to-5′ exonuclease (Figure 3.10). This is a property common to both *(S)*-HPMPA and CDV. Thus these primer extension assays have identified no particular effect of *(S)*-HPMPApp on vaccinia polymerase that can directly account for the enhanced activity *in vivo*.

These observations suggested that *(S)*-HPMPA exerts its effects on vaccinia replication in a more indirect manner than CDV, and in a way that is exacerbated by the relatively higher intracellular concentrations of *(S)*-HPMPApp versus CDVpp. A lower K_m for *(S)*-HPMPApp (versus CDVpp), combined with greater amounts of *(S)*-HPMPApp relative to dATP (compared to the CDVpp to dCTP ratio) and little effect of *(S)*-HPMPA on chain extension, would lead to relatively more incorporation of *(S)*-

HPMPA than CDV into viral DNA. One possible consequence might then be that more *(S)*-HPMPA can be misincorporated into DNA than CDV. However, we examined incorporation of CDV and *(S)*-HPMPA opposite all four dNMPs and found that both drugs are faithfully incorporated opposite dGMP and dTMP, respectively (Figure 3.13).

This led us to investigate what effects CDV and *(S)*-HPMPA have on DNA synthesis when located in the template strand. Most nucleoside and nucleotide analogues act as obligate chain terminators, whereas CDV, which still bears a 3′ hydroxyl, is generally classified as a non-obligate chain terminator. Since *(S)*-HPMPA can be incorporated into DNA without causing much chain termination (Figure 3.9), some of the antiviral effects of this drug could be explained by what happens at the next round of replication. Since there are currently no chemical methods for the synthesis of CDV- and (*S*)-HPMPA-containing DNAs, we developed an enzymatic approach using vaccinia DNA polymerase, or vaccinia DNA polymerase in combination with MMLV reverse transcriptase (Figure 3.14). A number of quality controls were conducted during the preparation of these substrates and one of the more interesting effects that we noted was that *(S)*-HPMPA-containing DNAs are also poorly labeled by TdT (Figure 3.15). This suggests that DNAs bearing a nucleoside phosphonate drug at the penultimate 3′ position likely exhibit some structural feature that is broadly inhibitory to many different nucleotidyl transferases. Since TdT plays a key role in the development of immune diversity (Thai and Kearney, 2005), this observation also raises some questions regarding what effect prolonged exposure to these drugs might have on immune responses to viral infection.

These new substrates were used to show that templates containing CDV and *(S)*-HPMPA cause a severe block in DNA extension. Although the polymerase can faithfully incorporate a nucleotide opposite either drug residue (Figure 3.16), further elongation is inhibited (Figure 3.17). Vaccinia DNA polymerase also rapidly degraded primers extending one nucleotide past the drug lesion in the presence of dNTPs, suggesting that such structures tend to be recognized as being mismatched by the 3′-to-5′ proofreading exonuclease. A similar experiment using HCMV DNA polymerase and a template DNA containing a single CDV residue showed that this drug greatly reduced the rate of

elongation and caused pausing at positions N – 1, N and N + 1 (Xiong *et al.*, 1997). Interestingly, CDV in the template did not cause an absolute block in DNA synthesis using the betaherpesvirus enzyme, as full-length extension products were still produced, although to a lesser extent than that seen using a control template containing dCMP (Xiong *et al.*, 1997). There have also been a few reports of similar effects caused by other polymerase inhibitors and using other enzymes. For example Mikita and Beardsley (Mikita and Beardsley, 1988) showed that arabinosylcytosine template residues partially blocked DNA elongation by Klenow, T4, and human α_2 DNA polymerases, although it did not efficiently inhibit avian myeoloblastosis virus reverse transcriptase. Satake *et al*. (1991; 1992) also noted that 5-trifluoromethyl-2′-deoxyuridine caused a strong arrest one nucleotide before or after the lesion site using Klenow polymerase and human DNA polymerase α, respectively. Collectively these results show that if nucleotide analogues are incorporated into the template strand, they can severely inhibit polymerase activity, much like some forms of DNA damage (Baynton and Fuchs, 2000). This mechanism of action is not relevant for common DNA polymerase inhibitors, since most are obligate or *de facto* chain terminators. However, *(S)*-HPMPApp is a good substrate and a not very effective chain terminator (Figure 3.9) and thus might well act more by inhibiting secondary rounds of DNA synthesis. Since both CDV and *(S)*-HPMPA block DNA synthesis to a similar extent, once incorporated into the template strand, the relatively greater efficacy of *(S)*-HPMPA is probably explained by a combination of factors related to higher intracellular levels of *(S)*-HPMPApp plus a greater likelihood that *(S)*-HPMPA would be incorporated into an irrepairable DNA lesion.

Viewed from this perspective, these new insights into this mode of drug action can shed new light on the genetics of drug-resistant poxviruses. We have shown that CDV-resistant vaccinia viruses exhibit cross-resistance to *(S)*-HPMPA, and acquire mutations in the E9L gene in both the DNA polymerase and 3′-to-5′ exonuclease domains (Andrei *et al.*, 2006). Substitution mutations located in the exonuclease domain are probably the primary determinant of resistance and likely act to enhance drug excision from DNA (Andrei *et al.*, 2006). However, it is less clear how the substitution mutations located in the polymerase domain promote drug resistance. We have suggested that they

might enhance the discrimination against CDVpp and *(S)*-HPMPApp during nucleotide selection (Andrei *et al.*, 2006). Based on the results of the present study, it is also possible that these mutations affect how mutant polymerases copy drug-containing (or otherwise damaged) templates and might help explain the weak mutator phenotype exhibited by virus encoding the A684V substitution mutation (Andrei *et al.*, 2006). Work is currently in progress to isolate these mutant polymerases so they can be used in biochemical assays to investigate the role of these mutations on DNA polymerase activity.

3.5 AUTHOR CONTRIBUTION TO DATA

Wendy Magee performed the plaque reduction assay and slot blot hybridization, prepared the CDV- and *(S)*-HPMPA-containing templates and performed all of the DNA polymerase and exonuclease assays. Kathy Aldern performed the studies on the cellular uptake and metabolism of *(S)*-HPMPA and CDV and their hexadecyloxypropyl esters.

3.6 REFERENCES

Aldern, K. A., S. L. Ciesla, K. L. Winegarden, and K. Y. Hostetler. 2003. Increased antiviral activity of 1-*O*-hexadecyloxypropyl-[2-^{14}C]cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism. Mol. Pharmacol. **63:** 678-681.

Andrei, G., D. B. Gammon, P. Fiten, E. De Clercq, G. Opdenakker, R. Snoeck, and D. H. Evans. 2006. Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice. J. Virol. **80:** 9391-9401.

Baker, R. O., M. Bray, and J. W. Huggins. 2003. Potential antiviral therapeutics for smallpox, monkeypox and other orthopoxvirus infections. Antiviral Res. **57:** 13-23.

Baynton, K., and R. P. P. Fuchs. 2000. Lesions in DNA: hurdles for polymerases. Trends Biochem. Sci. **25:** 74-79.

Beadle, J. R., W. B. Wan, S. L. Ciesla, K. A. Keith, C. Hartline, E. R. Kern, and K. Y. Hostetler. 2006. Synthesis and antiviral evaluation of alkoxyalkyl derivatives of 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)adenine against cytomegalovirus and orthopoxviruses. J. Med. Chem. **49:** 2010-2015.

Birkus, G., D. Rejman, M. Otmar, I. Votruba, I. Rosenberg, and A. Holy. 2004. The

substrate activity of (*S*)-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine diphosphate toward DNA polymerases α, δ and ε. Antiviral Chem. Chemother. **15:** 23-33.

Botros, S., S. William, O. Hammam, Z. Zídek, and A. Holý. 2003. Activity of 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine against *Schistosomiasis mansoni* in mice. Antimicrob. Agents Chemother. **47:** 3853-3858.

Bronson, J.J., I. Ghazzouli, M.J.M. Hitchcock, R.R. Webb II, E.R. Kern, J.C. Martin. 1989. Synthesis and antiviral activity of nucleotide analogues bearing the (*S*)-(3-hydroxy-2-phosphonylmethoxy)propyl moiety attached to adenine, guanine, and cytosine, p. 88-102. *In* J. C. Martin (ed.), Nucleotide analogues as antiviral agents. American Chemical Society, Washington, DC.

Challberg, M. D., and P. T. Englund. 1979. Purification and properties of the deoxyribonucleic acid polymerase induced by vaccinia virus. J. Biol. Chem. **254:** 7812-7819.

De Clercq, E. 2004. Antivirals and antiviral strategies. Nat. Rev. Microbiol. **2:** 704-720.

De Clercq, E., A. Holý, I. Rosenberg, T. Sakuma, J. Balzarini, and P. C. Maudgal. 1986. A novel selective broad-spectrum anti-DNA virus agent. Nature **323:** 464-467.

De Clercq, E., T. Sakuma, M. Baba, R. Pauwels, J. Balzarini, I. Rosenberg, and A. Holý. 1987. Antiviral activity of phosphonylmethoxyalkyl derivatives of purine and pyrimidines. Antiviral Res. **8:** 261-272.

de Vries, E., J. G. Stam, F. F. J. Franssen, H. Nieuwenhuijs, P. Chavalitshewinkoon, E. de Clercq, J. P. Overdulve, and P. C. van der Vliet. 1991. Inhibition of the growth of *Plasmodium falciparum* and *Plasmodium berghei* by the DNA polymerase inhibitor HPMPA. Mol. Biochem. Parasitol. **47:** 43-50.

Hostetler, K. Y., K. A. Aldern, W. B. Wan, S. L. Ciesla, and J. R. Beadle. 2006. Alkoxyalkyl esters of (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine are potent inhibitors of the replication of wild-type and drug-resistant human immunodeficiency virus type 1 in vitro. Antimicrob. Agents Chemother. **50:** 2857-2859.

Ito, J., and D. K. Braithwaite. 1991. Compilation and alignment of DNA polymerase sequences. Nucleic Acids Res. **19:** 4045-4057.

Kaminsky, R., B. Nickel, and A. Holý. 1998. Arrest of *Trypanosoma brucei rhodesiense* and *T. brucei brucei* in the S-phase of the cell cycle by (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine ((S)-HPMPA). Mol. Biochem. Parasitol. **93:** 91-100.

Kaminsky, R., C. Schmid, Y. Grether, A. Holý, E. De Clercq, L. Naesens, and R. Brun. 1996. (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA]: a

purine analogue with trypanocidal activity *in vitro* and *in vivo*. Trop. Med. Int. Health **1:** 255-263.

Kaminsky, R., E. Zweygarth, and E. De Clercq. 1994. Antitrypanosomal activity of phosphonylmethoxyalkylpurines. J. Parasitol. **80:** 1026-1030.

Keith, K. A., M. J. M. Hitchcock, W. A. Lee, A. Holý, and E. R. Kern. 2003. Evaluation of nucleoside phosphonates and their analogs and prodrugs for inhibition of orthopoxvirus replication. Antimicrob. Agents Chemother. **47:** 2193-2198.

Kern, E. R., C. Hartline, E. Harden, K. Keith, N. Rodriguez, J. R. Beadle, and K. Y. Hostetler. 2002. Enhanced inhibition of orthopoxvirus replication in vitro by alkoxyalkyl esters of cidofovir and cyclic cidofovir. Antimicrob. Agents Chemother. **46:** 991-995.

Kramata, P., I. Votruba, B. Otová, and A. Holý. 1996. Different inhibitory potencies of acyclic phosphonomethoxyalkyl nucleotide analogs toward DNA polymerases α, δ, and ε. Mol. Pharmacol. **49:** 1005-1011.

Lebeau, I., G. Andrei, F. Dal Pozzo, J. R. Beadle, K. Y. Hostetler, E. De Clercq, J. van den Oord, and R. Snoeck. 2006. Activities of alkoxyalkyl esters of cidofovir (CDV), cyclic cidofovir, and (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine against orthopoxviruses in cell monolayers and in organotypic cultures. Antimicrob. Agents Chemother. **50:** 2525-2529.

Magee, W. C., K. Y. Hostetler, and D. H. Evans. 2005. Mechanism of inhibition of vaccinia virus DNA polymerase by cidofovir diphosphate. Antimicrob. Agents Chemother. **49:** 3153-3162.

McDonald, W. F., and P. Traktman. 1994. Overexpression and purification of the vaccinia virus DNA polymerase. Protein Expr. Purif. **5:** 409-421.

Merta, A., I. Votruba, J. Jindřich, A. Holý, T. Cihlář, I. Rosenberg, M. Otmar, and T. Y. Herve. 1992. Phosphorylation of 9-(2-phosphonomethoxyethyl)adenine and 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)adenine by AMP(dAMP) kinase from L1210 cells. Biochem. Pharmacol. **44:** 2067-2077.

Merta, A., I. Votruba, I. Rosenberg, M. Otmar, H. Hřebabecký, R. Bernaerts, and A. Holý. 1990. Inhibition of herpes simplex virus DNA polymerase by diphosphates of acyclic phosphonylmethoxyalkyl nucleotide analogues. Antiviral Res. **13:** 209-218.

Mikita, T., and G. P. Beardsley. 1988. Functional consequences of the arabinosylcytosine structural lesion in DNA. Biochemistry **27:** 4698-4705.

Mul, Y. M., R. T. van Miltenburg, E. De Clercq, and P. C. van der Vliet. 1989.

Mechanism of inhibition of adenovirus DNA replication by the acyclic nucleoside triphosphate analogue (*S*)-HPMPApp: influence of the adenovirus DNA binding protein. Nucleic Acids Res. **17:** 8917-8929.

Palú, G., S. Stefanelli, M. Rassu, C. Parolin, J. Balzarini, and E. De Clercq. 1991. Cellular uptake of phosphonylmethoxyalkylpurine derivatives. Antiviral Res. **16:** 115-119.

Pauwels, R., J. Balzarini, D. Schols, M. Baba, J. Desmyter, I. Rosenberg, A. Holy, and E. De Clercq. 1988. Phosphonomethoxyethyl purine derivatives, a new class of anti-human immunodeficiency virus agents. Antimicrob. Agents Chemother. **32:** 1025-1030.

Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Satake, H., S. Takeda, A. Matsumura, M. Sasaki, N. Sugimoto, and Y. Wataya. 1992. Action of 5-trifluoromethyl-2'-deoxyuridine on DNA synthesis. Nucleic Acids Symp. Ser. **27:** 189-190.

Satake, H., S. Takeda, and Y. Wataya. 1991. Inhibition of *in vitro* DNA chain elongation of 5-trifluoromethyl-2'-deoxyuridine residue in the template. Nucleic Acids Symp. Ser. **25:** 37-38.

Smeijsters, L. J. J. W., F. F. J. Franssen, L. Naesens, E. de Vries, A. Holý, J. Balzarini, E. de Clercq, and J. P. Overdulve. 1999. Inhibition of the in vitro growth of *Plasmodium falciparum* by acyclic nucleoside phosphonates. Int. J. Antimicrob. Agents **12:** 53-61.

Smeijsters, L. J. J. W., N. M. Zijlstra, J. Veenstra, B. E. Verstrepen, C. Heuvel, J. P. Overdulve, and E. de Vries. 2000. *Plasmodium falciparum* clones resistant to (*S*)-9-(3-hydroxy-2-phosphonylmethoxy-propyl)adenine carry amino acid substitutions in DNA polymerase δ. Mol. Biochem. Parasitol. **106:** 175-180.

Snoeck, R., A. Holý, C. Dewolf-Peeters, J. Van Den Oord, E. De Clercq, and G. Andrei. 2002. Antivaccinia activities of acyclic nucleoside phosphonate derivatives in epithelial cells and organotypic cultures. Antimicrob. Agents Chemother. **46:** 3356-3361.

Thai, T.-H., and J. F. Kearney. 2005. Isoforms of terminal deoxynucleotidyltransferase: developmental aspects and function. Adv. Immunol. **86:** 113-136.

Veselý, J., A. Merta, I. Votruba, I. Rosenberg, and A. Holý. 1990. The cytostatic effects and mechanism of action of antiviral acyclic adenine nucleotide analogs in L1210 mouse leukemia cells. Neoplasma **37:** 105-110.

Votruba, I., R. Bernaerts, T. Sakuma, E. De Clercq, A. Merta, I. Rosenberg, and A.

Holý. 1987. Intracellular phosphorylation of broad-spectrum anti-DNA virus agent (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine and inhibition of viral DNA synthesis. Mol. Pharmacol. **32:** 524-529.

Xiong, X., J. L. Smith, and M. S. Chen. 1997. Effect of incorporation of cidofovir into DNA by human cytomegalovirus DNA polymerase on DNA elongation. Antimicrob. Agents Chemother. **41:** 594-599.

Yao, X.-D.,D.H. Evans. 2004. Construction of recombinant vaccinia viruses using leporipoxvirus-catalyzed recombination and reactivation of orthopoxvirus DNA, p. 51-64. *In* S. N. Isaacs (ed.), Vaccinia virus and poxvirology: methods and protocols. Humana Press, Totowa, NJ.

Yokota, T., K. Konno, E. Chonan, S. Mochizuki, K. Kojima, S. Shigeta, and E. De Clercq. 1990a. Comparative activities of several nucleoside analogs against duck hepatitis B virus in vitro. Antimicrob. Agents Chemother. **34:** 1326-1330.

Yokota, T., S. Mochizuki, K. Konno, S. Mori, S. Shigeta, and E. De Clercq. 1990b. Phosphonylmethoxyalkyl derivatives of purine as inhibitors of human hepatitis B virus DNA synthesis. Nucleic Acids Symp. Ser. 17-18.

CHAPTER FOUR

INHIBITION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 AND MOLONEY MURINE LEUKEMIA VIRUS REVERSE TRANSCRIPTASES BY CIDOFOVIR DIPHOSPHATE AND *(S)*-9-[3-HYDROXY-(2-PHOSPHONOMETHOXY)PROPYL]ADENINE DIPHOSPHATE[1]

Wendy C. Magee, Karl Y. Hostetler and David H. Evans

4.1 INTRODUCTION

The nucleoside phosphonates *(S)*-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine [*(S)*-HPMPC], also known as cidofovir (CDV), and *(S)*-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine [*(S)*-HPMPA] are acyclic analogs of dCMP and dAMP, respectively. Collectively these drugs are effective inhibitors of a wide range of DNA viruses and retroviruses (De Clercq *et al.*, 1986; reviewed in De Clercq and Holý, 2005). We have previously used the poxvirus vaccinia as a model system to investigate the mechanism of action of these compounds (Chapters 2 and 3; Magee *et al.*, 2008; Magee *et al.*, 2005). Our results have shown that the active intracellular metabolites of these drugs, CDV diphosphate (CDVpp) and *(S)*-HPMPA diphosphate [*(S)*-HPMPApp], respectively, are substrates for vaccinia virus DNA polymerase and, once incorporated into the penultimate 3′-end of the primer strand, inhibit the enzyme's polymerase and 3′-to-5′ proofreading exonuclease activities. Further, we found that when these drugs are incorporated into the template strand, they profoundly inhibit replication across the drug lesion. These results suggest that this class of antiviral agents has a complex mechanism of action, inhibiting DNA synthesis when present in both the primer and template strands and blocking 3′-to-5′ exonuclease activity when located in the primer strand.

During the course of these studies and in other experiments in our laboratory, we determined that both CDVpp and *(S)*-HPMPApp are also used by the Moloney murine

---

[1] This study was supported by a Canadian Institutes of Health Research (CIHR) grant to Dr. D.H. Evans.

leukemia virus (MMLV) reverse transcriptase (RT) as substrates and are incorporated into DNA by this enzyme (Chapter 3; Hamilton *et al.*, 2007; Magee *et al.*, 2008). In the original description of the nucleoside phosphonate class of drugs, *(S)*-HPMPA was found to be inhibitory to the transformation of mouse embryo cells by Moloney murine sarcoma virus (MMSV) (De Clercq *et al.*, 1986). Further work showed the efficacy of this compound against the transformation of murine fibroblast cells by this virus (Pauwels *et al.*, 1988). *(S)*-HPMPA however, was not effective against human immunodeficiency virus type 1 (HIV-1) in its original form (Pauwels *et al.*, 1988). Interestingly, CDV was able to inhibit HIV-1 in epithelioid HeLa-CD4 cells, but not in the T-lymphocyte cell line MT-2, a result partially explained by differences in the metabolism of CDV to CDVpp in the two cells lines (Srinivas *et al.*, 1997). The influence of cellular metabolism on the efficacy of these drugs was strengthened by data showing that alkoxyalkyl ester derivatives of both CDV and *(S)*-HPMPA are inhibitory to HIV-1 in MT-2 cells (Hostetler *et al.*, 2006; Valiaeva *et al.*, 2008). These prodrugs of CDV and *(S)*-HPMPA are taken up by cells and metabolized to the active CDVpp and *(S)*-HPMPApp metabolites to a greater extent than the underivatized forms (Chapter 3; Aldern *et al.*, 2003; Magee *et al.*, 2008).

Based on these data, we were interested in extending our studies on the mechanism of action of CDV and *(S)*-HPMPA to reverse transcriptases to determine if a pattern of inhibition similar to that seen with vaccinia virus DNA polymerase was observed. Although both retroviruses, MMLV and HIV-1 belong to different genera of the *Retroviridae* family and the structures of their RTs are different (reviewed in Goff, 2007 and Prasad, 1993). MMLV is a simple gammaretrovirus with a monomeric enzyme of 75 kDa (Das and Georgiadis, 2004). In contrast, HIV-1 is a complex lentivirus and its RT is an asymmetric heterodimer consisting of 66 kDa and 51 kDa subunits (Le Grice, 1993). We used steady state primer extension analyses to determine the effects of CDVpp and *(S)*-HPMPApp on the purified forms of these two enzymes. Our data indicate that both MMLV RT and HIV-1 RT can use CDVpp and *(S)*-HPMPApp as substrates and incorporate them into DNA using both RNA and DNA templates. In addition, both enzymes are inhibited by the drugs when they are incorporated into the template strand.

However, the degree of inhibition by CDV and *(S)*-HPMPA, both in the primer strand and in the template strand, differed between the two enzymes. Further, differences were observed in the ability of CDV and *(S)*-HPMPA to block DNA elongation when in the template strand. These results are discussed in terms of the known antiviral activity of CDV and *(S)*-HPMPA against these two types of retroviruses.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals. CDVpp and *(S)*-HPMPApp were prepared by custom synthesis by TriLink Biotechnologies. Radiolabeled [γ-^{32}P]ATP and cordycepin triphosphate ([α-^{32}P]3′-deoxyATP) was purchased from PerkinElmer, and unlabeled deoxynucleoside triphosphates (dNTPs) were from Fermentas. DNA and RNA oligonucleotides were purchased from Sigma-Genosys or Integrated DNA Technologies.

4.2.2 Enzymes. MMLV reverse transcriptase was purchased from Fermentas and Invitrogen. Wild-type clade B HIV-1 reverse transcriptase produced in *Escherichia coli* was a generous gift from Dr. M. Götte (McGill). T4 polynucleotide kinase, the Klenow fragment of DNA polymerase I, uracil-DNA glycosylase (UDG), and terminal deoxynucleotidyl transferase (TdT) were obtained from Fermentas.

4.2.3 Preparation of CDV- and *(S)*-HPMPA-containing templates. CDV- and *(S)*-HPMPA-containing templates were prepared by enzymatic synthesis as described previously (Chapter 3; Magee *et al.*, 2008) using the primer-template pairs shown in Figure 4.1. Control templates containing dCMP and dAMP, respectively, were prepared also as described previously (Chapter 3; Magee *et al.*, 2008).

4.2.4 Reverse transcriptase and DNA polymerase assays. Oligonucleotide primer-template pairs (Figure 4.1) were used as substrates for reverse transcriptase and DNA polymerase assays. Reverse transcriptase assays utilized RNA oligonucleotide templates and DNA oligonucleotide templates were used in the DNA polymerase assays. The primers were first end-labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase prior to

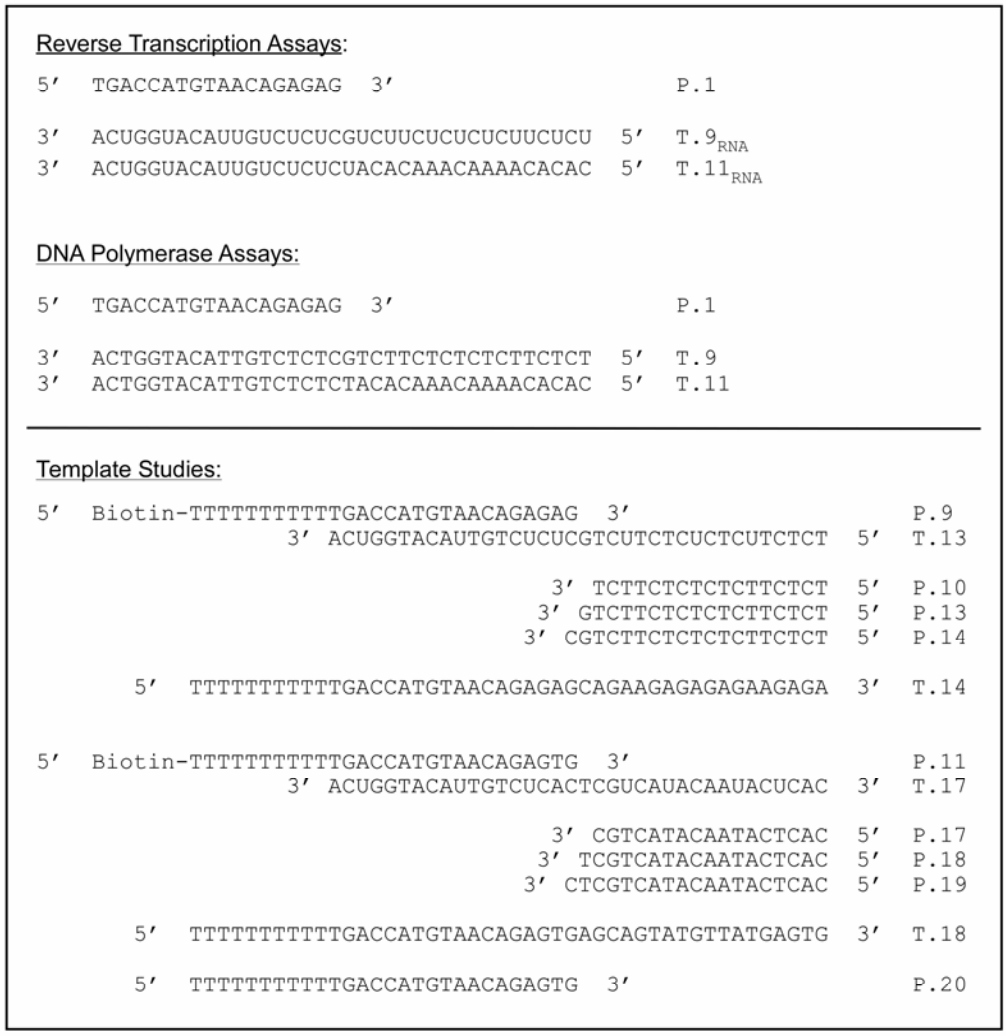

Figure 4.1 Oligonucleotide primer–template pairs used in this study. Primer P1 was previously described by Xiong *et al.* (1997). Template T9 was originally described by Magee *et al.* (2005; Chapter 2) and primers P9, P10, P11, P13, P14, P17, P18, P19 and templates T11, T13, T14, T17, and T18 were previously described by Magee *et al.* (2008; Chapter 3). The RNA oligonucleotides $T9_{RNA}$ and $T11_{RNA}$ have a sequence corresponding to the DNA oligonucleotides T9 and T11, respectively.

annealing to the template strand. Various combinations of dNTPs and CDVpp or *(S)*-HPMPA were added as indicated. MMLV RT was used at a final concentration of 2 U/µl in the manufacturer's recommended buffer (Fermentas) and HIV-1 was used at a final concentration of 50 nM in a solution containing 50 mM Tris·HCl, pH 7.8; 50 mM NaCl; and 6 mM $MgCl_2$. After incubation at 37°C for 5 min, reaction mixtures were stopped by the addition of gel loading buffer [80% (v/v) formamide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue]. Reaction products were resolved on 10% polyacrylamide gels and analyzed by phosphorimager analysis as previously described (Chapter 2; Magee *et al.*, 2005) using a Typhoon 9400 phosphorimager. DNA polymerase assays using the CDV- or *(S)*-HPMPA-containing templates were performed as previously described (Chapter 3, Magee *et al.*, 2008), except that MMLV RT (2 U/µl) and HIV-1 RT (50 nM) in their respective buffers were used in place of vaccinia virus DNA polymerase. Size standards were generated by using the Klenow fragment and dideoxy sequencing reactions (Sambrook and Russell, 2001).

4.3 RESULTS

4.3.1 The addition of CDVpp or *(S)*-HPMPApp to a primer extension assay catalyzed by MMLV RT results in a greater inhibition of DNA elongation when using a DNA template rather than an RNA template.

To determine the mechanism of inhibition of CDVpp and *(S)*-HPMPApp against a simple monomeric retrovirus reverse transcriptase, we performed a primer extension assay using MMLV RT and all four dNTPs. Because retroviruses synthesize DNA using both RNA and DNA templates, we performed these assays using both types of templates to represent first strand and second strand syntheses, respectively. For reactions involving CDVpp, primer-template pairs P1-$T9_{RNA}$ and P1-T9 were used, and for those reactions designed to study *(S)*-HPMPApp, primer-template pairs P1-$T11_{RNA}$ and P1-T11 were used (Figure 4.1). The 18 nucleotide primer strands were first 5′-end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase prior to annealing to the template strand. The results of this experiment are shown in Figures 4.2 and 4.3. When 10 µM CDVpp is added to a reaction mixture containing 10 µM dATP, 5 µM dCTP, 10 µM dGTP, 10 µM dTTP, and primer-template pair P1-$T9_{RNA}$,

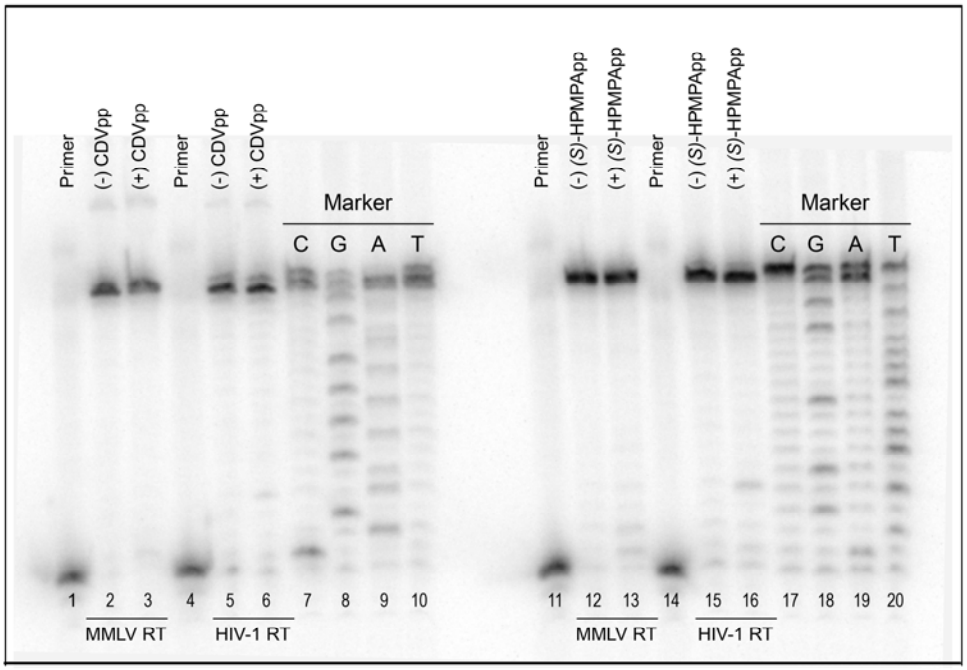

Figure 4.2 Primer extension analysis of MMLV RT and HIV-1 RT using RNA templates. **Left panel.** Primer extension reactions incubated in the presence or absence of CDVpp. Each 10-µl reaction contained ^{32}P labeled primer P1 annealed to template $T9_{RNA}$, 5 µM dCTP, 10 µM dATP, 10 µM dGTP, 10 µM dTTP, and either 20 U of MMLV RT or 50 nM of HIV-1 RT. Lanes marked "(+) CDVpp" were further supplemented with 10 µM CDVpp. After incubation at 37°C for 5 min, each reaction was stopped by the addition of gel loading buffer, reaction products were separated on a 10% polyacrylamide gel and then analyzed by phosphorimaging. Lanes marked "Primer" show the position of the labeled primer. Size markers are shown in lanes 7 to 10. **Right panel.** Primer extension reactions incubated in the presence or absence of *(S)*-HPMPApp. Each 10-µl reaction consisted of ^{32}P labeled primer P1 annealed to template $T11_{RNA}$, 5 µM dATP, 10 µM dCTP, 10 µM dGTP, 10 µM dTTP and either 20 U of MMLV RT or 50 nM of HIV-1 RT. Lanes marked "(+) *(S)*-HPMPApp" also contained 10 µM *(S)*-HPMPApp. Reactions were incubated at 37°C for 5 min, stopped by the addition of gel loading buffer and subjected to phosphorimager analysis after running reaction products on a 10% polyacrylamide gel. Lanes marked "Primer" show the position of the labeled primer. Size markers are shown in lanes 17 to 20.

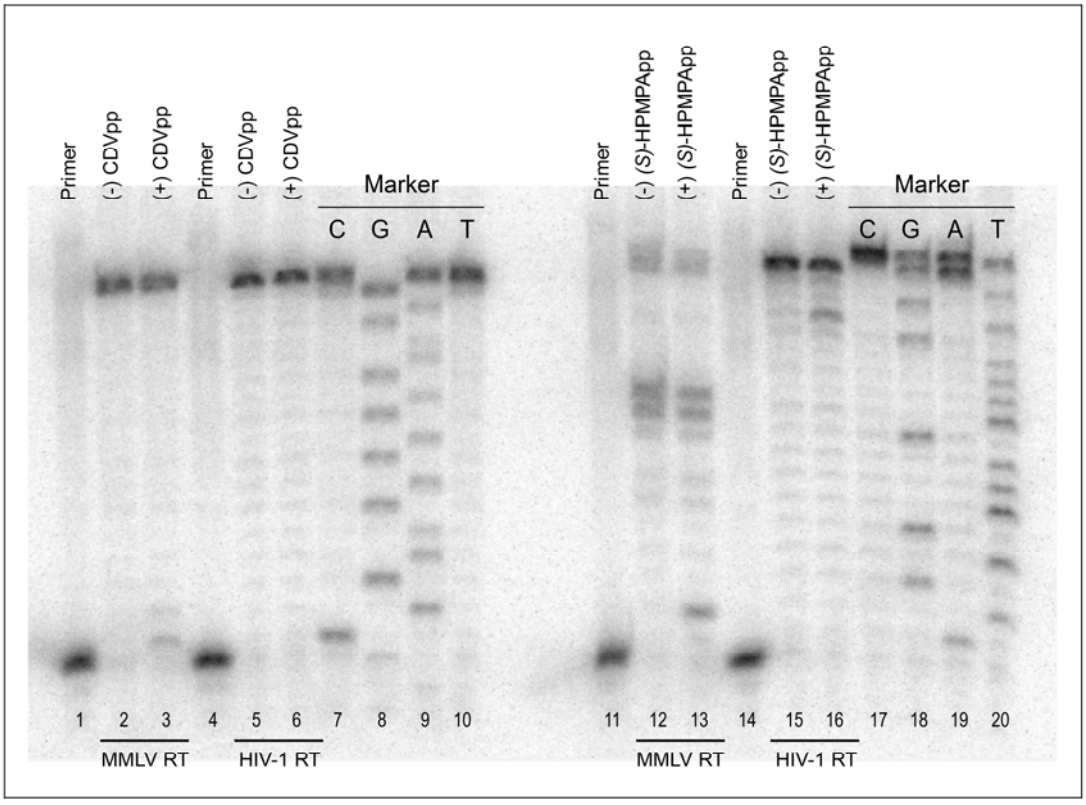

Figure 4.3 Primer extension analysis of MMLV RT and HIV-1 RT using DNA templates. Reactions were prepared and analyzed as indicated in the legend to Figure 4.2, except that templates T9 and T11 replaced templates $T9_{RNA}$ and $T11_{RNA}$, respectively.

no substantial difference in reaction products were seen compared to a reaction incubated in the absence of drug (Figure 4.2, lanes 2 and 3). Both reactions resulted in the production of full-length extension products. Faint bands of a size larger than the full-length extension products were also observed in these lanes. As retroviral reverse transcriptases do not possess terminal transferase activity (Marcus and Sarkar, 1978), these bands may be a result of DNA synthesis occurring after slippage of the primer-template. A very faint band was also seen in the products of the reaction incubated with CDVpp; this band was located at position N, where N is the expected site of incorporation of CDV opposite a template dG. When a reaction mixture containing primer-template pair P1-$T11_{RNA}$ and all four dNTPs (5 μM dATP, 10 μM dCTP, 10 μM dGTP and 10 μM dTTP) was further supplemented with 10 μM *(S)*-HPMPApp, the reaction products were also similar to those produced in the absence of drug (Figure 4.2, lanes 12 and 13). However, the addition of *(S)*-HPMPApp resulted in the presence of two very faint bands at positions N and N + 1. These results suggest that CDVpp and *(S)*-HPMPApp do not greatly inhibit MMLV RT DNA synthesis when using an RNA template.

In contrast, when the DNA templates T9 and T11 were used in these same primer extension assays, the inhibition of DNA synthesis by these drugs was more pronounced. The addition of 10 μM CDVpp to a P1-T9 reaction mixture resulted in a strong stop at position N and a weaker stop at position N + 1 (Figure 4.3, compare lanes 2 and 3). Similarly, a strong stop at the N + 1 position was observed when a P1-T11 reaction mixture was incubated with 10 μM *(S)*-HPMPApp (Figure 4.3, lanes 12 and 13). Other pause sites were also seen in this reaction mixture (weak stops corresponding to extension products of 24, 25 and 27 nucleotides and strong stops corresponding to extension products of 28 and 29 nucleotides). These latter pause sites were also seen in the control reaction incubated without *(S)*-HPMPApp and most likely represent template sequence effects on the enzyme. Interestingly, these sites all occurred at the beginning of runs of dA in the template strand. MMLV RT has been reported to exhibit a strong stop within an oligo$(dA)_{16}$ tract, suggesting that this enzyme has difficulty copying runs of dA (Williams *et al.*, 1990).

4.3.2 CDVpp and *(S)*-HPMPApp do not inhibit HIV-1 RT when using either RNA or DNA templates. The same primer extension assays as those performed in Section 4.3.1 were repeated using HIV-1 RT in place of MMLV RT to see if there were differences in the interactions of these drugs with the two enzymes. When 10 μM CDVpp or 10μM *(S)*-HPMPApp were added to reaction mixtures containing the primer-template pairs P1-$T9_{RNA}$ and P1-$T11_{RNA}$, respectively, little difference was observed compared to those reactions incubated in the absence of drug (Figure 4.2, lanes 5 and 6 and lanes 15 and 16, respectively). A very weak band at position N + 3, where N is the expected site of incorporation of drug opposite dG and dT, respectively, was seen in each drug-containing reaction. The occurrence of this band in these reactions was reproducible; however, the weak intensity of these bands suggests that these drugs were not inhibiting DNA synthesis to a great extent. The reactions containing the P1-$T9_{RNA}$ primer-template pair also produced reaction products of a length greater than full-length extension products, similar to that observed in reactions catalyzed by the MMLV RT. CDVpp and *(S)*-HPMPApp also did not appear to inhibit DNA synthesis catalyzed by HIV-1 RT when using a DNA template (Figure 4.3, lanes 5 and 6 and 15 and 16, respectively). The reaction products produced in the absence and presence of 10 μM CDVpp were indistinguishable using this assay. The reaction products formed in the absence and presence of 10 μM *(S)*-HPMPApp were also similar. However, there was a strong pause site corresponding to a 33 base pair (bp) product in the reaction mixture containing *(S)*-HPMPApp that was not seen in the reaction incubated without drug. As this pause site is located far away (14 nucleotides) from the expected incorporation site of *(S)*-HPMPA, its relevance is unknown.

4.3.3 MMLV RT can incorporate CDV and *(S)*-HPMPA into DNA and extend primers containing these substrates. We next wanted to investigate the stepwise incorporation and extension of CDV and *(S)*-HPMPA into DNA by MMLV RT compared to the incorporation and extension of the corresponding natural nucleotides, dCTP and dATP, respectively. The results of the experiment examining the incorporation and extension of CDVpp and dCTP opposite RNA and DNA templates are shown in

Figure 4.4. When using the primer-template pair P1-$T9_{RNA}$, MMLV RT could incorporate CDVpp into DNA to an extent similar to that seen with dCTP (Figure 4.4, lanes 2 and 6). When these reaction mixtures were supplemented with the next templated nucleotide, dATP, the primer was extended by one nucleotide in each case with no apparent difference in the formation of this product between the two reaction mixtures (Figure 4.4, lanes 3 and 7). A band at position N was also seen in each of these lanes. There were also two additional bands seen in the control reaction sample. These corresponded to DNA products of 23 and 25 bp in length and likely represent extension products resulting from the misincorporation of dCTP and/or dATP. We and others have previously observed that MMLV RT is able to incorporate and extend non-complementary nucleotides (Chapter 3; Huang *et al.*, 1992; Magee *et al.*, 2008). When the remaining two nucleotides (dGTP and dTTP) were added, full-length and larger than full-length extension products were observed in each reaction mixture. There were also two stops at positions N and N + 1 seen in the CDVpp reaction lane (Figure 4.4, lane 8). When the DNA primer-template pair P1-T9 was used in these same reactions, similar results were obtained, although no larger than full-length extension products were observed and the stops at positions N and N + 1 were stronger than those obtained using the DNA-RNA primer-template pair (Figure 4.4, lane 20). Additionally, in the sample that was incubated with CDVpp, dATP and a DNA template, the N and N + 1 bands were of a similar intensity (Figure 4.4, lane 19), whereas in the reaction using an RNA template, the N + 1 band was the stronger band (Figure 4.4, lane 7). These results suggest that MMLV RT has difficulty extending primers terminating in CDV and in CDV + 1, and this difficulty is more pronounced when using a DNA template.

The incorporation and extension of *(S)*-HPMPApp relative to dATP was also investigated. As shown in Figure 4.5 (left panel), MMLV RT had little difficulty incorporating and extending a primer containing *(S)*-HPMPA when using the RNA template $T11_{RNA}$. *(S)*-HPMPApp was utilized as a substrate and incorporated into DNA to an extent similar to that seen in the control reaction containing dATP (Figure 4.5, lanes 2 and 6). These primers were then extended one nucleotide after the addition of the next templated nucleotide, dTTP, with no obvious difference in incorporation between the two

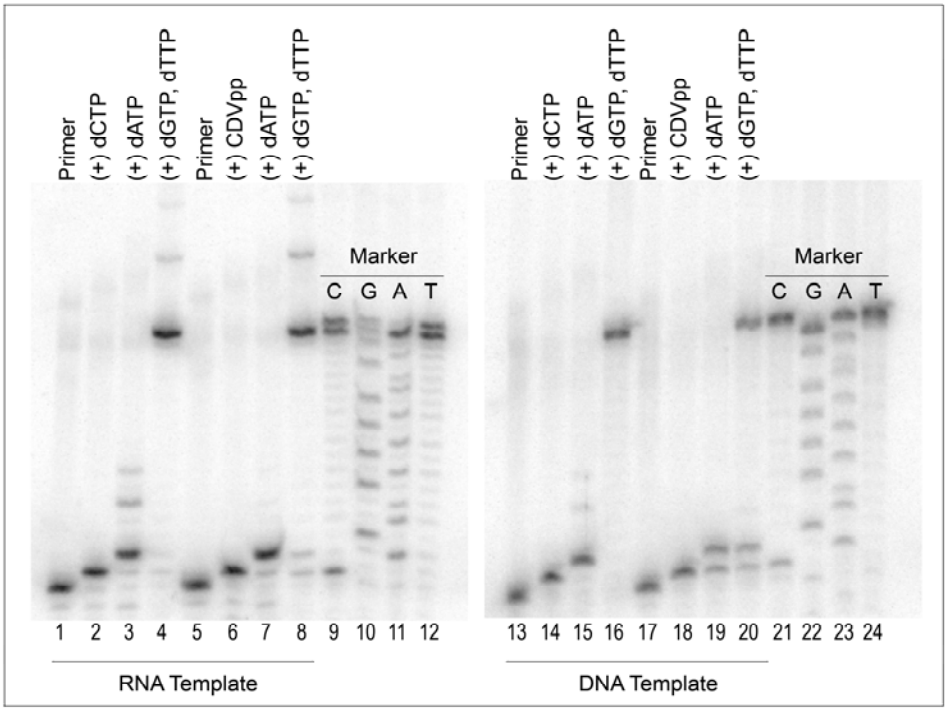

Figure 4.4 MMLV RT can use CDVpp as a substrate and extend primers containing CDV opposite RNA and DNA templates. **Left panel.** The incorporation and extension of CDV opposite an RNA template. Labeled primer P1 was annealed to template $T9_{RNA}$ and incubated with 2 U/µl of MMLV RT in the presence of 10 µM dCTP (lanes 2 to 4) or 10 µM CDVpp (lanes 6 to 8) for 5 min at 37°C. After sampling, the reaction mixtures were adjusted to contain 10 µM dATP, incubated for another 5 min, and another sample taken. The reaction mixtures were further adjusted to contain 10 µM each dGTP and dTTP and the reactions incubated for a final 5 min. **Right panel.** The incorporation and extension of CDV opposite a DNA template. Reactions were prepared as indicated for the left panel, except that the DNA template T9 was used. dCTP reactions are shown in lanes 14 to 16 and CDVpp reactions are shown in lanes 18 to 20. Lanes marked "Primer" show labeled primer P1. Size standards are shown in lanes 9 to 12 and 21 to 24.

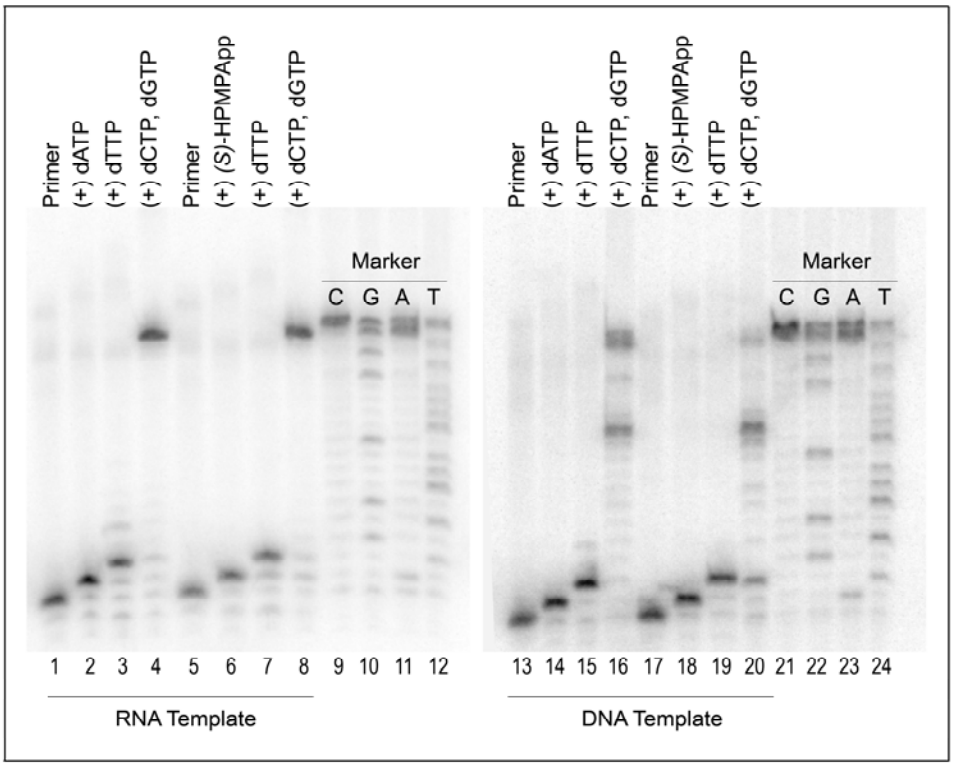

Figure 4.5 MMLV RT can incorporate *(S)*-HPMPA opposite RNA and DNA templates and extend primers containing this drug. **Left panel.** The incorporation and extension of *(S)*-HPMPA opposite an RNA template. ^{32}P-labeled primer P1 was annealed to template $T11_{RNA}$ and incubated at 37°C for 5 min with 2 U/µl MMLV RT in the presence of 10 µM dATP (lanes 2 to 4) or 10 µM *(S)*-HPMPApp (lanes 6 to 8). A sample was taken, each reaction mixture was adjusted to contain 10 µM dTTP, and the reactions incubated for a further 5 min. Another sample was taken and the reaction mixtures further adjusted to include 10 µM dCTP and 10 µM dGTP. After an additional 5 min incubation, a final sample was taken. **Right panel.** The incorporation and extension of *(S)*-HPMPA opposite a DNA template. Reaction mixtures were prepared and analyzed as indicated in the description for the left panel, except that the DNA oligonucleotide T11 was used as the template. The reactions using dATP are shown in lanes 14 to 16 and those using *(S)*-HPMPApp are shown in lanes 18 to 20. The position of the labeled primer P1 is shown in the lanes marked "Primer". Dideoxy sequencing reaction products used as size markers are shown in lanes 9 to 12 and 21 to 24.

reactions (Figure 4.5, lanes 3 and 7), although there were some faint bands in the control reaction lane that, again, were most likely due to misincorporation. When the remaining two nucleotides, dCTP and dGTP, were added, the products formed by the two reaction mixtures were indistinguishable (Figure 4.5, lanes 4 and 8). In contrast, when these experiments were repeated using the DNA template T11, differences between the two reactions were observed. Although MMLV RT can incorporate *(S)*-HPMPA and extend this *(S)*-HPMPA-terminated primer by an additional nucleotide to an extent similar to that seen in the control reaction using dATP (Figure 4.5, compare lanes 18 and 19 to lanes 14 and 15), the addition of dCTP and dGTP resulted in the appearance of a strong stop at position N + 1 (Figure 4.5, lane 20). This strong stop was also observed in the primer extension assay shown in Figure 4.3.

4.3.4 HIV-1 RT can also incorporate CDV and *(S)*-HPMPA into DNA and extend primers containing these substrates. The ability of HIV-1 RT to use CDVpp and *(S)*-HPMPApp as substrates and incorporate them into DNA was also investigated using both RNA and DNA templates. Using the primer-template pair P1-$T9_{RNA}$, HIV-1 used CDVpp as a substrate, extended this primer by one more nucleotide, and then extended this N + 1 primer out to the end of the RNA template (Figure 4.6, lanes 6 to 8). The reaction products formed were comparable to those observed in the control reaction in which dCTP replaced CDVpp (Figure 4.6, compare lanes 6 to 8 with 2 to 4). Both sets of reaction products showed evidence of misincorporation, although this was seen to a greater extent in the control reaction lanes. Interestingly, a stop at position N + 3 was seen in the products of the reaction mixture containing CDVpp, dATP, dGTP and dTTP. This stop was at the same position as the weak stop observed in the primer extension assay shown in Figure 4.2. In this assay however, this stop was also seen in the control reaction; the latter reaction also produced an additional stop product at the N + 4 position. Since the N + 3 stop was seen in both reaction mixtures, it is not likely due to a drug-specific effect. When the DNA template T9 was used in the reaction, the incorporation and extension of CDVpp by HIV-1 RT was similar to that seen with dCTP, although the latter reaction produced some additional products, again presumably due to

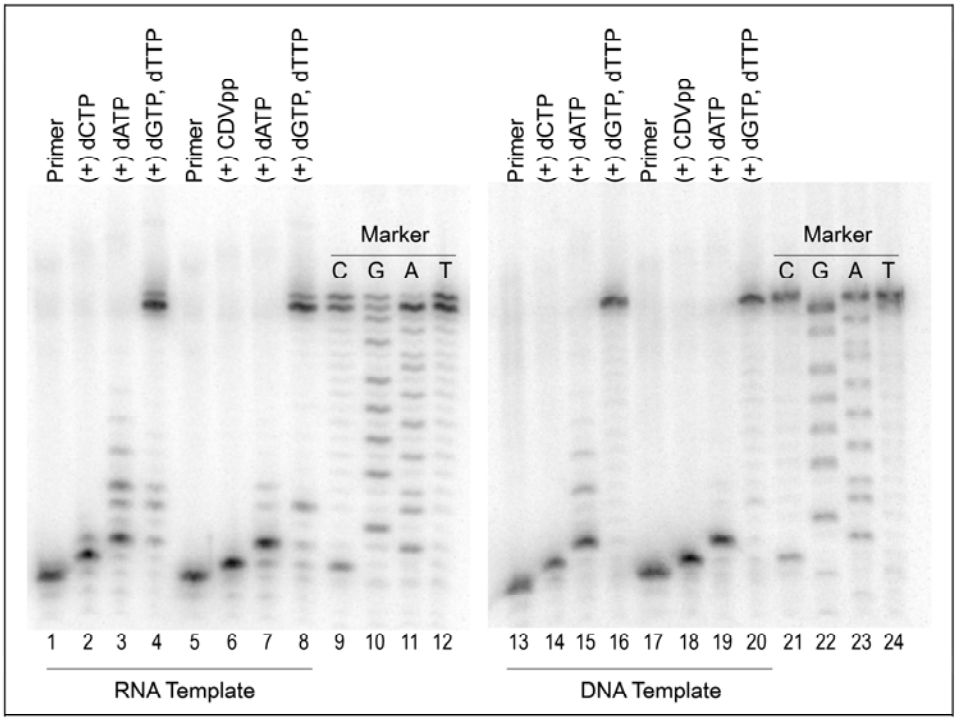

Figure 4.6 HIV-1 RT can incorporate and extend molecules of CDV opposite RNA and DNA templates. Reaction mixtures were prepared as described in the legend to Figure 4.4, except that 50 nM HIV-1 RT was used in place of MMLV RT.

misincorporation (Figure 4.6, right panel). No pause sites were detected in the products of the reactions containing CDVpp.

The incorporation and extension of *(S)*-HPMPApp by HIV-1 were also analyzed; these results are shown in Figure 4.7. The stepwise incorporation and extension of *(S)*-HPMPApp opposite RNA was very similar to that seen in the control reaction containing dATP instead of drug (Figure 4.7, lanes 2 to 4 and 6 to 8). As observed previously, there was evidence of misincorporation products in the control reaction lanes. The N + 3 stop, previously observed in the primer extension assay containing *(S)*-HPMPApp (Figure 4.2), was also seen in this incorporation assay (Figure 4.7, lane 8). However, this stop was also seen in the control reaction containing dATP, although this band was much weaker in the latter reaction (Figure 4.7, lane 4). The incorporation and extension of *(S)*-HPMPApp by HIV-1 RT using a DNA template was also very similar to that observed in the control reaction in which dATP replaces *(S)*-HPMPApp. The only differences observed between the products formed in these two reaction mixtures was the extent and nature of the misincorporation products produced (Figure 4.7, compare lanes 15 and 19) and the presence of pause sites in the *(S)*-HPMPApp reaction mixture corresponding to products greater than 27 nucleotides in length. These pause sites included the strong stop at an oligonucleotide length of 33 nucleotides that was observed in the primer extension analysis shown in Figure 4.3.

4.3.5 MMLV RT and HIV-1 RT are inhibited by CDV and *(S)*-HPMPA in the template strand. Since both MMLV RT and HIV-1 RT can utilize CDVpp and *(S)*-HPMPApp and incorporate them into DNA, we next examined the ability of these enzymes to copy DNA templates containing each of these drugs. The drug-containing templates were prepared as described previously (Chapter 3; Magee *et al.*, 2008), and annealed to ^{32}P-labeled primers designed to terminate one nucleotide prior to the drug lesion (P10 and P17), at the drug lesion (P13 and P18), or one nucleotide past the drug lesion (P14 and P19) (Figure 4.1) (Chapter 3; Magee *et al.*, 2008). The results of these experiments are shown in Figure 4.8. In each of the control reaction lanes, in which dCMP or dAMP is present in place of CDV and *(S)*-HPMPA, respectively, both MMLV

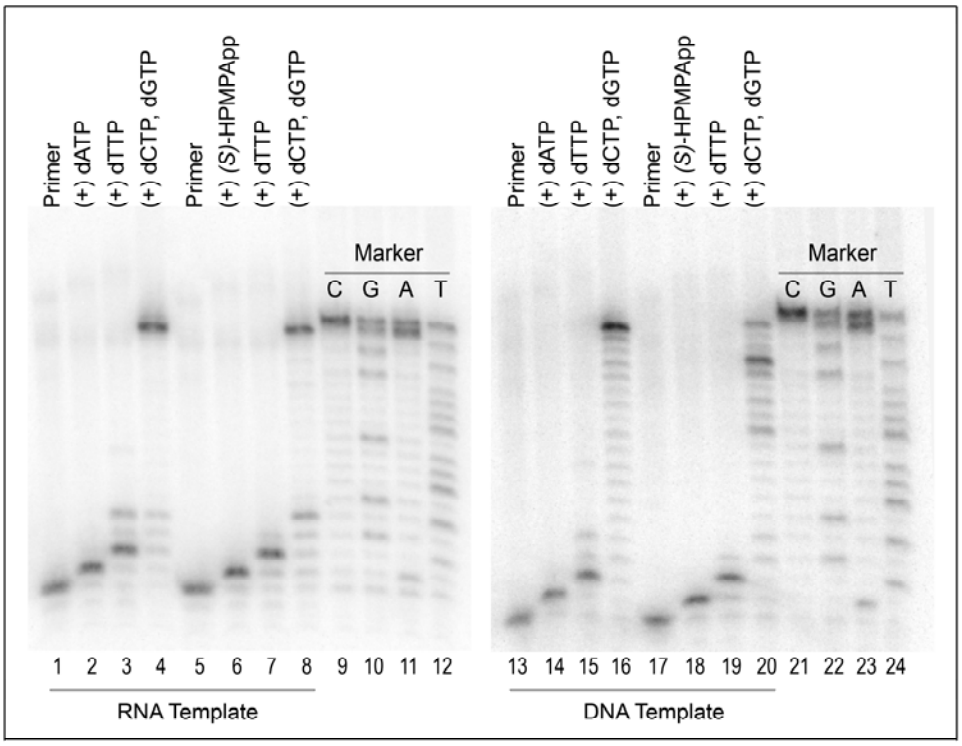

Figure 4.7 The incorporation of *(S)*-HPMPA by HIV-1 RT opposite RNA or DNA templates does not cause chain termination. Reaction mixtures were prepared and analyzed as described in the legend to Figure 4.5, except that 50 nM HIV-1 RT was used in place of 2 U/µl MMLV RT.

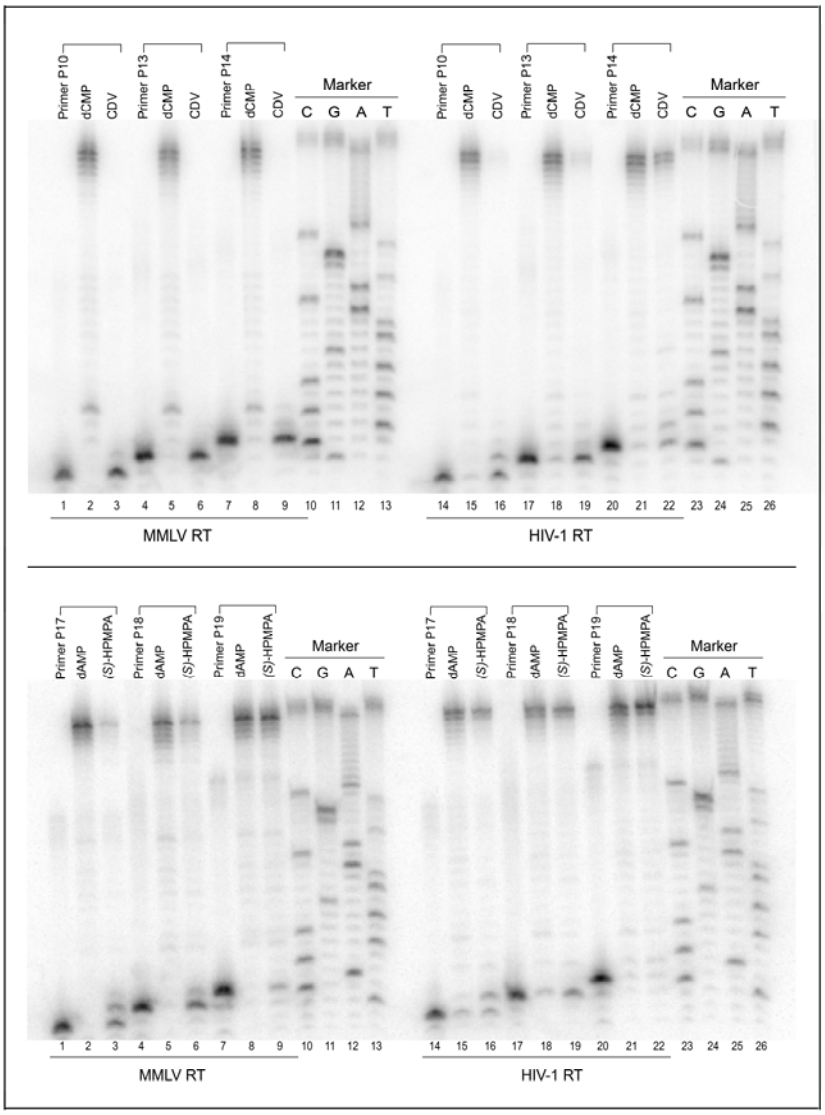

Figure 4.8 Effects of templates bearing CDV and *(S)*-HPMPA on DNA synthesis catalyzed by MMLV RT and HIV-1 RT. Templates containing dCMP/CDV (top panel) or dAMP/*(S)*-HPMPA (bottom panel) were prepared and annealed to the indicated ^{32}P labeled primers. These primers terminate one nucleotide prior to (P10 and P17), at (P13 and P18), or one nucleotide after (P14 and P19) the drug lesion. The primer-template pairs were incubated with 200 μM dNTPs and either 2 U/μl MMLV RT or 50 nM HIV-1 RT for 5 min at 37°C. The reaction products were recovered with magnetic beads and analyzed by phosphorimager analysis after separation on a 10% polyacrylamide gel. Lanes marked "Primer" show the position of each primer on the gel and dideoxy sequencing reaction size markers are shown to the right of each set of reactions.

RT and HIV-1 RT were able to extend each primer out to the end of the template. There was an array of these fully-extended products seen in each of these reaction mixtures; the nature of these products is unknown but may be a result of the biotin tag located at the 5′-end of the template strand. The dCMP control template reactions incubated with MMLV RT also exhibited a pause site at a position corresponding to an oligonucleotide 22 nucleotides in length (Figure 4.8 top panel, lanes 2, 5, and 8).

The presence of CDV or *(S)*-HPMPA in the template strand resulted in the inhibition of DNA synthesis across the drug lesions. This inhibition was more pronounced with the CDV-containing templates relative to the *(S)*-HPMPA-containing templates and in reactions catalyzed by MMLV RT relative to those reactions catalyzed by HIV-1 RT. In each of the three reactions catalyzed by MMLV RT in which the CDV-containing templates were used, no full-length extension products were produced (Figure 4.8, top panel, lanes 3, 6, and 9). In the reaction containing primer P10, the products consisted of a mixture of primer and primer + 1 products; the latter product was weak compared to the former. In the reaction utilizing primer P13, there appeared to be no net DNA synthesis, and in the reaction containing primer P14, there was a mixture of primer, primer + 1, and primer + 2 products; the primer product was the predominant band in this lane. The presence of *(S)*-HPMPA in the template strand did not result in as strong of a block to MMLV RT relative to that seen with the CDV-containing templates. Some full-length extension products were observed in all three reaction mixtures, although relatively little were produced when primers P17 and P18 were used relative to P19 (Figure 4.8 bottom panel, lanes 3, 6 and 9). In addition to these full-length products, two prominent bands corresponding to primer and primer + 1 were seen in the reactions incubated with primers P17 and P18. A stop at the primer position is also observed in the reaction utilizing primer P19.

When these primer-template pairs were incubated with HIV-1 RT, some inhibition of DNA synthesis was observed, but not as great as that seen in reactions incubated with MMLV RT. For the CDV-containing templates, the use of primers P10 and P13 resulted in faint smearing located at the position of full-length extension products. The strongest products found in each of these reactions however, were primer

and primer + 1 and primer, respectively (Figure 4.8 top panel, lanes 16 and 19). In contrast, when primer P14 was annealed to this template, distinct full-length extension products were seen along with two stop sites located at positions primer and primer + 1 (Figure 4.8 top panel, lane 22). The presence of *(S)*-HPMPA in the template strand resulted in even less of a block to DNA synthesis. In all three reactions catalyzed by HIV-1 RT full-length extension products were observed (Figure 4.8 bottom panel, lanes 16, 19 and 22). Although stop sites were observed at positions primer + 1 and primer for reactions containing P17 and P18, respectively (and relative to the control reactions), there were no stop sites seen when primer P19 was used. These results suggest that once the newly-synthesized DNA is immediately past the *(S)*-HPMPA drug lesion, HIV-1 can continue synthesis unimpeded.

4.4 DISCUSSION

The nucleoside phosphonate class of antiviral drugs are effective against a wide range of DNA viruses and retroviruses (De Clercq *et al.*, 1986; reviewed in De Clercq and Holý, 2005). Reports also indicate that some of these drugs are effective against hepatitis C virus (HCV) and hepatitis B virus (HBV) (Wyles *et al.*, 2009). Although CDV has been approved for the treatment of human cytomegalovirus retinitis in AIDS patients and has been used off-label for the treatment of a variety of other DNA virus infections (De Clercq, 2004), *(S)*-HPMPA has not been used in a clinical setting. These two drugs possess a 3′-hydroxyl moiety that can theoretically be used for subsequent DNA chain elongation. This property separates CDV and *(S)*-HPMPA from all other nucleoside/nucleotide reverse transcriptase inhibitors approved for the treatment of HIV-1 infections; the latter compounds are all obligate chain terminators lacking this functional group. In contrast, the hepatitis B drug entecavir, an analog of deoxyguanosine, is not an obligate chain terminator as it possesses the 3′-hydroxyl group. Reports have indicated that this drug also has activity against HIV-1 (McMahon *et al.*, 2007), and biochemical studies using purified HIV-1 RT have demonstrated that this drug acts as a delayed chain terminator (Tchesnokov *et al.*, 2008). The results presented in this

report suggest that CDV and *(S)*-HPMPA inhibit HIV-1 replication by a different mechanism, namely by blocking DNA synthesis when in the template strand.

The addition of CDVpp or *(S)*-HPMPApp to a primer extension assay containing all four dNTPs indicated that the RTs utilize these drugs as substrates differently depending on whether or not the template strand is RNA or DNA. For example, very weak stop sites associated with drug incorporation were observed when MMLV RT catalyzed a reaction in which RNA was used as a template (Figure 4.2), but strong stops were observed when a DNA template of the same sequence was used (Figure 4.3). The opposite occurred in HIV-1 catalyzed reactions. No inhibition of DNA synthesis was observed when using a DNA template (with the exception of a stop site 14 nucleotides away from the expected incorporation site of *(S)*-HPMPA) (Figure 4.3), but a weak stop was observed at position N + 3 in both drug-containing reaction products when using an RNA template (Figure 4.2). Discrimination between RNA and DNA templates by HIV-1 RT has previously been observed, although mainly in the context of replication fidelity (Boyer *et al.*, 1992; Kerr and Anderson, 1997). However, because this stop product was also seen in the control reaction lanes when analyzing the stepwise incorporation and extension by HIV-1 RT, its relevance to the inhibition of DNA synthesis is difficult to assess. The presence of this reaction product is intriguing, since it is located at the same position as the delayed chain termination site caused by entecavir incorporation into DNA by HIV-1 RT (Tchesnokov *et al.*, 2008).

Because these primer extension assays contained all four dNTPs, it was possible that where no drug effect was seen, the enzymes were simply not using the drugs as substrates. We therefore performed stepwise incorporation and extension assays and supplied each reaction with drug first, then the next template nucleotide, followed by the remaining two nucleotides. Under these conditions, it was apparent that both MMLV RT and HIV-1 RT could use CDVpp and *(S)*-HPMPApp as substrates, incorporate them into DNA, and then extend primers terminating in either of these drugs, although in certain cases the presence of the drugs caused pausing to occur (Figures 4.4 to 4.7) In all cases however, full-length extension products were seen, indicating that these drugs are not causing an absolute block in DNA synthesis. It is unclear from these studies, however,

the efficiency with which these RTs utilize CDVpp and *(S)*-HPMPApp relative to the natural nucleotides, dCTP and dATP, respectively. Although Cherrington *et al.* (1996) calculated a K_i value of 0.83 μM for CDVpp against HIV-1 RT (relative to the K_m value of 0.14 μM for dCTP), the K_m values of CDVpp and *(S)*-HPMPA for MMLV RT and HIV-1 RT have not been determined. We hypothesize that these K_m values, at least for HIV-1 RT, will be large relative to the natural nucleotides. This hypothesis is based on the observation that CDV and *(S)*-HPMPA are not effective against HIV-1 in a T-lymphocyte cell culture, in contrast to their prodrugs, which achieve higher intracellular diphosphoryl metabolite concentrations, are effective.

This work helps to explain the known antiviral activities of CDV and *(S)*-HPMPA against these two types of retroviruses. Although no data are available for the activity of CDV against MMLV, *(S)*-HPMPA was shown to have activity against a related retrovirus, MMSV (De Clercq *et al.*, 1986; Pauwels *et al.*, 1988). In contrast, CDV and *(S)*-HPMPA are ineffective against HIV-1 in a CD4+ T-lymphocyte cell line (Pauwels *et al.*, 1988; Srinivas *et al.*, 1997), although alkoxyalkyl ester prodrug derivatives do show efficacy against this virus (Hostetler *et al.*, 2006; Valiaeva *et al.*, 2008). Both CDV and *(S)*-HPMPA inhibit MMLV RT when being incorporated into DNA (at least during second strand synthesis), as well as when in the template strand. In contrast, HIV-1 RT does not appear to be inhibited by either drug when being incorporated into DNA, but it is inhibited by drug in the template strand. This inhibition is not an absolute block to replication, however. Although the impact of the efficiency with which these two enzymes use these drugs relative to the natural substrates remains to be determined, the antiviral efficacy of each drug can be explained by their relative effects on incorporation during first strand synthesis, on incorporation during second strand synthesis, and on trans-lesion synthesis during second strand synthesis. Unlike MMLV RT, HIV-1 RT does not show an absolute block at any of these stages and may require a large amount of incorporated drug before any antiviral activity is seen, a condition that could be achieved by using the alkoxyalkyl ester prodrugs. Our findings suggest that CDV and *(S)*-HPMPA affect diverse viral polymerases by a similar, complex mechanism. With the antiviral spectrum of these compounds recently expanded to include HCV and HBV (Wyles *et al.*,

2009), it will be of interest to determine if CDVpp and *(S)*-HPMPApp inhibit the polymerases encoded by these two viruses in a similar manner.

4.5 AUTHOR CONTRIBUTION TO DATA

Wendy Magee performed all of the experiments described in this study.

4.6 REFERENCES

Aldern, K. A., S. L. Ciesla, K. L. Winegarden, and K. Y. Hostetler. 2003. Increased antiviral activity of 1-*O*-hexadecyloxypropyl-[2-^{14}C]cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism. Mol. Pharmacol. **63:** 678-681.

Boyer, J. C., K. Bebenek, and T. A. Kunkel. 1992. Unequal human immunodeficiency virus type 1 reverse transcriptase error rates with RNA and DNA templates. Proc. Natl. Acad. Sci. USA **89:** 6919-6923.

Cherrington, J. M., M. D. Fuller, A. S. Mulato, S. J. W. Allen, S. C. Kunder, M. A. Ussery, Z. Lesnikowski, R. F. Schinazi, J.-P. Sommadossi, and M. S. Chen. 1996. Comparative kinetic analyses of interactions of inhibitors with Rauscher murine leukemia virus and human immunodeficiency virus reverse transcriptases. Antimicrob. Agents Chemother. **40:** 1270-1273.

Das, D., and M. M. Georgiadis. 2004. The crystal structure of the monomeric reverse transcriptase from Moloney murine leukemia virus. Structure **12:** 819-829.

De Clercq, E. 2004. Antivirals and antiviral strategies. Nat. Rev. Microbiol. **2:** 704-720.

De Clercq, E., and A. Holý. 2005. Acyclic nucleoside phosphonates: a key class of antiviral drugs. Nat. Rev. Drug Discov. **4:** 928-940.

De Clercq, E., A. Holý, I. Rosenberg, T. Sakuma, J. Balzarini, and P. C. Maudgal. 1986. A novel selective broad-spectrum anti-DNA virus agent. Nature **323:** 464-467.

Goff, S.P. 2007. *Retroviridae*: the retroviruses and their replication, p. 1999-2065. *In* D. M. Knipe, P. M. Howley (eds.), Fields Virology, fifth edition. Lippincott Williams & Wilkins, a Wolters Kluwer Business, Philadelphia.

Hamilton, M. D., A. A. Nuara, D. B. Gammon, R. M. Buller, and D. H. Evans. 2007. Duplex strand joining reactions catalyzed by vaccinia DNA polymerase. Nucleic Acids Res. **35:** 143-151.

Hostetler, K. Y., K. A. Aldern, W. B. Wan, S. L. Ciesla, and J. R. Beadle. 2006. Alkoxyalkyl esters of (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine are potent inhibitors of the replication of wild-type and drug-resistant human immunodeficiency virus type 1 in vitro. Antimicrob. Agents Chemother. **50:** 2857-2859.

Huang, P., D. Farquhar, and W. Plunkett. 1992. Selective action of 2',3'-didehydro-2',3'-dideoxythymidine triphosphate on human immunodeficiency virus reverse transcriptase and human DNA polymerases. J. Biol. Chem. **267:** 2817-2822.

Kerr, S. G., and K. S. Anderson. 1997. RNA dependent DNA replication fidelity of HIV-1 reverse transcriptase: evidence of discrimination between DNA and RNA substrates. Biochemistry **36:** 14056-14063.

Le Grice, S.F.J. 1993. Human immunodeficiency virus reverse transcriptase, p. 163-191. *In* A. M. Skalka, S. P. Goff (eds.), Reverse Transcriptase. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Magee, W. C., K. A. Aldern, K. Y. Hostetler, and D. H. Evans. 2008. Cidofovir and *(S)*-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine are highly effective inhibitors of vaccinia virus DNA polymerase when incorporated into the template strand. Antimicrob. Agents Chemother. **52:** 586-597.

Magee, W. C., K. Y. Hostetler, and D. H. Evans. 2005. Mechanism of inhibition of vaccinia virus DNA polymerase by cidofovir diphosphate. Antimicrob. Agents Chemother. **49:** 3153-3162.

Marcus, S. L., and N. H. Sarkar. 1978. Retroviral "terminal deoxynucleotidyl transferase" activity is reverse transcription. Virology **84:** 247-259.

McMahon, M. A., B. L. Jilek, T. P. Brennan, L. Shen, Y. Zhou, M. Wind-Rotolo, S. Xing, S. Bhat, B. Hale , R. Hegarty, C. R. Chong, J. O. Liu, R. F. Siliciano, and C. L. Thio. 2007. The HBV drug entecavir - effects on HIV-1 replication and resistance. N. Engl. J. Med. **356:** 2614-2621.

Pauwels, R., J. Balzarini, D. Schols, M. Baba, J. Desmyter, I. Rosenberg, A. Holy, and E. De Clercq. 1988. Phosphonomethoxyethyl purine derivatives, a new class of anti-human immunodeficiency virus agents. Antimicrob. Agents Chemother. **32:** 1025-1030.

Prasad, V.R. 1993. Genetic analysis of retroviral reverse transcriptase structure and function, p. 135-162. *In* A. M. Skalka, S. P. Goff (eds.), Reverse Transcriptase. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Srinivas, R. V., M. Connely, and A. Fridland. 1997. *(S)*-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) inhibits HIV-1 replication in epithelial cells, but not T-lymphocytes. Antiviral Res. **35:** 23-27.

Tchesnokov, E. P., A. Obikhod, R. F. Schinazi, and M. Götte. 2008. Delayed chain termination protects the anti-hepatitis B virus drug entecavir from excision by HIV-1 reverse transcriptase. J. Biol. Chem. **283:** 34218-34228.

Valiaeva, N., K. A. Aldern, J. Trahan, J. R. Beadle, and K. Y. Hostetler. 2008. Synthesis and evaluation of octadecyloxyethyl esters of five 3-hydroxy-2-(phosphonomethoxy)propyl nucleoside phosphonates in HIV-1 infected cells. Antiviral Research **78:** A49-A50.

Williams, K. J., L. A. Loeb, and M. Fry. 1990. Synthesis of DNA by human immunodeficiency virus reverse transcriptase is preferentially blocked at template oligo(deoxyadenosine) tracts. J. Biol. Chem. **265:** 18682-18689.

Wyles, D. L., K. A. Kaihara, B. E. Korba, R. T. Schooley, J. R. Beadle, and K. Y. Hostetler. 2009. ODE-(S)-HPMPA is a potent and selective inhibitor of hepatitis C virus replication in genotype 1A, 1B and 2A replicons. Antimicrob. Agents Chemother. Published ahead of print on 16 March 2009. doi:10.1128/AAC.01546-08.

Xiong, X., J. L. Smith, and M. S. Chen. 1997. Effect of incorporation of cidofovir into DNA by human cytomegalovirus DNA polymerase on DNA elongation. Antimicrob. Agents Chemother. **41:** 594-599.

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY OF RESULTS

The mechanism of action of two antiviral agents, CDV and *(S)*-HPMPA, was examined using steady state analyses with three different viral polymerases, vaccinia virus DNA polymerase and HIV-1 and MMLV reverse transcriptases. These drugs are part of the nucleoside phosphonate class of antiviral agents and are structurally similar in that they differ only in the structure of their base moiety. The three viral enzymes each play a key role in the life cycle of their respective viruses and are responsible for the replication of the viral genome. CDV and *(S)*-HPMPA were shown to inhibit these enzymes in several different ways. These two drugs are first taken up into cells and then phosphorylated by cellular enzymes to their active intracellular metabolites, CDVpp and *(S)*-HPMPApp, respectively (Cihlar and Chen, 1996; Merta *et al.*, 1992). Although the amount of CDV taken up by MRC-5 cells is similar to that of *(S)*-HPMPA, *(S)*-HPMPA is phosphorylated to *(S)*-HPMPApp to a greater extent than is CDV to CDVpp (Chapter 3; Aldern *et al.*, 2003). This increased amount of *(S)*-HPMPApp in cells results in a larger pool of active metabolite that can be used by the viral enzymes. Further, the addition of an alkoxyalkyl moiety to either CDV or *(S)*-HPMPA results in greater uptake relative to the underivatized forms, leading to larger pools of the diphosphoryl metabolites (Chapter 3; Aldern *et al.*, 2003). The importance of drug uptake and metabolism for antiviral efficacy is clearly illustrated by the inhibition of HIV-1 by CDV, *(S)*-HPMPA and their alkoxyalkyl ester derivatives. Early studies of *(S)*-HPMPA indicated that this drug exhibits some inhibitory effects on HIV-1 replication, but only at levels approaching cytotoxicity (Pauwels *et al.*, 1988). In addition, CDV was shown to inhibit HIV-1 replication in epithelioid HeLa-CD4 cells, but not in the T-lymphocyte cell line MT-2 (Srinivas *et al.*, 1997). These latter results were due, at least in part, to differences in the metabolism of CDV in the two cell types. The uptake of CDV is similar in MT-2 cells and HeLa-CD4 cells, but the levels of CDVpp are ~three- to ten-fold lower in the former

relative to the latter. In contrast, alkoxyalkyl ester derivatives of CDV and *(S)*-HPMPA are effective in inhibiting HIV-1 replication in the subnanomolar to nanomolar range (Hostetler *et al.*, 2006; Valiaeva *et al.*, 2008). As the difference between these derivatives and the parent compounds is only in the amount of drug taken up and the extent of metabolism to the active diphosphoryl compounds, these results suggest that high levels CDVpp or *(S)*-HPMPApp need to be present in the cell in order for inhibition of the HIV-1 RT to occur. Indeed, kinetic studies with HIV-1 RT and a series of antiviral agents including CDVpp, AZT triphosphate, stauvidine triphosphate, lamivudine triphosphate and ddATP showed that the K_i value for CDVpp is ~nine- to 110-fold higher than for the other antiretroviral drugs (Cherrington *et al.*, 1996).

CDVpp and *(S)*-HPMPApp can be used as substrates by all three polymerases and be incorporated into the 3′-terminus of the growing DNA strand. *(S)*-HPMPApp was shown to be a more efficient substrate for vaccinia virus DNA polymerase than was CDVpp (Chapters 2 and 3). Indeed, *(S)*-HPMPApp was shown to be as good as, if not a better substrate than, the natural substrate, dATP. In contrast, CDVpp was a less efficient substrate for this enzyme than dCTP. This difference in the efficiency of use between the two substrates in part explains the greater antiviral efficacy of *(S)*-HPMPA versus CDV against poxviruses. The K_m and V_{max} values of these substrates for the HIV-1 and MMLV RTs have not yet been determined. Because little work has been done on the effects of CDV and *(S)*-HPMPA on MMLV replication, it is difficult to make predictions about the efficiency of use of these substrates by MMLV RT relative to the natural nucleotides. However, the need for large pools of CDVpp and *(S)*-HPMPApp in the cell, coupled with the higher K_i value of CDVpp for HIV-1 RT (Cherrington *et al.*, 1996), suggests that these are inefficient substrates for the HIV-1 enzyme. This prediction is strengthened by the findings of Franguel *et al.* (2008), who measured a K_d value of CDVpp for HIV-1 RT using pre-steady state kinetic analysis. This value was determined to be 180 μM, 23-fold higher than the K_d of dCTP (7.9 μM).

The drug-terminated primers could also be extended by each polymerase, indicating that the hydroxymethyl moiety on each drug can act as a 3′-hydroxyl group to initiate nucleophilic attack on the incoming dNTP. The presence of drug in the primer

strand did however, lead to the slowing or pausing of DNA synthesis when vaccinia virus DNA polymerase or MMLV RT were used to catalyze the reaction (Chapters 2, 3 and 4). The incorporation of a single CDV or *(S)*-HPMPA residue by vaccinia virus DNA polymerase resulted in pausing at the N + 1 position, where N is the expected site of incorporation of the drug. When two consecutive molecules of CDV or *(S)*-HPMPA were incorporated into DNA by this enzyme, a slowing of DNA synthesis relative to control reactions in the absence of drug was also observed; pause sites were located at positions N, N + 1, N + 2, and N + 3. This slowing of DNA synthesis was more profound when CDV was incorporated in the primer strand than when *(S)*-HPMPA was present, particularly when two molecules were incorporated. MMLV RT was more strongly inhibited by the incorporation of these drugs when DNA templates, rather than RNA templates, were used. Further, the pause sites produced in these reactions were observed at positions N and N + 1 (CDV) and N [*(S)*-HPMPA]. In contrast, HIV-1 RT was not inhibited when these drugs were incorporated into the primer strand; drug-containing reaction products were similar to those obtained in the absence of drug and using the natural substrates (Chapter 4). In reactions catalyzed by all three enzymes however, the production of full-length DNA strands was observed. These results indicate that CDV and *(S)*-HPMPA do not act as functional chain terminators and can be incorporated into DNA allowing subsequent DNA synthesis to occur.

The effect of CDV and *(S)*-HPMPA on the 3′-to-5′ exonuclease activity of vaccinia virus DNA polymerase was also investigated. Although CDV and *(S)*-HPMPA could be excised from DNA bearing these drugs at the primer terminus, oligonucleotides containing either drug at the penultimate position of this terminus, equivalent to the N + 1 structure described above, were refractory to exonuclease activity (Chapters 2 and 3).

The ability of CDV and *(S)*-HPMPA to be incorporated into DNA without causing chain termination prompted an examination of the effects of these drugs when positioned in the template strand. Although not relevant for most nucleoside and nucleotide analogs (because these are either obligate or functional chain terminators), some of the antiviral effects seen by CDV and *(S)*-HPMPA may in fact be mediated during subsequent rounds of DNA synthesis, after drug has been incorporated into DNA.

Indeed, the presence of CDV or *(S)*-HPMPA in the template resulted in inhibition of DNA synthesis catalyzed by vaccinia virus DNA polymerase, MMLV RT or HIV-1 RT. The extent of this inhibition varied depending on the enzyme and, in the case of the reverse transcriptases, the drug used (Chapters 3 and 4). Vaccinia virus DNA polymerase was completely inhibited by drug in the template; nucleotide could be incorporated opposite the drug lesion but further extension did not occur. This inhibition of extension was observed even if oligonucleotides terminating one nucleotide past the drug residue were used to prime DNA synthesis. Interestingly, these latter primers were rapidly degraded by one nucleotide by the vaccinia virus DNA polymerase 3′-to-5′ exonuclease activity, suggesting that these structures are not favored substrates. Similarly, when MMLV RT was used to catalyze DNA synthesis using a template containing CDV, no full-length extension products were observed and synthesis was terminated at, or a few nucleotides past, the drug lesion, depending on the site of the primer 3′-terminus. The presence of *(S)*-HPMPA in the template strand did not result in as great an inhibition of MMLV RT as did CDV in the template; full-length extension product were observed, although the predominant products were pause sites again located at, or a few nucleotides past, the drug lesion. HIV-1 RT was not as strongly inhibited by CDV and *(S)*-HPMPA in the template strand as was MMLV RT. Full-length extension products were observed in all reaction mixtures, although some pausing was seen around the drug lesions. The results obtained with HIV-1 RT help to explain those obtained by Franguel *et al.* (2008), who were unable to measure the inhibition of HIV-1 RT by CDVpp at concentrations up to 2 mM and concluded that this drug is therefore not active against HIV-1 RT. These authors used a filter-based DNA polymerase assay, which measured the incorporation of radiolabeled dAMP into an activated calf-thymus DNA substrate. This type of experiment measures only the first round of DNA synthesis and its inhibition by drug incorporated into the primer strand. Our results indicate that CDV and *(S)*-HPMPA do not inhibit DNA synthesis by HIV-1 RT when incorporated into DNA and exert their inhibitory effects only when in the template strand, which would not be measured by this assay. These results also indicate that the type of assay used to assess inhibition is important in determining the mechanism of action of drugs and suggest that different

assays, like filter-based DNA polymerase assays and primer extension assays, be used in parallel to understand and detect inhibition of viral polymerases by antiviral drugs. In fact, Franguel *et al.* were able to show that CDVpp can be used as a substrate, albeit an inefficient one, by HIV-1 RT and is incorporated into the 3′-end of a DNA primer by this enzyme (Frangeul *et al.*, 2008).

The mechanism of action of CDVpp was previously examined by Xiong *et al.* using HCMV DNA polymerase (Xiong *et al.*, 1997). These authors demonstrated that CDVpp can be used by HCMV DNA polymerase as a substrate and is incorporated into DNA. The incorporation of a single CDV molecule does not block DNA synthesis, although it does result in a pause site at the N + 1 position, the same pause site observed when the vaccinia virus DNA polymerase is used. Two incorporated CDV molecules impede further DNA synthesis, regardless of whether these two molecules are incorporated consecutively or with an intervening natural nucleotide. Further, the presence of CDV at the primer terminus blocks the HCMV DNA polymerase's 3′-to-5′ proofreading exonuclease activity. These authors also investigated the effect of CDV when in the template strand. They observed that the rate of DNA synthesis is slowed by the drug lesion. The three major products seen in this reaction are DNAs terminated one residue prior to, at, and one residue past the CDV lesion. However, full-length DNA products are still formed. These authors noted that the degree of inhibition of HCMV DNA polymerase is greater when CDV is in the template strand than when it is in the primer strand (Xiong *et al.*, 1997).

5.2 MODEL OF THE MECHANISM OF ACTION OF CDV AND *(S)*-HPMPA

Taken together, these results indicate that the inhibition of virus polymerases by CDV and *(S)*-HPMPA is due to a complex interplay of uptake, metabolism to the diphosphoryl derivatives, the efficiency of substrate usage, the ability to incorporate the drugs into DNA, inhibition of DNA synthesis and 3′-to-5′ exonuclease activity (if present) by drug in the primer strand, and inhibition of DNA synthesis by drug in the template strand (Figure 5.1). The impact of each of these factors will determine the relative susceptibility of a virus to these nucleotide analogs. For example, the greater

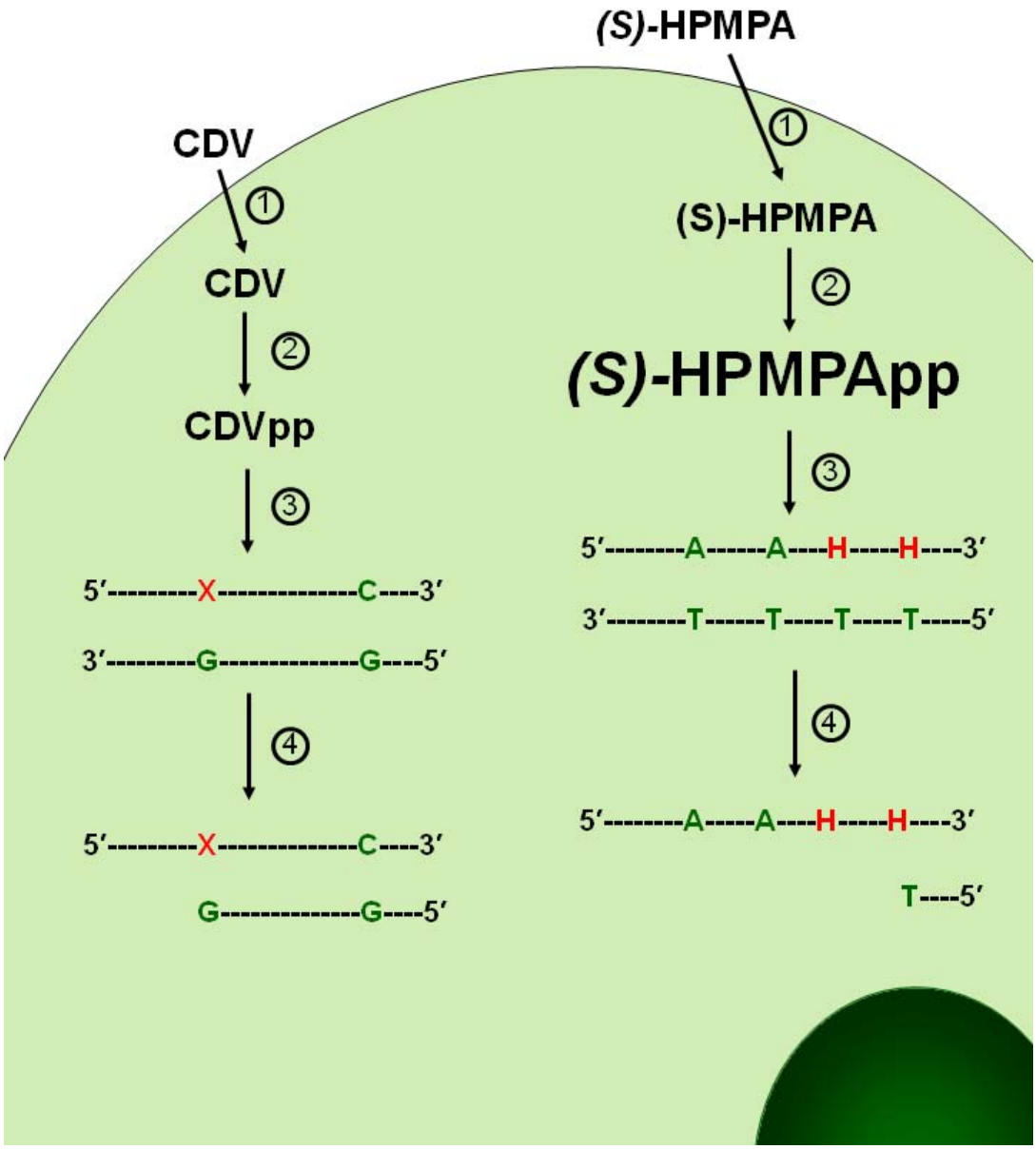

Figure 5.1 The mechanism of action of CDV and *(S)*-HPMPA. CDV and *(S)*-HPMPA are taken up by cells by endocytosis (1) and then converted to their active intracellular metabolites, CDVpp and *(S)*-HPMPApp, respectively, by cellular enzymes (2). Although similar amounts of both drugs are taken up into cells, *(S)*-HPMPA is phosphorylated to a greater extent than is CDV. Both CDVpp (X) and *(S)*-HPMPApp (H) can be used as substrates by viral polymerases and incorporated into viral nucleic acid (3). Although this incorporation of drug may slow elongation, it does not terminate synthesis. The drugs' inhibitory effects take place during the subsequent rounds of synthesis, where drug in the template strand inhibits synthesis past the lesion (4).

efficacy of *(S)*-HPMPA versus CDV against vaccinia virus can be explained by differences in metabolism, the efficiency with which each drug is utilized as a substrate, and the inhibition of DNA synthesis upon incorporation of the drug in the primer strand. *(S)*-HPMPA is metabolized to *(S)*-HPMPApp to a greater extent than is CDV to CDVpp. *(S)*-HPMPApp is also used by vaccinia virus DNA polymerase more efficiently than is CDVpp; incorporation of the former also leads to less inhibition of DNA synthesis than incorporation of the latter. These properties will result in a greater amount of *(S)*-HPMPA being incorporated into viral DNA than CDV, resulting in a more profound inhibition of DNA synthesis by drug in the template strand, even though single molecules of CDV or *(S)*-HPMPA similarly inhibit vaccinia virus DNA polymerase when located in the template strand.

Similarly, the inhibition of simple retroviruses like MMLV and MMSV relative to the lentivirus HIV-1 by these drugs can also be explained, at least in part, by differences in the inhibition of DNA synthesis by the presence of drug in the primer strand or in the template strand. The transformation of murine cells by MMSV is inhibited by *(S)*-HPMPA (De Clercq *et al.*, 1986; Pauwels *et al.*, 1988). In contrast, HIV-1 replication in T-lymphocytes is not inhibited by CDV or *(S)*-HPMPA (Hostetler *et al.*, 2006; Srinivas *et al.*, 1997; Valiaeva *et al.*, 2008). The MMLV RT was inhibited by CDV and *(S)*-HPMPA when incorporated into the primer strand and was strongly inhibited by both drugs when in the template strand. HIV-1 RT, in contrast, exhibited no inhibition of DNA synthesis by either drug in the primer strand and was inhibited by these drugs in the template strand to a much lesser extent than that seen with MMLV RT. The relative efficiencies with which MMLV RT and HIV-1 RT utilize CDVpp and *(S)*-HPMPApp as substrates may also play a role in the inhibition of each virus by these drugs. However, the steady state kinetic values for these substrates and these enzymes have not been determined at present. Further, pre-steady state experiments examining the effects of CDVpp and *(S)*-HPMPApp on these three viral enzymes will provide a more detailed determination of the interactions of these drugs with the enzymes' active sites.

The mechanism of action of CDV and *(S)*-HPMPA is of interest in that the dominant inhibitory effect appears to occur when these drugs are positioned in the

template strand. The inhibition by drug in the template strand is not relevant for most other antiviral nucleoside analogs because they act as either obligate or functional chain terminators. The antiviral drug entecavir is the most closely related to CDV and *(S)*-HPMPA based on the mechanism of inhibition. Entecavir is a 2′-deoxyguanosine analog that possesses a 3′-hydroxyl moiety. Entecavir 5′-triphosphate can be used as a substrate by HBV DNA polymerase and HIV-1 RT and can be incorporated into DNA by both of these enzymes. Studies using the HBV DNA polymerase indicated that this drug acts as a delayed chain terminator and that this termination is more profound after dG rich sequences (Langley *et al.*, 2007; Seifer *et al.*, 1998). The effect of entecavir in the template strand has not been examined using this enzyme. The inhibition of HIV-1 RT by this drug is a result of three different mechanisms: pausing after the incorporation of drug, pausing at a position three nucleotides after the incorporation of drug, and inhibition of DNA synthesis by drug in the template (Domaoal *et al.*, 2008; Tchesnokov *et al.*, 2008). However, the pausing at the N + 3 position and not inhibition of DNA synthesis by drug in the template was shown to be the dominant mechanism of inhibition (Tchesnokov *et al.*, 2008).

The effects of nucleotide analogs in the template strand on DNA replication have not been extensively studied, presumably because most of these analogs are obligate or functional chain terminators. As described above, entecavir in the template strand inhibits DNA synthesis by HIV-1 RT (Tchesnokov *et al.*, 2008). Pausing of the enzyme was observed at two positions: at the site of incorporation of nucleotide opposite the entecavir residue and at one nucleotide past this residue. However, with extended reaction times, these pause products were chased into full-length products, albeit to a lesser extent than that seen in control reactions using templates containing dG. The presence of 5-trifluoromethyl-2′-deoxyuridine in a template strand was also described to block DNA synthesis by human DNA polymerase α and the *Escherichia coli* DNA polymerase I Klenow fragment, although no data were shown (Satake *et al.*, 1992; Satake *et al.*, 1991). Further, a cytosine arabinoside (araC) residue in the template strand slows DNA synthesis past the drug lesion catalyzed by the Klenow fragment, T4 DNA polymerase and human DNA polymerase α_2 (Mikita and Beardsley, 1988). Interestingly, avian

myeloblastosis virus RT exhibited only a weak pause site opposite the araC residue and DNA synthesis was not inhibited appreciably (Mikita and Beardsley, 1988).

The ability of CDV and *(S)*-HPMPA to be incorporated into DNA by viral polymerases without causing chain termination has implications for cellular DNA synthesis. Rat DNA polymerases α, δ, and ε can use *(S)*-HPMPApp as a substrate and incorporate it into DNA (Birkus *et al.*, 2004; Kramata *et al.*, 1996). Primer extension analyses using cellular DNA polymerases have not been conducted using CDVpp as a substrate, but its similarity to *(S)*-HPMPApp and the ability of diverse viral polymerases to use both drugs as substrates suggest that it too will be incorporated into the DNA of a cell. These drugs may then be recognized as DNA damage. Indeed, Vortuba *et al.* showed that the treatment of cells with a related analog, *(S)*-HPMPazaC, resulted in the up-regulation of several genes involved in the DNA damage response (Votruba *et al.*, 2008). The repair of the drug-induced lesions by one of the various DNA repair pathways could protect cells from the deleterious effects of the drugs (Hakem, 2008; Harper and Elledge, 2007).

The complex mechanism of action of CDV and *(S)*-HPMPA on viral polymerases also has implications for the development of resistance to these drugs. The *in vitro* selection of resistance to CDV and/or *(S)*-HPMPA has been described for vaccinia virus (Andrei *et al.*, 2006; Becker *et al.*, 2008; Kornbluth *et al.*, 2006), HCMV (Cihlar *et al.*, 1998) and HSV-1 (Andrei *et al.*, 2000). Resistance in each case has been mapped to the viral polymerase genes and the specific residues conferring resistance have been located in both the polymerase domain and the 3′-to-5′ exonuclease domain of these enzymes. Although more work is needed to examine the interactions of CDVpp and *(S)*-HPMPApp with the resistant polymerases, it is possible that mutated residues in the polymerase domain allow the enzyme to select against incorporation of the drugs into DNA or to bypass drug lesions in the template strand while mutations in the 3′-to-5′ exonuclease domain facilitate removal of the drug after incorporation. The dominant inhibitory effect of the drugs in the template strand also helps to explain the difficulty in generating high level resistance in poxviruses to these compounds. These viruses will not be able to completely exclude the incorporation of either drug into DNA, even in the presence of

resistance mutations. Further, mutant DNA polymerases that are able to both faithfully replicate the viral genome and replicate past the drug lesions are most likely difficult to develop, as evidenced by the occurrence of specialized bypass polymerases in cells. And finally, poxviruses lack specialized lesion repair systems and due to their cytoplasmic site of replication, do not have access to cellular repair machinery located in the nucleus. Interestingly, Hostetler *et al.* (2006) showed that the alkoxyalkyl ester derivatives of *(S)*-HPMPA retained full activity against HIV-1 resistant to azidothymidine and lamivudine, and although there was significant cross-resistance noted with a virus resistant to tenofovir, this virus was still susceptible to the *(S)*-HPMPA derivatives at clinically relevant concentrations. It will be of interest to determine the types of mutations that will develop upon the *in vitro* selection of resistance of HIV-1 to CDV and *(S)*-HPMPA. Since these two drugs exert their effects on the HIV-1 RT only when present in the template strand, we predict that resistance will be more difficult to achieve than that obtained with other antiretroviral compounds that act as obligate chain terminators and that novel mutations will develop.

5.3 FUTURE DIRECTIONS

The results from this work point to several avenues for future research. For example, the structural basis for the inhibition of viral polymerases by CDV and *(S)*-HPMPA has not been elucidated. The presence of either drug in nucleic acid is predicted to cause perturbations in the DNA or RNA structure around the site of the drug residue, leading to inefficient synthesis. This perturbation of nucleic acid structure has previously been observed in a duplex DNA containing ganciclovir residues, another acyclic nucleoside analog (Foti *et al.*, 1997; Marshalko *et al.*, 1995). The overall nuclear magnetic resonance (NMR) structure of this duplex is consistent with the B-form of DNA, but there is a kink observed in the sugar-phosphate backbone at a position 3′ to the ganciclovir lesion site (Foti *et al.*, 1997). Dr. James Beadle, a chemist in the laboratory of our collaborator, Dr. Karl Hostetler (University of California, San Diego), has been developing a method to chemically synthesize the phosphoramidite precursors of CDV and *(S)*-HPMPA so that they can be used to prepare oligonucleotides bearing these drugs,

thus eliminating the need for enzymatic synthesis. These oligonucleotides will then be used to determine the NMR structures of DNAs containing CDV and *(S)*-HPMPA and compared with the structures of DNA duplexes containing dCMP or dAMP, respectively, at the same positions. The drug molecules will be located at positions equivalent to those that cause pausing of the various polymerases. These NMR studies have been initiated in collaboration with Dr. Brian Sykes (Department of Biochemistry, University of Alberta). Further, the thermodynamic properties of these drug-containing duplexes can be measured and compared to those of control duplexes as well as ganciclovir and araC-containing DNA duplexes (Beardsley *et al.*, 1988; Marshalko *et al.*, 1995) to determine the relative impact of these drugs on DNA stability.

Further determination of the structural basis of inhibition by these drugs will include elucidating the structures of vaccinia virus DNA polymerase, MMLV RT, and HIV-1 RT with DNAs containing CDV and *(S)*-HPMPA in both the primer strand and in the template strand. At present, a crystal structure of the vaccinia virus DNA polymerase has not been determined. This protein cannot be purified from virus-infected cells in amounts required for crystallography studies, and attempts to express the protein in heterologous systems like *E. coli* or yeast have been unsuccessful (McDonald and Traktman, 1994; Appendix). However, the crystal structures of other B family DNA polymerase members, like the HSV-1 and the bacteriophage RB69 DNA polymerases, have been determined (Franklin *et al.*, 2001; Liu *et al.*, 2006; Shamoo and Steitz, 1999; Wang *et al.*, 1997). These structures could be used as a basis for modeling the vaccinia virus enzyme with DNAs containing the two drugs, as the overall architecture of this family of polymerases is similar (Liu *et al.*, 2006). The structures of the DNAs would be obtained from the NMR studies described above. These types of modeling experiments have been performed previously with the HCMV DNA polymerase, based on the RB69 DNA polymerase crystal structure, in the analysis of this enzyme's resistance to ganciclovir, CDV and foscarnet (Shi *et al.*, 2006).

Several crystal structures of the MMLV RT and HIV-1 RT have been solved, including structures of HIV-1 RT in complex with the nucleoside/nucleotide analog inhibitors AZT and tenofovir (Arnold *et al.*, 1992; Das and Georgiadis, 2004; Ding *et al.*,

1998; Georgiadis *et al.*, 1995; Huang *et al.*, 1998; Jacobo-Molina *et al.*, 1993; Pata *et al.*, 2004; Sarafianos *et al.*, 2002; Tuske *et al.*, 2004). Comparison of the structures of the MMLV RT and HIV-1 RT complexed with DNAs containing CDV and *(S)*-HPMPA will provide insights into the differential susceptibility of these enzymes to these drugs.

Further work on CDV and *(S)*-HPMPA will include a determination of the mechanism of inhibition of the diphosphoryl derivatives of these drugs on the polymerases of HBV and HCV. These experiments will help to determine if the mechanism of action that we have observed against vaccinia virus DNA polymerase, MMLV RT and HIV-1 RT is more broadly applicable to these other virus systems. In addition, it would be of interest to test the efficacy of CDV, *(S)*-HPMPA and their alkoxyalkyl ester derivatives against other viruses, like influenza, measles, or respiratory syncytial virus, to determine the full antiviral activity spectrum of these drugs. Both the parent compounds and their derivatives should be tested, in case high levels of drug are required for activity, as was seen in the case of HIV-1.

5.4 CONCLUSIONS

The novel mechanism of action that has been described for CDV and *(S)*-HPMPA, namely inhibition of viral polymerases by drug incorporation in the template strand, points to a new avenue for targeted antiviral drug design. Nucleoside and nucleotide analogs can be developed that are efficient and selective substrates for viral polymerases and that cause little inhibition of nucleic acid synthesis when being incorporated into DNA or RNA. These analogs would instead exert their effects in subsequent rounds of replication. Modifications to these drugs could then be made, if necessary, to improve oral bioavailability, uptake, and metabolism. These data also stress the importance of using multiple screening assays in the discovery of new antiviral compounds. The focus on chain terminating nucleoside and nucleotide analogs and the use of assays that examine only the inhibitory effects of drug incorporation into the growing nucleic acid have the potential to miss useful candidate drugs that act through novel mechanisms.

5.5 REFERENCES

Aldern, K. A., S. L. Ciesla, K. L. Winegarden, and K. Y. Hostetler. 2003. Increased antiviral activity of 1-*O*-hexadecyloxypropyl-[2-^{14}C]cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism. Mol. Pharmacol. **63:** 678-681.

Andrei, G., D. B. Gammon, P. Fiten, E. De Clercq, G. Opdenakker, R. Snoeck, and D. H. Evans. 2006. Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice. J. Virol. **80:** 9391-9401.

Andrei, G., R. Snoeck, E. De Clercq, R. Esnouf, P. Fiten, and G. Opdenakker. 2000. Resistance of herpes simplex virus type 1 against different phosphonylmethoxyalkyl derivatives of purines and pyrimidines due to specific mutations in the viral DNA polymerase gene. J. Gen. Virol. **81:** 639-648.

Arnold, E., A. Jacobo-Molina, R. G. Nanni, R. L. Williams, X. Lu, J. Ding, Jr. A. D. Clark, A. Zhang, A. L. Ferris, P. Clark, A. Hizi, and S. H. Hughes. 1992. Structure of HIV-1 reverse transcriptase/DNA complex at 7 Å resolution showing active site locations. Nature **357:** 85-89.

Beardsley, G. P., T. Mikita, M. M. Klaus, and A. L. Nussbaum. 1988. Chemical synthesis of DNA oligomers containing cytosine arabinoside. Nucleic Acids Res. **16:** 9165-9176.

Becker, M. N., M. Obraztsova, E. R. Kern, D. C. Quenelle, K. A. Keith, M. N. Prichard, M. Luo, and R. W. Moyer. 2008. Isolation and characterization of cidofovir resistant vaccinia viruses. Virology J. **5:** 58.

Birkus, G., D. Rejman, M. Otmar, I. Votruba, I. Rosenberg, and A. Holy. 2004. The substrate activity of (*S*)-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine diphosphate toward DNA polymerases α, δ and ε. Antiviral Chem. Chemother. **15:** 23-33.

Cherrington, J. M., M. D. Fuller, A. S. Mulato, S. J. W. Allen, S. C. Kunder, M. A. Ussery, Z. Lesnikowski, R. F. Schinazi, J.-P. Sommadossi, and M. S. Chen. 1996. Comparative kinetic analyses of interactions of inhibitors with Rauscher murine leukemia virus and human immunodeficiency virus reverse transcriptases. Antimicrob. Agents Chemother. **40:** 1270-1273.

Cihlar, T., and M. S. Chen. 1996. Identification of enzymes catalyzing two-step phosphorylation of cidofovir and the effect of cytomegalovirus infection on their activities in host cells. Mol. Pharmacol. **50:** 1502-1510.

Cihlar, T., M. D. Fuller, A. S. Mulato, and J. M. Cherrington. 1998. A point mutation

in the human cytomegalovirus DNA polymerase gene selected *in vitro* by cidofovir confers a slow replication phenotype in cell culture. Virology **248:** 382-393.

Das, D., and M. M. Georgiadis. 2004. The crystal structure of the monomeric reverse transcriptase from Moloney murine leukemia virus. Structure **12:** 819-829.

De Clercq, E., A. Holý, I. Rosenberg, T. Sakuma, J. Balzarini, and P. C. Maudgal. 1986. A novel selective broad-spectrum anti-DNA virus agent. Nature **323:** 464-467.

Ding, J., K. Das, Y. Hsiou, S. G. Sarafianos, A. D. Clark Jr., A. Jacobo-Molina, C. Tantillo, S. H. Hughes, and E. Arnold. 1998. Structure and functional implications of the polymerase active site region in a complex of HIV-1 RT with a double-stranded DNA template-primer and an antibody Fab fragment at 2.8 Å resolution. J. Mol. Biol. **284:** 1095-1111.

Domaoal, R. A., M. McMahon, C. L. Thio, C. M. Bailey, J. Tirado-Rives, A. Obikhod, M. Detorio, K. L. Rapp, R. F. Siliciano, R. F. Schinazi, and K. S. Anderson. 2008. Pre-steady-state kinetic studies establish entecavir 5'-triphosphate as a substrate for HIV-1 reverse transcriptase. J. Biol. Chem. **283:** 5452-5459.

Foti, M., S. Marshalko, E. Schurter, S. Kumar, G. P. Beardsley, and B. I. Schweitzer. 1997. Solution structure of a DNA decamer containing the antiviral drug ganciclovir: combined use of NMR, restrained molecular dynamics, and full relaxation matrix refinement. Biochemistry **36:** 5336-5345.

Frangeul, A., C. Bussetta, J. Deval, K. Barral, K. Alvarez, and B. Canard. 2008. Gln151 of HIV-1 reverse transcriptase acts as a steric gate towards clinically relevant acyclic phosphonate nucleotide analogues. Antiviral Ther. **13:** 115-124.

Franklin, M. C., J. Wang, and T. A. Steitz. 2001. Structure of the replicating complex of a pol α family DNA polymerase. Cell **105:** 657-667.

Georgiadis, M. M., S. M. Jessen, C. M. Ogata, A. Telesnitsky, S. P. Goff, and W. A. Hendrickson. 1995. Mechanistic implications from the structure of a catalytic fragment of Moloney murine leukemia virus reverse transcriptase. Structure **3:** 879-892.

Hakem, R. 2008. DNA-damage repair; the good, the bad, and the ugly. EMBO J. **27:** 589-605.

Harper, J. W., and S. J. Elledge. 2007. The DNA damage response: ten years later. Mol. Cell **28:** 739-745.

Hostetler, K. Y., K. A. Aldern, W. B. Wan, S. L. Ciesla, and J. R. Beadle. 2006. Alkoxyalkyl esters of (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine are potent inhibitors of the replication of wild-type and drug-resistant human immunodeficiency

virus type 1 in vitro. Antimicrob. Agents Chemother. **50:** 2857-2859.

Huang, H., R. Chopra, G. L. Verdine, and S. C. Harrison. 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science **282:** 1669-1675.

Jacobo-Molina, A., J. Ding, R. G. Nanni, Jr. A. D. Clark, X. Lu, C. Tantillo, R. L. Williams, G. Kamer, A. L. Ferris, P. Clark, A. Hizi, S. H. Hughes, and E. Arnold. 1993. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. Proc. Natl. Acad. Sci. USA **90:** 6320-6324.

Kornbluth, R. S., D. F. Smee, R. W. Sidwell, V. Snarsky, D. H. Evans, and K. Y. Hostetler. 2006. Mutations in the E9L polymerase gene of cidofovir-resistant vaccinia virus strain WR are associated with the drug resistance phenotype. Antimicrob. Agents Chemother. **50:** 4038-4043.

Kramata, P., I. Votruba, B. Otová, and A. Holý. 1996. Different inhibitory potencies of acyclic phosphonomethoxyalkyl nucleotide analogs toward DNA polymerases α, δ, and ε. Mol. Pharmacol. **49:** 1005-1011.

Langley, D. R., A. W. Walsh, C. J. Baldick, B. J. Eggers, R. E. Rose, S. M. Levine, A. J. Kapur, R. J. Colonno, and D. J. Tenney. 2007. Inhibition of hepatitis B virus polymerase by entecavir. J. Virol. **81:** 3992-4001.

Liu, S., J. D. Knafels, J. S. Chang, G. A. Waszak, E. T. Baldwin, Jr. M. R. Deibel, D. R. Thomsen, F. L. Homa, P. A. Wells, M. C. Tory, R. A. Poorman, H. Gao, X. Qiu, and A. P. Seddon. 2006. Crystal structure of herpes simplex virus 1 DNA polymerase. J. Biol. Chem. **281:** 18193-18200.

Marshalko, S. J., B. I. Schweitzer, and G. P. Beardsley. 1995. Chiral chemical synthesis of DNA containing (*S*)-9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) and effects on thermal stability, duplex structure, and thermodynamics of duplex formation. Biochemistry **34:** 9235-9248.

McDonald, W. F., and P. Traktman. 1994. Overexpression and purification of the vaccinia virus DNA polymerase. Protein Expr. Purif. **5:** 409-421.

Merta, A., I. Votruba, J. Jindřich, A. Holý, T. Cihlář, I. Rosenberg, M. Otmar, and T. Y. Herve. 1992. Phosphorylation of 9-(2-phosphonomethoxyethyl)adenine and 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)adenine by AMP(dAMP) kinase from L1210 cells. Biochem. Pharmacol. **44:** 2067-2077.

Mikita, T., and G. P. Beardsley. 1988. Functional consequences of the

arabinosylcytosine structural lesion in DNA. Biochemistry **27:** 4698-4705.

Pata, J. D., W. G. Stirtan, S. W. Goldstein, and T. A. Steitz. 2004. Structure of HIV-1 reverse transcriptase bound to an inhibitor active against mutant reverse transcriptases resistant to other nonnucleoside inhibitors. Proc. Natl. Acad. Sci. USA **101:** 10548-10553.

Pauwels, R., J. Balzarini, D. Schols, M. Baba, J. Desmyter, I. Rosenberg, A. Holy, and E. De Clercq. 1988. Phosphonomethoxyethyl purine derivatives, a new class of anti-human immunodeficiency virus agents. Antimicrob. Agents Chemother. **32:** 1025-1030.

Sarafianos, S. G., Jr. A. D. Clark, K. Das, S. Tuske, J. J. Birktoft, P. Ilankumaran, A. R. Ramesha, J. M. Sayer, D. M. Jerina, P. L. Boyer, S. H. Hughes, and E. Arnold. 2002. Structures of HIV-1 reverse transcriptase with pre- and post-translocation AZTMP-terminated DNA. EMBO J. **21:** 6614-6624.

Satake, H., S. Takeda, A. Matsumura, M. Sasaki, N. Sugimoto, and Y. Wataya. 1992. Action of 5-trifluoromethyl-2'-deoxyuridine on DNA synthesis. Nucleic Acids Symp. Ser. **27:** 189-190.

Satake, H., S. Takeda, and Y. Wataya. 1991. Inhibition of *in vitro* DNA chain elongation of 5-trifl uoromethyl-2'-deoxyuridine residue in the template. Nucleic Acids Symp. Ser. **25:** 37-38.

Seifer, M., R. K. Hamatake, R. J. Colonno, and D. N. Standring. 1998. In vitro inhibition of hepadnavirus polymerases by the triphosphates of BMS-200475 and lobucavir. Antimicrob. Agents Chemother. **42:** 3200-3208.

Shamoo, Y., and T. A. Steitz. 1999. Building a replisome from interacting pieces: sliding clamp complexed to a peptide from DNA polymerase and a polymerase editing complex. Cell **99:** 155-166.

Shi, R., A. Azzi, C. Gilbert, G. Boivin, and S.-X. Lin. 2006. Three-dimensional modeling of cytomegalovirus DNA polymerase and preliminary analysis of drug resistance. Proteins **64:** 301-307.

Srinivas, R. V., M. Connely, and A. Fridland. 1997. *(S)*-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) inhibits HIV-1 replication in epithelial cells, but not T-lymphocytes. Antiviral Res. **35:** 23-27.

Tchesnokov, E. P., A. Obikhod, R. F. Schinazi, and M. Götte. 2008. Delayed chain termination protects the anti-hepatitis B virus drug entecavir from excision by HIV-1 reverse transcriptase. J. Biol. Chem. **283:** 34218-34228.

Tuske, S., S. G. Sarafianos, A. D. Clark Jr., J. Ding, L. K. Naeger, K. L. White, M.

D. Miller, C. S. Gibbs, P. L. Boyer, P. Clark, G. Wang, B. L. Gaffney, R. A. Jones, D. M. Jerina, S. H. Hughes, and E. Arnold. 2004. Structures of HIV-1 RT-DNA complexes before and after incorporation of the anti-AIDS drug tenofovir. Nat. Struct. Mol. Biol. **11:** 469-474.

Valiaeva, N., K. A. Aldern, J. Trahan, J. R. Beadle, and K. Y. Hostetler. 2008. Synthesis and evaluation of octadecyloxyethyl esters of five 3-hydroxy-2-(phosphonomethoxy)propyl nucleoside phosphonates in HIV-1 infected cells. Antiviral Res. **78:** A49-A50.

Votruba, I., M. Hájek, H. Kaiserová, E. Tloust'ová, M. Krecmerová, and A. Holý. 2008. Biochemical evaluation of a new potential antiviral drug HPMP-5-azacytosine. Antiviral Res. **78:** A66-A67.

Wang, J., A. K. M. A. Sattar, C. C. Wang, J. D. Karam, W. H. Konigsberg, and T. A. Steitz. 1997. Crystal structure of a pol α family replication DNA polymerase from bacteriophage RB69. Cell **89:** 1089-1099.

Xiong, X., J. L. Smith, and M. S. Chen. 1997. Effect of incorporation of cidofovir into DNA by human cytomegalovirus DNA polymerase on DNA elongation. Antimicrob. Agents Chemother. **41:** 594-599.

APPENDIX

PRODUCTION AND CHARACTERIZATION OF ANTI-VACCINIA DNA POLYMERASE ANTIBODIES[1,2]

Wendy C. Magee, Soraya Shahhosseini, Y.-C. James Lin, Mavanur R. Suresh and David H. Evans

A.1 INTRODUCTION

Vaccinia virus is a large DNA virus defined taxonomically as belonging to the poxvirus subfamily *Chordopoxvirinae*. It is one of a number of viruses belonging to the genus *Orthopoxvirus*, which also includes *Variola virus* (the causative agent of smallpox), *Cowpox virus,* and *Ectromelia virus*. There are seven other genera of *Chordopoxvirinae*, including the *Leporipoxviruses* (*Mxyoma* and *Shope fibroma virus)*, the *Yatapoxviruses* (e.g. *Tanapox virus)*, and the *Avipoxviruses* (e.g. *Fowlpox virus*) (Moss, 2001). Although these viruses show considerable differences with respect to host range, genome size, gene complement, and antigenicity, they all share a similar replication cycle and a genome structure consisting of a large duplex DNA bearing hairpin ends (Baroudy *et al.*, 1982). The replication cycle is unusual for a DNA virus, in that it takes place within the cytoplasm of infected cells in special structures called either virosomes or factories (reviewed in Moss, 2001). Probably because of this mode and site of replication, poxviruses encode an unusually large complement of proteins needed to catalyze DNA replication and nucleotide biogenesis. These proteins include a DNA polymerase, processivity factor, DNA ligase, single-strand DNA binding protein, a DNA-independent nucleoside triphosphatase that possesses DNA primase activity, Holliday junction resolvase, and uracil glycosylase plus biosynthetic enzymes like a thymidine

---

[1] A version of this chapter has been accepted for publication in the Journal of Virological Methods.

[2] This study was supported by Canadian Institutes of Health Research (CIHR) and Natural Sciences and Engineering Research Council of Canada (NSERC) grants to Dr. D. H. Evans.

kinase and ribonucleotide reductase (Garcia *et al.*, 2000; reviewed in Beaud, 1995 and Traktman, 1996). Although most of the proteins required for vaccinia virus DNA replication have probably now been identified, the actual mechanism of poxvirus replication remains obscure. A nick-dependent rolling hairpin model was proposed over twenty-five years ago (Moyer and Graves, 1981), but needs to be updated since it was discovered that the vaccinia virus D5 protein, the DNA-independent nucleoside triphosphatase, encodes a DNA primase activity (De Silva *et al.*, 2007). The D5 primase could potentially support bidirectional replication with lagging strand DNA synthesis and without requiring replication initiation from a nick.

The vaccinia virus encoded DNA polymerase plays a central role in catalyzing virus replication. The enzyme belongs to the DNA polymerase B family and possesses both 5′-to-3′ polymerase and 3′-to-5′ proofreading exonuclease activities (Challberg and Englund, 1979; Ito and Braithwaite, 1991). The E9L gene encodes the vaccinia virus enzyme, and E9 has been widely used as a surrogate for the homologous variola virus polymerase in studies concerning the development of new smallpox therapeutics (Andrei *et al.*, 2006; Magee *et al.*, 2008; Magee *et al.*, 2005). E9 also plays a key role in catalyzing virus recombination (Colinas *et al.*, 1990; Willer *et al.*, 1999; Yao and Evans, 2001). E9 comprises the core of a multi-protein replication complex, consisting of the polymerase plus A20 and D4 proteins, and it exhibits markedly different enzymatic properties when complexed in this state (Stanitsa *et al.*, 2006). However, it is still unclear whether these three components suffice to form a vaccinia replication machine, or whether E9+A20+D4 simply comprise the core of an even larger holoenzyme. For example, it has been suggested that A20 also interacts with H5 and H5 with G2 (Black *et al.*, 1998; Ishii and Moss, 2002; McCraith *et al.*, 2000). The recent demonstration of the recruitment by vaccinia DNA ligase of cellular topoisomerase II to sites of viral replication and assembly also raises questions regarding what other host proteins might promote virus replication (Lin *et al.*, 2008).

Antibodies provide a powerful tool for dissecting these kinds of complexes and for mapping the distribution of virus antigens in infected cells, yet only one polyclonal antibody directed against E9 protein has been described to date (McDonald *et al.*, 1992).

In this communication the properties of two new monoclonal antibodies and a complementary new polyclonal antibody, which recognize the amino-terminal exonuclease-encoding domain of vaccinia virus DNA polymerase, are described. These antibodies collectively support Western blotting, immunofluorescence microscopy and immunoprecipitation analyses and should prove a useful tool for further investigating the systems catalyzing vaccinia virus DNA replication.

A.2 MATERIALS AND METHODS

A.2.1 Cells and viruses. SP2/0 myeloma cells and BSC40 African green monkey kidney cells were obtained from the American Type Culture Collection (ATCC). Buffalo green monkey kidney (BGMK) cells were obtained from Diagnostic Hybrids (Maryland, USA) and QM5 quail muscle cells from Dr. M. Barry (University of Alberta). SP2/0 cells were cultured in RPMI-1640 medium containing 5% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma or Invitrogen-GIBCO) and 1% (v/v) penicillin-streptomycin-glutamine. BSC40 cells were cultured in minimum essential medium (MEM) containing 5% FBS, 1% (v/v) non-essential amino acids, 2 mM L-glutamine and 1% (v/v) antibiotic plus antimycotic. BGMK cells were grown under the same conditions except using 10% FBS. QM5 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated FBS, 2 mM L-glutamine and 1% antibiotic/antimycotic. All cells were maintained at 37°C in a 5% CO_2 atmosphere and all media and components were purchased from Invitrogen-GIBCO, unless otherwise noted.

Vaccinia virus (strain Western Reserve), myxoma virus (Lausanne), and Shope fibroma virus (Kasza) were obtained from ATCC. Fowlpox virus was obtained from Dr. É. Nagy (University of Guelph) and ectromelia virus (Moscow) and cowpox virus (Brighton Red) were from Dr. M. Barry. Tanapox virus (Kenya) was provided by Dr. C. Brunetti (Trent University).

A.2.2 Expression and purification of full-length vaccinia DNA polymerase from *Escherichia coli*. A full-length copy of the 3,018 bp vaccinia (strain Western Reserve) E9L gene was resynthesized by Geneart (Toronto), bearing codons optimized for

expression in *E. coli*. This codon-optimized gene was used as a template DNA in PCR reactions designed to amplify E9L for cloning into *E. coli* expression vectors bearing carboxy-terminal His_6 (pETBlue-2; Novagen) or amino-terminal glutathione-S-transferase (GST) (pDEST™15; Invitrogen) fusion sequences.

For cloning into pETBlue-2, the 3,018 bp E9L fragment was amplified using the primers $E9L_{Fwd}$ (5′ GAAGGAGATATACC**ATG**GATGTGCGCTGCATCAAC 3′) and $E9L_{Rev}$ (5′ GTGGTGGTGGTGCTCGAACGCTTCATAGAAGGTCGG 3′); the ATG start codon is shown bolded. These primers also incorporate sequences homologous to the pETBlue-2 cloning vector at their 5′ ends (underlined). After amplification and gel purification, the PCR fragments were recombined into *Nco*I and *Xho*I digested pETBlue-2 using the In-Fusion™ cloning method (Clontech) to yield plasmid pETBlue2-E9L. The DNA was used to transform *E. coli* NovaBlue cells (Novagen), followed by plating onto Luria-Bertani (LB) medium containing 50 µg/ml carbenicillin (Sigma) and 12.5 µg/ml tetracycline (Sigma). Positive clones were confirmed by sequencing using the DYEnamic™ ET Dye Terminator Cycle Sequencing kit (Amersham Biosciences) and the primers listed in Table A.1.

To express the E9 protein from pETBlue2-E9L, *E. coli* pLacI Tuner cells (Novagen) were transformed with the plasmid and cultured in Luria broth containing 50 µg/ml carbenicillin, 34 µg/ml chloramphenicol (ICN Biomedicals, Inc.) and 1% (w/v) glucose. Protein expression was induced by the addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen) for 3 h at 37°C. The cells were then harvested, resuspended in extraction buffer (50 mM sodium phosphate, pH 7.0; 300 mM NaCl) and treated with 0.75 mg of chicken egg white lysozyme (USB Corporation) per ml for 1 h at 4°C. The cell suspension was Dounce homogenized and centrifuged to prepare supernatant and pellet fractions. The supernatant fraction was applied to TALON metal affinity resin (BD Biosciences), pre-equilibrated with extraction buffer. The resin was washed twice with extraction buffer, twice with 25 mM imidazole (Sigma) in extraction buffer and once with 100 mM imidazole in extraction buffer. Aliquots of collected samples were analyzed by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (section A.2.8).

Table A.1 Oligonucleotide sequencing primers

Primer	Sequence (5′ to 3′)	Source
pETBlueUP	TCACGACGTTGTAAAACGAC	Novagen
pETBlueDOWN	GTTAAATTGCTAACGCAGTCA	Novagen
E9L Seq1 WM	TCAATAACGGCTGCTATCAT	Sigma-Genosys
E9L Seq2 WM	CGTAACCAGAGCAGCCATAA	Sigma-Genosys
E9L Seq3 WM	TCTGTGGGAATACTACGGCG	Sigma-Genosys
E9L Seq4 WM	GCGGAACGTGCGCGCTATAA	Sigma-Genosys
E9L Seq5 WM	AATATACCACCATGAAATAC	Sigma-Genosys
E9L Seq6 WM	TTCAAACGCCTGACCAGCGA	Sigma-Genosys
E9L Seq7 WM	ATCAAATTCGCTACGCAGAT	Sigma-Genosys
E9L Seq8 WM	CAAAGCGCAGCATACCATTG	Sigma-Genosys
E9L Seq9 WM	AAGATCAGCACGTTGTTGCT	Sigma-Genosys
E9L Seq10 WM	TTTACCTTTCGCATCGGTGG	Sigma-Genosys
E9L Seq11 WM	CCTGAATTTCCTGTTCGGTC	Sigma-Genosys
E9L Seq12 WM	TGATAGATTTCATCGGTCAC	Sigma-Genosys

Gateway® technology (Invitrogen) was used to clone E9L into pDEST15. The E9L coding sequence was amplified using primers pENTR-E9L$_{Fwd}$ (5′ CACC**ATG**GATGTGCGCTGCATCAAC 3′) and pENTR-E9L$_{Rev}$ (5′ TTACGCTTCATAGAAGGTCGGT 3′); the sequence required for directional TOPO® cloning is underlined and the ATG start codon is again shown in bold. The amplified fragment was cloned into pENTR™/TEV/D-TOPO® (Invitrogen) according to the manufacturer's recommendations, transformed into *E. coli* One Shot® TOP10 cells (Invitrogen), and plated onto LB medium containing 50 μg/ml kanamycin (ICN Biomedicals, Inc.) to yield plasmid pENTR-E9L. The E9L coding sequence was recombined into pDEST15 using LR Clonase™ II Enzyme mix (Invitrogen) according to the manufacturer's recommendations to yield plasmid pDEST-E9L. The recombination reaction was transformed into *E. coli* DH5α followed by selection on LB medium containing 100 μg/ml ampicillin (ICN Biomedicals, Inc.).

To express E9 protein from pDEST-E9L, *E. coli* DHE 142 cells (Zhang and Evans, 1995) were transformed with the plasmid and cultured in Luria broth containing 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol. IPTG was added to a final concentration of 0.5 mM and the culture induced at 37°C for 2 h. The cells were harvested and resuspended in phosphate buffered saline, pH 7.3. The cells were then lysed by incubating with 1 mg/ml chicken egg white lysozyme for 1 h at 4°C, followed by Dounce homogenization. Triton X-100 (ICN Biomedicals, Inc.) was added to a final concentration of 1% (v/v), the lysates were incubated at 4°C for 1 h, and then centrifuged to prepare supernatant and pellet samples.

A.2.3 Expression and purification of an amino-terminal fragment of vaccinia DNA polymerase from *E. coli.* The PCR was used to amplify a 1,351 bp fragment encoding the first 450 amino acids of the E9 protein using two primers [E9L$_{Fwd}$ (5′ GAAGGAGAT ATACC**ATG**GATGTGCGCTGCATCAAC 3′) and E9L$_{N450Rev}$ (5′ GTGGTGGTG GTGGTGCTCGAT*GTT*CAGGTTATAATC 3′); the ATG start codon is indicated in bold, the anti-codon 450 is italicized, and sequences homologous to the pETBlue-2 cloning vector are underlined] and the codon-optimized E9L gene as a template. After

amplification, this fragment was recombined into pETBlue-2 and plated as described above (Section A.2.2) to yield plasmid pETBlue2-E9L$_{1\text{-}N450}$. Positive clones were confirmed by sequencing also as described above (Section A.2.2).

Expression of the E9$_{1\text{-}N450}$ protein was performed by transforming *E. coli* pLacI Tuner cells with plasmid pETBlue2-E9L$_{1\text{-}N450}$ and culturing in Luria broth containing 50 μg/ml carbenicillin, 34 μg/ml chloramphenicol and 1% (w/v) glucose. IPTG was added to a final concentration of 0.5 mM and the cells incubated with shaking at 37°C for 3 h. The protein formed inclusion bodies, which were purified using two different methods. In the first method, crude inclusion bodies were isolated using a BugBuster kit (Novagen) as per the manufacturer's recommendations, resuspended in phosphate buffered saline, pH 7.4 (PBS) and the E9$_{1\text{-}N450}$ concentration estimated by comparison with a protein of known concentration on an SDS-PAGE gel. In the second method, the inclusion bodies were isolated and solubilized using an iFold^{TM} kit (Novagen) again according to the manufacturer's recommendations. The buffer was exchanged with binding buffer (20 mM sodium phosphate; 0.5 M sodium chloride; 20 mM imidazole, pH 7.4; 0.06% (v/v) sarkosyl) using a HiPrep 26/10 column (GE Healthcare), and the tagged protein then applied to a HisTrap column (GE Healthcare) also equilibrated in binding buffer. The protein was eluted using binding buffer containing 500 mM imidazole, dialyzed against PBS containing 10 mM β-mercaptoethanol, and the protein content determined by using a Bradford assay (Bio-Rad). The purity of these proteins is shown in Figure A.1.

A.2.4 Expression and purification of vaccinia DNA polymerase from vaccinia virus-infected cells. Full-length recombinant vaccinia DNA polymerase was purified from vaccinia virus infected cells as described previously (McDonald and Traktman, 1994). The concentration of the purified protein was determined using a Bradford assay (Bio-Rad). The purity of this protein is shown in Figure A.1.

A.2.5 Production of mouse hybridoma cell lines. Five 6-8 week old BALB/c mice were immunized intraperitoneally. Two different forms of the E9 protein were used as antigens. The first was the E9$_{1\text{-}N450}$ crude inclusion body preparation and the second was

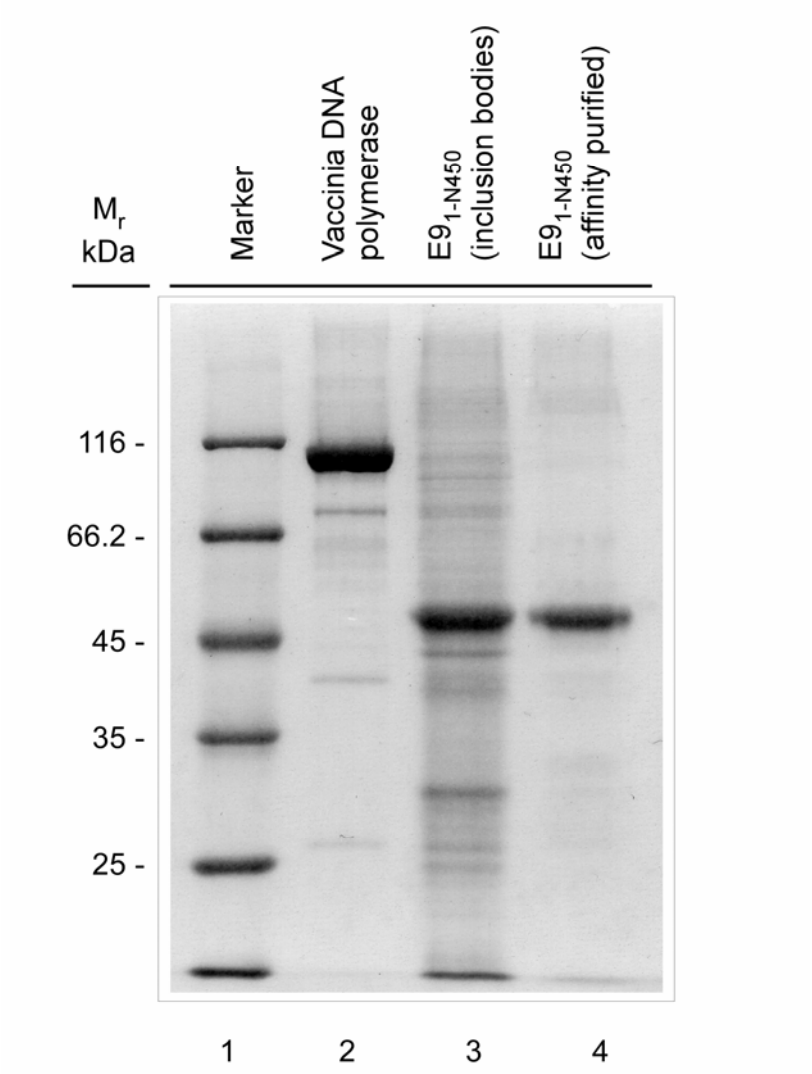

Figure A.1 Vaccinia virus DNA polymerase antigens. Samples of full-length vaccinia DNA polymerase purified from virus-infected cells, $E9_{1\text{-}N450}$ inclusion bodies and affinity-purified $E9_{1\text{-}N450}$ (~1 μg each) were resolved on a 10% SDS-PAGE gel and stained with GelCode Blue (Pierce).

the full-length protein purified from vaccinia virus-infected cells. The first immunization took place on day 0 and consisted of 50 µg/mouse of $E9_{1\text{-}N450}$ antigen in complete Freund's adjuvant (CFA; Sigma). This was followed by 25 µg/mouse of $E9_{1\text{-}N450}$ in incomplete Freund's adjuvant (IFA; Sigma) on day 14, 50 µg/mouse of $E9_{1\text{-}N450}$ in PBS on day 28, and then 10 µg/mouse of full-length polymerase in PBS on days 37 and 53. The mouse serum antibody titres were assessed by indirect ELISA (Section A.2.7) using both protein antigens. The mouse with the highest antibody titres was euthanized and its spleen removed on day 56. The splenocytes were fused to SP2/0 cells and the resulting hybridomas cultured as described (Köhler and Milstein, 1975, Shahhosseini *et al.*, 2007). Vaccinia DNA polymerase was used as the antigen in an indirect ELISA (Section A.2.7) and positive cell lines recloned by limiting dilution. Large-scale (~300 ml) cultures of these monoclonal cell lines were grown to high cell density, allowed to die off, and the supernatant recovered. The isotype of each antibody was determined using the collected supernatants and an IsoStrip kit (Roche). Where described, antibodies were affinity purified using a HiTrap Protein G HP column (GE Healthcare), as per the manufacturer's guidelines, dialyzed against 50% glycerol in PBS and stored at -20°C.

A.2.6 Production of a rabbit polyclonal antibody. Affinity purified $E9_{1\text{-}N450}$ was used to immunize four rabbits through a commercial venue (ProSci Inc). Each rabbit was first immunized with 200 µg of antigen in CFA and then boosted twice with 100 µg of antigen in IFA at two-week intervals. Western blot analysis (Section A.2.8) was used to identify the sera with highest titre and specificity.

A.2.7 Indirect ELISA. Indirect ELISAs were performed in 96-well flat bottom plates [MaxiSorp and C8 Starwell Maxi (Nunc)]. The plates were coated overnight at 4°C with 3 µg/well of antigen in 100 µl of PBS, washed 3 times with PBS, and blocked with 2% (w/v) bovine serum albumin (BSA) in PBS. Test samples were added (100 µl/well) and incubated for 2 h at 20°C or overnight at 4°C. The wells were washed three times with PBS, incubated for 1 h at 20°C with 100 µl/well of 1:10,000 diluted goat anti-mouse IgG conjugated to horseradish peroxidise (Sigma) in 2% BSA, washed 3 times with PBS, and

incubated with 100 µl/well of peroxidase substrate [tetramethylbenzidine (TMB); KPL] for 10 minutes at 20°C. The reaction was stopped by adding 100 µl/well of TMB stop solution (KPL), incubated 5 min at 20°C, and the absorbance determined at 450 nm using a FLUOstar OPTIMA plate reader (BMG LABTECH) using PBS or RPMI 1640 media as a blank. Hybridoma culture supernatants yielding absorbance values greater than 0.5 were determined to be positive.

A.2.8 Western blotting. Proteins were size fractionated using 10% SDS-PAGE gels and transferred to membranes by electrophoresis at 25V overnight or at 65V for 2 to 2.5 h. Two types of detection methods were used, chemiluminescent and infrared. For chemiluminescent detection, proteins were transferred to Immobilon-P membranes (Millipore) and the membranes treated with 1% (w/v) Membrane Blocking Agent (Amersham Biosciences) in PBS-T [0.1% Tween 20 (ICN Biomedicals, Inc.) in PBS]. The membranes were washed and incubated with antibody for 1 h at 20°C [1:1,000 diluted anti-His_6 monoclonal antibody (Roche) or 1:100 diluted anti-GST monoclonal antibody (Shahhosseini *et al.*, 2006)], washed, and incubated with a 1:50 000 diluted goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Bio-Rad). After washing, the membrane was incubated with Super Signal West Pico Chemiluminescent substrate (Pierce) for 5 min followed by exposure to BioMax XAR film (Kodak) and developing using an x-ray film developer (Kodak).

For infrared detection, proteins were transferred to NitroBind (GE Water & Process Technologies) or Immobilon-FL (Millipore) membranes. Where indicated, the membranes were stained with copper phthalocyanine 3,4′,4″,4‴-tetrasulfonic acid tetrasodium salt (CPTS; Sigma) to detect proteins (Bickar and Reid, 1992). The membranes were treated using 5% (w/v) skim milk in PBS-T or Odyssey blocking buffer (Li-Cor), incubated with antibody for 2 h at 20°C [undiluted culture supernatant, a 1:10,000 diluted monoclonal antibody recognizing vaccinia I3 protein (Lin *et al.*, 2008), or 1:2,000 diluted rabbit polyclonal anti-vaccinia DNA polymerase serum], washed, and incubated for 1 h at 20°C with the appropriate IRDye-labeled secondary antibody (Li-Cor) that had been diluted 1:20,000 in PBS-T containing 0.01% (w/v) SDS. The blots

were washed twice with PBS-T and twice with PBS and then visualized using an Odyssey imaging system (Li-Cor).

A.2.9 Immunofluorescence microscopy. Cells were seeded on flamed coverslips in 24-well plates and infected with virus at a multiplicity of infection (moi) of ~5 plaque-forming units (pfu) per cell. The cells were incubated at 37˚C [for 4 h (vaccinia), 6 h (cowpox, ectromelia, myxoma, Shope fibroma, and fowlpox viruses), or 10 h (tanapox virus)], fixed with 4% (w/v) paraformaldehyde (Sigma) in PBS, and then permeabilized and blocked with 0.1% (v/v) Tween 20 and 3% (w/v) BSA in PBS. The cells were incubated at 20°C with undiluted hybridoma culture supernatant for 4 h, with 1:2,000 diluted purified 1F5 or 3C11 monoclonal antibodies for 2 h, or with 1:1,000 diluted rabbit polyclonal antibody for 2 h. The cells were washed with PBS-T, and then incubated with 1:2,000 diluted Alexa Fluor® 488 goat anti-mouse (or anti-rabbit) IgG (Molecular Probes), followed by staining for DNA with 20 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). The coverslips were washed, mounted in Mowiol media (Harlow and Lane, 1999), and imaged with a Zeiss Axioskop2 fluorescence microscope using 40 msec (DAPI) and 600 msec (Alexa Fluor® 488) exposure times.

A.2.10 Immunoprecipitation analysis. BSC40 cells were infected (or mock infected) with vaccinia virus at a moi of 10 pfu/cell in 150 mm dishes. After 4 h, the cells were washed and scraped into 5 ml/dish of cold PBS. The cells were centrifuged at 800 x *g* for 5 minutes at 4°C and resuspended in 1 ml ice cold lysis buffer [125 mM sodium chloride; 50 mM Tris, pH 7.5; 5 mM EDTA; 0.2% (v/v) Tween 20] containing protease inhibitors (Roche). The lysate was held on ice for 20 min, centrifuged at 20,000 x *g* for 20 min at 4°C and 0.8 ml of supernatant transferred to a fresh tube. The purified 1F5 or 3C11 monoclonal antibodies (5 µl), or polyclonal rabbit antiserum (10 µl), were added to each lysate and gently mixed overnight at 4°C. The immune complexes were retrieved by adding Protein G (monoclonal) or Protein A (polyclonal) Sepharose 4 Fast Flow beads (GE Heathcare) and mixed for 2 h at 4˚C. The beads were washed 5 times with cell lysis buffer and resuspended in SDS-PAGE loading buffer. The samples were boiled for 5

min, centrifuged at 18,000 x *g* for 10 seconds, and size fractionated using 10% SDS-PAGE gels. The gels were stained using a Silver Stain Plus kit (Bio-Rad) or used in Western blots (Section A.2.8).

A.3 RESULTS

A.3.1 Production of mouse monoclonal antibodies. Five BALB/c mice were immunized with vaccinia polymerase as a first step in monoclonal antibody production. This enzyme is normally purified using a vaccinia-based expression system (McDonald and Traktman, 1994), but the method cannot economically generate the large amounts of antigen needed for immunization. As a result, the protein was expressed in *E. coli*. All attempts to express the full-length codon-optimized protein bearing either His_6 or glutathione-S-transferase tags were unsuccessful because the protein was either not expressed or was degraded, respectively, as judged by Western blot analysis (Figure A.2). A faint band of a size between 110 and 130 kDa, consistent with the expected size of the His_6-tagged E9 protein (~117 kDa), can be observed in the total cell lysate lane of the anti-His_6 Western blot (Figure A.2, left panel). However, attempts to purify this protein by affinity chromatography (Figure A.2, left panel) as well as subsequent mass spectroscopy analysis did not indicate the presence of soluble His_6-tagged E9 protein in these induced cultures (significant hits were to bacterial 2-oxoglutarate dehydrogenase E1 components). Therefore, a portion of the protein encoding the first 450 amino acids ($E9_{1-N450}$) (Figure A.1) and spanning the predicted proofreading exonuclease domain (Blanco *et al.*, 1992) was expressed. This His_6-tagged form of the protein expressed very well, although it was insoluble and formed inclusion bodies. The inclusion bodies were used to immunize mice three times, followed by two injections using full-length vaccinia DNA polymerase purified from virus-infected cells. This approach reduced the amount of polymerase needed for the immunizations, but was expected to favor selection for antibodies recognizing native epitopes. Indeed, higher serum antibody titers were detected in ELISAs using $E9_{1-N450}$ *versus* vaccinia DNA polymerase antigens, after the first three immunizations, whereas the antibody titers favored vaccinia DNA polymerase over the $E9_{1-N450}$ antigen after the last two booster injections (Figure A.3). Splenocytes

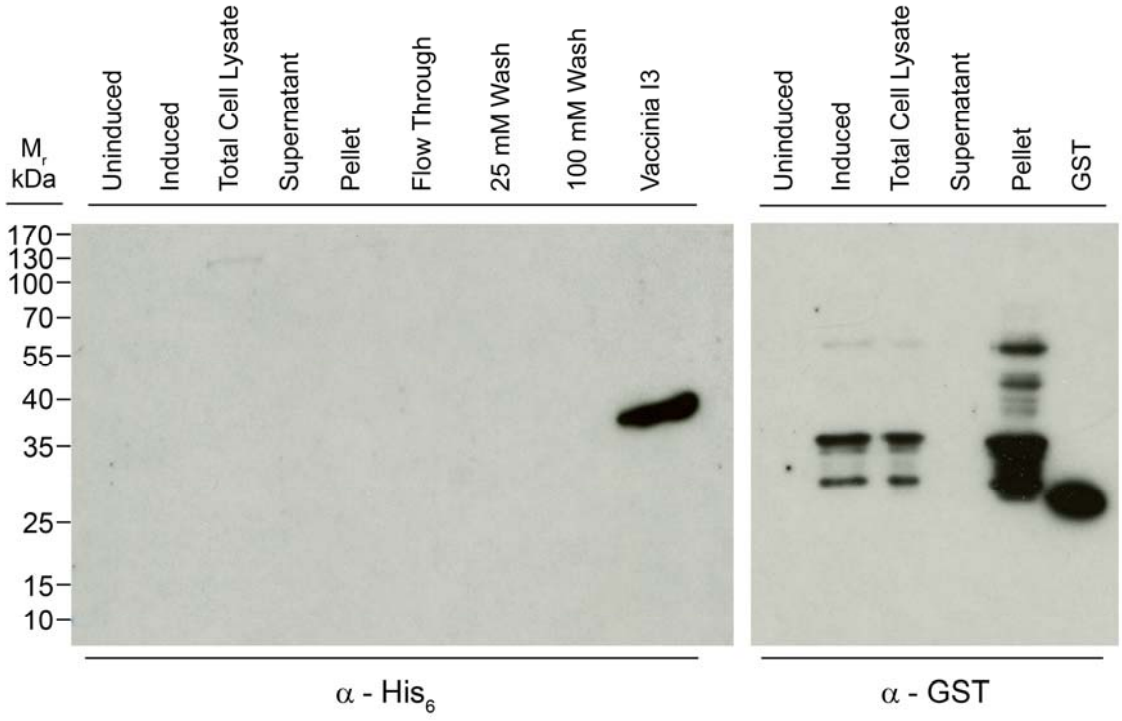

Figure A.2 Western blot analysis of vaccinia DNA polymerase (E9) expressed in *E. coli*. **Left panel.** Protein samples taken during the process of purification of a culture induced to express His_6-tagged E9 were fractionated on a 10% SDS-PAGE gel and transferred to an Immobilon-P membrane. The membrane was used for Western blot analysis using an anti-His_6 monoclonal antibody. The ~35 kDa His_6-tagged vaccinia I3 single-stranded DNA binding protein was run as a positive control. **Right panel.** Protein samples taken from a culture induced to express GST-tagged E9 were fractionated and transferred to a membrane as in the left panel. This membrane was used for a Western blot using an anti-GST monoclonal antibody. The 27.7 kDa GST protein was run as a positive control.

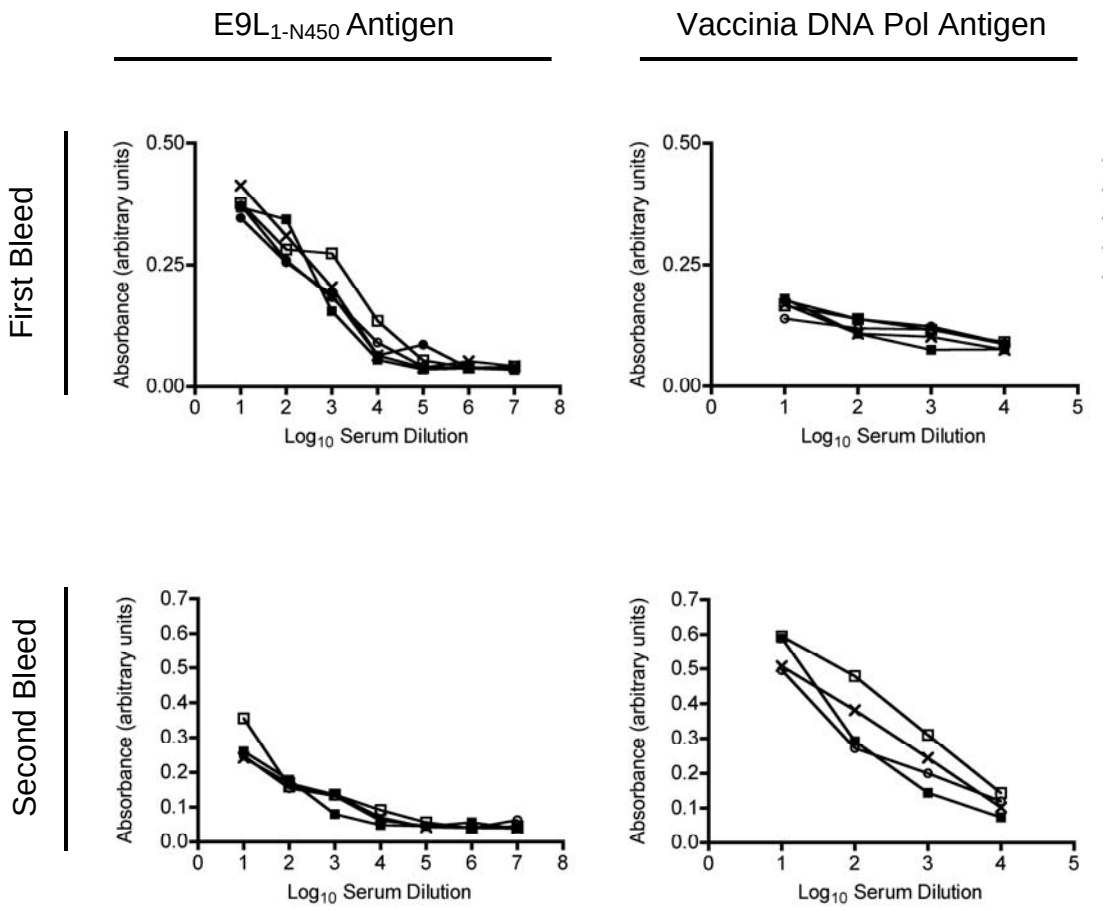

Figure A.3 Serum antibody titers after immunization of mice with three injections of purified $E9_{1\text{-}N450}$ inclusion bodies (first bleed) and two booster injections of purified vaccinia DNA polymerase (second bleed). Blood samples were collected from the tail vein of each mouse and the serum separated from each sample. Ten-fold serial dilutions of each serum sample in PBS were added to flat-well microtiter plates coated with either $E9_{1\text{-}N450}$ inclusion bodies (left) or vaccinia DNA polymerase (right) and blocked with BSA. Bound antibody was detected using goat anti-mouse IgG (Fc-specific) horseradish peroxidase conjugated secondary antibody and TMB Microwell Peroxidase Substrate System. The absorbance of each sample was read using a plate reader. Five mice were initially immunized [#1 (-■-), #2 (-□-), #3 (-●-), #4 (-○-), #5 (-x-)]; however, one (#3) became ill and was euthanized prior to receiving the last booster.

from the mouse with the highest serum antibody titers (#5) against vaccinia DNA polymerase were fused with SP2/0 myeloma cells to form hybridomas. The culture supernatants were screened using an indirect ELISA and positive hybridoma cells cloned by limiting dilution. Five stable lines were obtained: 1F5, 1G5, 3C11, 3E9 and 4E5 and isotyped using a commercial kit (Table A.2). Two of these hybridomas, 1F5 and 3C11, were subsequently purified from the culture supernatants using Protein G affinity chromatography.

A.3.2 Western blotting applications. The monoclonal antibodies were first tested to see if they would be suitable for Western blotting applications. Western blots were performed using infected cell lysates as well as the purified vaccinia DNA polymerase as antigens. As a positive control an antibody recognizing vaccinia I3 single-strand DNA binding protein was used, and mock-infected cell lysates were used as negative controls. Although the anti-I3 monoclonal antibody readily detected the ~35 kDa I3 protein band in infected cell lysates, none of the five new antibodies could detect the ~116 kDa DNA polymerase band (Figure A.4). These results show that these reagents are not suitable for Western blot analysis.

Because of this difficulty, a rabbit polyclonal anti-vaccinia DNA polymerase antibody was also prepared. An affinity-purified preparation of His_6-tagged $E9_{1\text{-}N450}$ was used as an antigen for this process, rather than the crude inclusion bodies, to favor production of a more specific antibody. Two of the four rabbits yielded sera recognizing both the $E9_{1\text{-}N450}$ immunogen and purified vaccinia DNA polymerase, and one was boosted with more $E9_{1\text{-}N450}$ due to the low non-specific background in Western blots. This preparation of polyclonal antibody worked very well in blots, readily detecting <20 ng of full-length purified vaccinia DNA polymerase (Figure A.5). Little or no cross-reacting proteins were detected in mock-infected cells. Using a dilution of 1:2,000 serum, the antibody exhibited sufficient sensitivity to detect native levels of polymerase present in 40 μg of infected cell extract.

Table A.2 Isotypes of monoclonal antibodies against vaccinia DNA polymerase

Antibody	Isotype
1F5	IgG_1, kappa light chain
1G5	IgG_1, lambda light chain
3C11	IgG_{2b}, kappa light chain
3E9	IgG_1, kappa light chain
4E5	IgG_1, kappa light chain

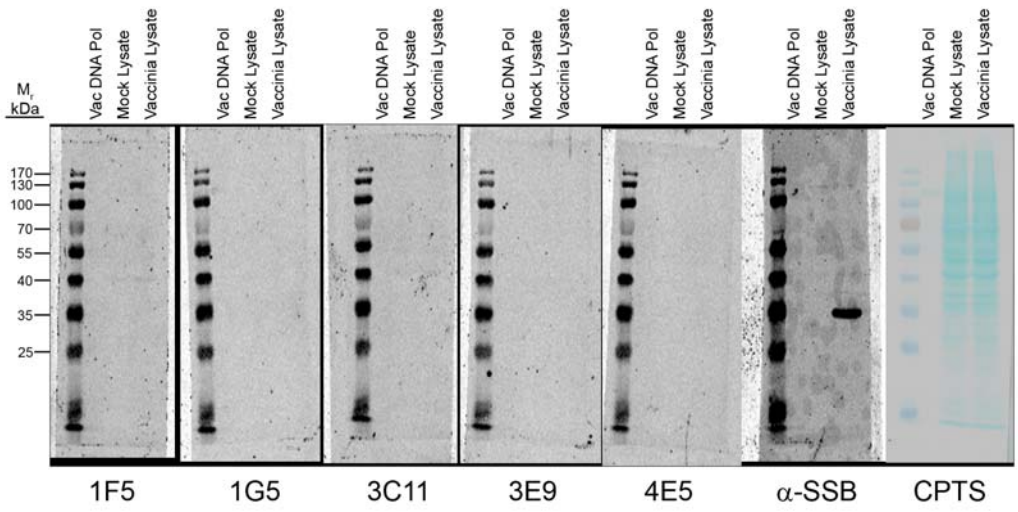

Figure A.4 Immunoblots using the five monoclonal antibodies. Purified vaccinia DNA polymerase and lysates prepared from BSC40 cells mock infected or infected with vaccinia WR at an multiplicity of infection (moi) of 10 pfu per cell for 4 hours were run on 10% SDS-PAGE gels and the proteins transferred to nitrocellulose. Culture supernatants from the five monoclonal hybridomas were used as the primary antibody, and a monoclonal antibody recognizing the vaccinia single-strand DNA binding protein (α-SSB) was used as a positive control. Bound primary antibodies were detected using goat anti-mouse IRDye® 800CW secondary antibodies (LI-COR). Membranes were then scanned using an Odyssey® infrared imaging system. A portion of the nitrocellulose membrane stained with the protein stain copper phthalocyanine 3,4′,4″,4‴-tetrasulfonic acid tetrasodium salt (CPTS) is shown on the right.

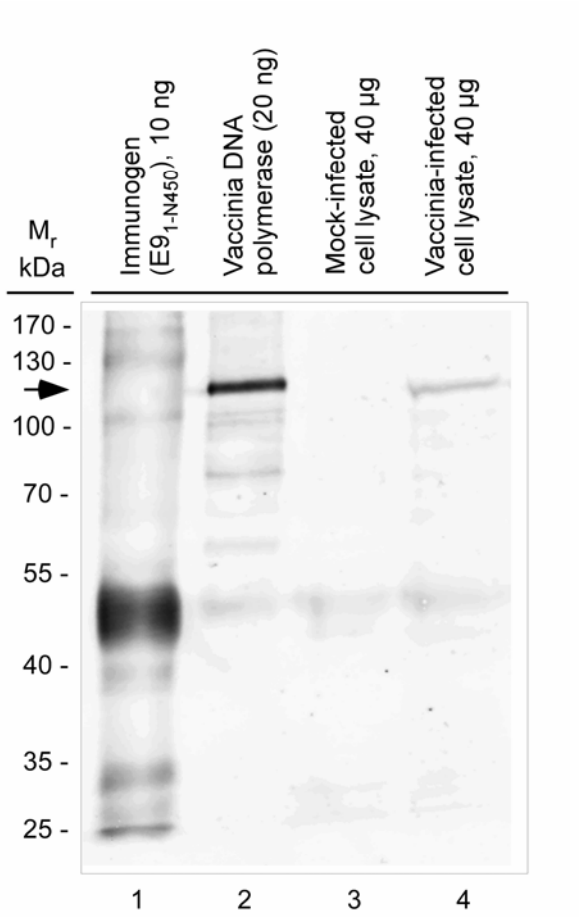

Figure A.5 Western blot detection of vaccinia virus DNA polymerase. SDS-PAGE was used to fractionate the indicated samples and then, after transfer to a PVDF membrane, the proteins were Western blotted using a rabbit polyclonal antibody raised against an affinity-purified $E9_{1-450}$ immunogen (lane 1). The blot was hybridized to a goat anti-rabbit IRDye® 800CW secondary antibody and then scanned using an Odyssey® infrared imaging system. Full-length vaccinia DNA polymerase (116 kDa) is indicated with an arrow.

A.3.3 Characterization of antibodies by immunofluorescence microscopy. These antibodies were also tested to determine whether they would be useful in indirect immunofluorescence applications. Cells were seeded on coverslips and infected (or mock infected) with vaccinia virus. The cells were fixed and stained 4 h post-infection with culture supernatants from each of the five monoclonal hybridomas, or with the polyclonal antibody. The cells were also counterstained with DAPI to visualize nuclei and viral factories. The results are shown in Figure A.6. The 1F5, 3C11 and 3E9 monoclonal antibodies worked well in this application as evidenced by specific staining of the virus factories. Interestingly the 3C11 antibody also exhibited some additional diffuse cytoplasmic staining seen only in infected cells. The 1G5 antibody also stained the virus factories; however, this staining was faint compared to that seen using the 1F5, 3C11 and 3E9 antibodies. The 4E5 antibody did not stain the virus factories. This antibody instead stained the cytoplasm surrounding these viral structures. No immunofluorescence was detected using any of the monoclonal antibodies in mock-infected cells, nor was any immunofluorescence detected in the media controls. The polyclonal antibody exhibited only a very weak and non-specific staining pattern with the fluorescence dispersed throughout all cells in both mock- and vaccinia-infected specimens. Based on these results, the 1F5 and 3C11 monoclonal antibodies were chosen for further analysis and purified.

The specificity of the 1F5 and 3C11 antibodies were also tested using a selection of additional chordopoxviruses. For these experiments, BSC40 cells were infected with vaccinia and tanapox viruses; BGMK cells were infected with cowpox, ectromelia, myxoma, or Shope fibroma viruses; and QM5 cells were infected with fowlpox virus. For vaccinia infections, the cells were fixed 4 h post-infection and for the cowpox, ectromeila, myxoma and Shope fibroma infections the cells were fixed 6 h post-infection. However, the tanapox-infected cells were fixed 10 h post-infection because this virus exhibits a longer replication cycle (Knight *et al.*, 1989, Mediratta and Essani, 1999). The 1F5 monoclonal antibody appears to be highly specific for vaccinia virus, as it did not stain the factories formed by any other poxvirus (Figure A.7). In contrast, the 3C11 antibody seems to recognize an epitope common to vaccinia, cowpox and ectromelia

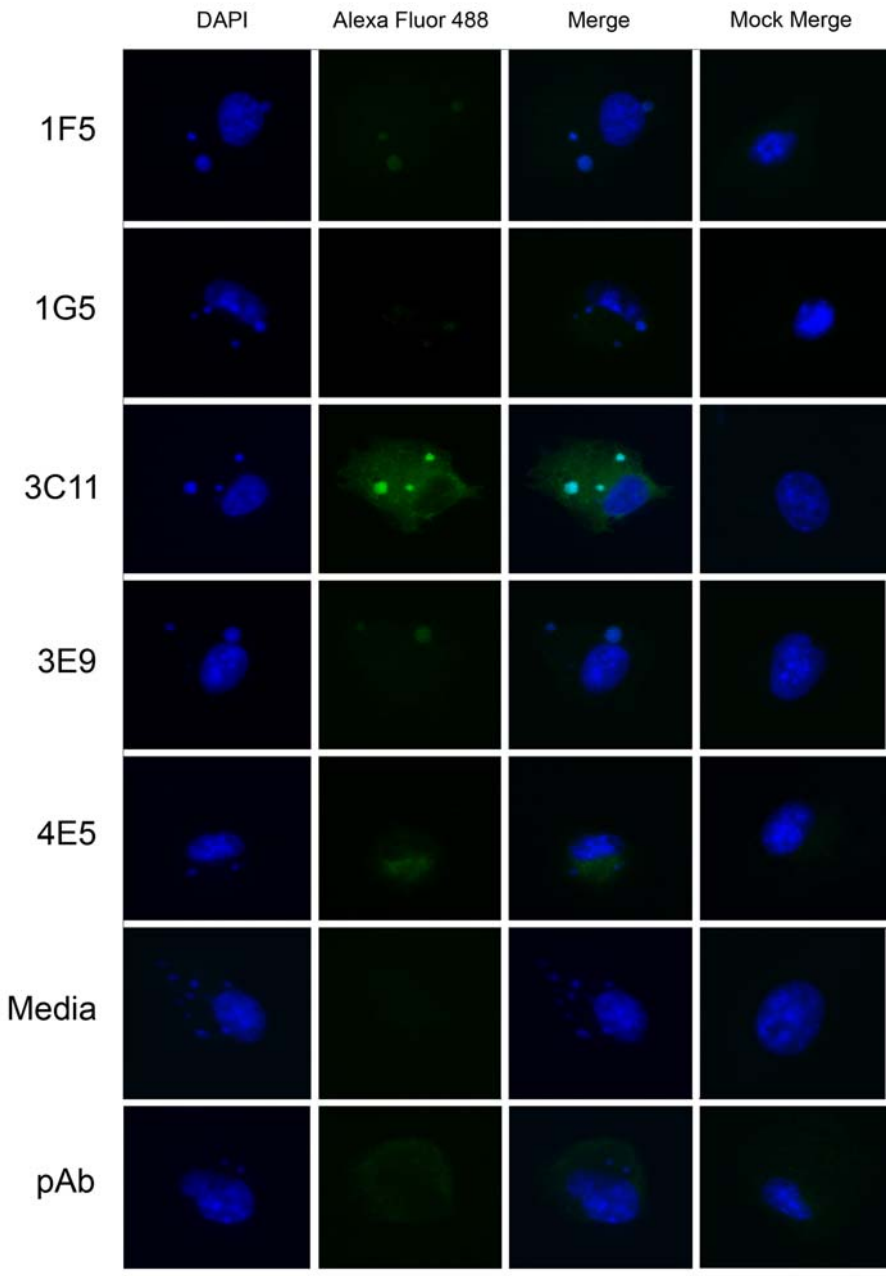

Figure A.6 Immunofluorescence microscopy of vaccinia infected cells. BSC40 cells seeded on coverslips were mock infected or infected with vaccinia WR at an moi of 5 pfu per cell for 4 hours. After fixing, the cells were stained using monoclonal hybridoma culture supernatants, hybridoma culture media or the rabbit anti-vaccinia DNA polymerase polyclonal antibody, followed by staining with appropriate Alexa Fluor488-conjugated secondary antibodies. The cells were counterstained using DAPI to visualize nuclei and viral factories. The samples were imaged using a Zeiss Axioskop 2 microscope and 40 millisecond (DAPI) and 600 millisecond (Alexa Fluor488) exposures.

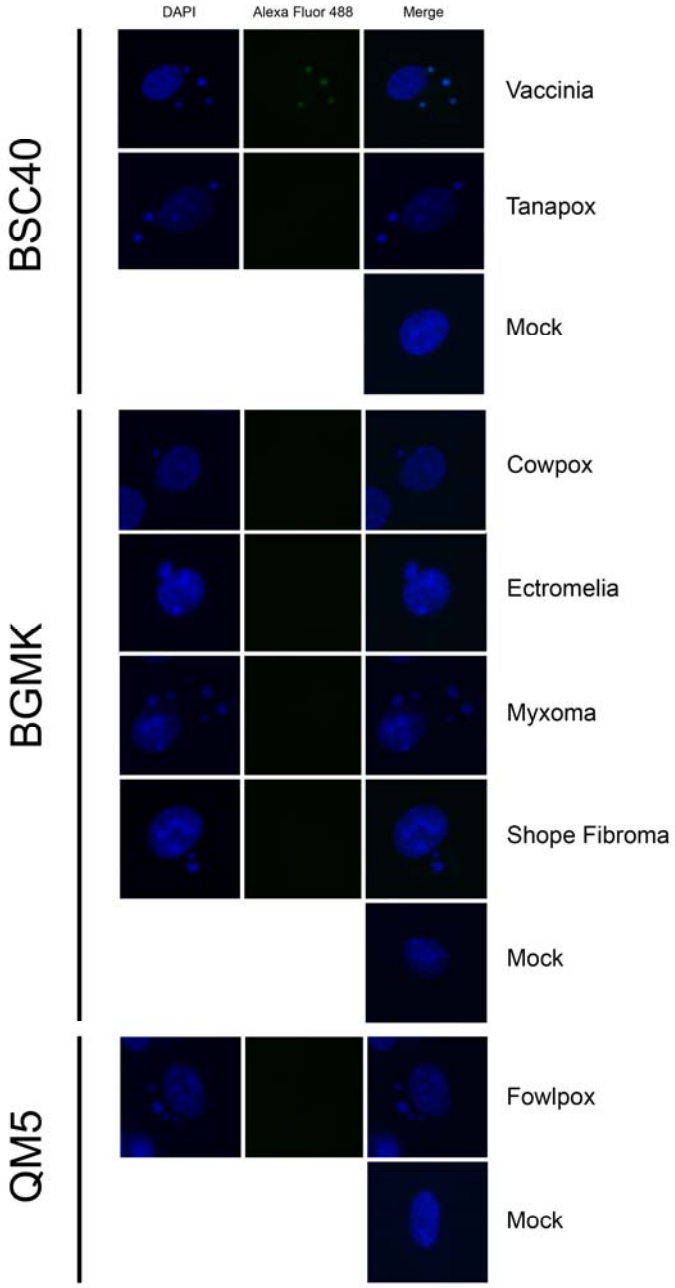

Figure A.7 Immunofluorescence microscopy of poxvirus infected cells using the purified 1F5 monoclonal antibody. Coverslips were prepared bearing the indicated combinations of cell and viruses and then fixed, stained with the purified 1F5 antibody, counterstained with DAPI, and imaged as described in Figure A.6. 1F5 appears to recognize only vaccinia antigens, and not antigens of the other poxviruses. These other poxviruses did establish successful infections, however, as judged by the presence of DAPI-stained virus factories.

viruses (Figure A.8), but not produced in cells infected with myxoma, Shope fibroma, tanapox, or fowlpox viruses (Figure A.8). The pattern was similar to that seen in vaccinia infections with bright staining of factories and more diffuse staining across the cytoplasm.

A.3.4 Characterization of antibodies by immunoprecipitation analysis. Finally, these antibodies were tested to determine whether they can be used to immunoprecipitate vaccinia DNA polymerase from infected cell extracts. BSC40 cells were infected (or mock infected) with vaccinia virus at a multiplicity of infection of 10 pfu/cell for 4 h. The cells were then harvested, lysed, and incubated with purified 1F5 or 3C11 monoclonal antibodies or with the polyclonal antibody. Protein A or G beads were then used to collect the immune complexes and the bound proteins size fractionated using duplicate SDS-PAGE gels. The gels were either silver stained or Western blotted using the polyclonal rabbit antibody. All three antibodies retrieved a ~120 kDa protein, present only in infected cells, and that co-migrated with authentic vaccinia DNA polymerase (116 kDa, Figure A.9A, closed arrow). The Western blot confirmed that this large protein is vaccinia virus DNA polymerase (Figure A.9B, closed arrow). Close inspection of the silver-stained proteins did detect at least one additional protein band that co-immunoprecipitated with E9 when the 3C11 antibody was used; this protein exhibited a mass of ~35 kDa (Figure A.9A, open arrow). This does not match the mass of proteins previously shown to bind E9 [A20 (49 kDa) and D4 (25 kDa)] although these would have been obscured on our gels by the heavy and light chain bands.

A.4 DISCUSSION

Many of the proteins required for poxvirus DNA replication have been identified using a combination of biochemical, genetic, and molecular genetic methods (reviewed in Moss, 2001 and Traktman, 1996). Some of the interactions between these proteins have also been mapped using yeast two-hybrid and co-immunoprecipitation methods (Ishii and Moss, 2002; McCraith *et al.*, 2000; Stanitsa *et al.*, 2006), and other studies have provided insights into how these processes relate to the process of factory formation (Cairns, 1960;

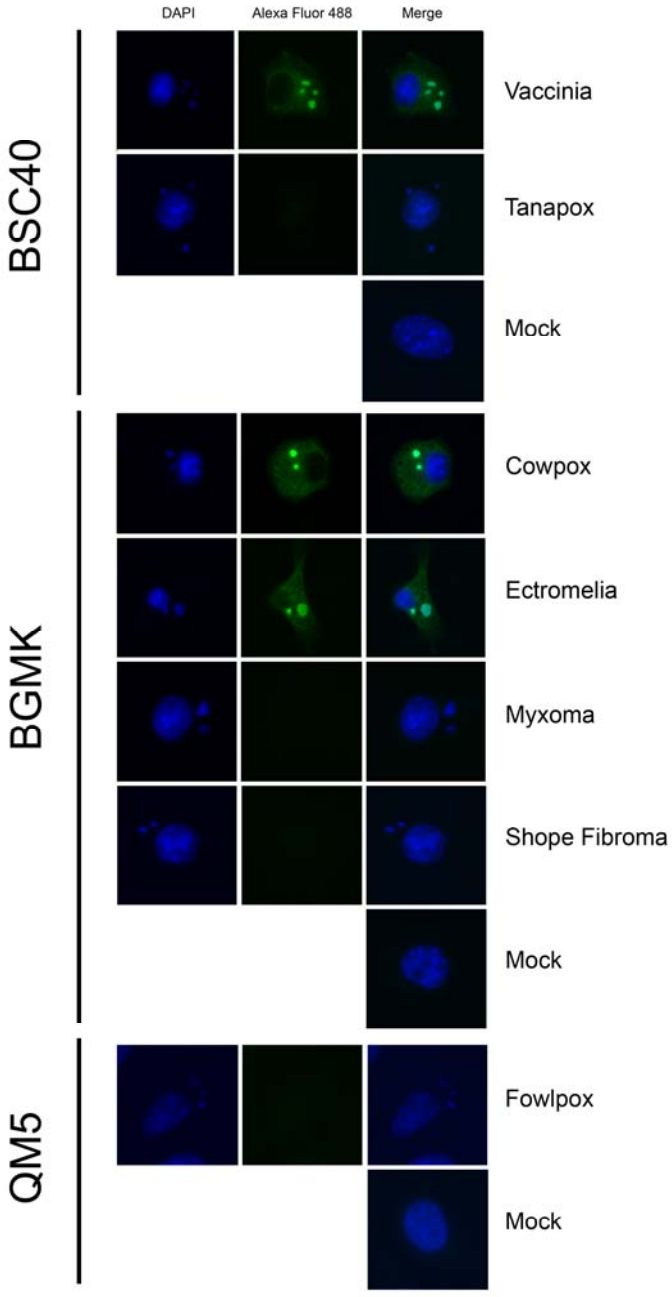

Figure A.8 Immunofluorescence microscopy of poxvirus infected cells using the purified 3C11 monoclonal antibody. Coverslips were prepared bearing the indicated combinations of cell and viruses and then fixed, stained with the purified 3C11 antibody, counterstained with DAPI, and imaged as described in Figure A.6. 3C11 stains vaccinia virus infected cells, and also stains cells infected with the orthopoxviruses ectromelia and cowpox. This antibody does not recognize the antigens of other non-orthopoxviruses.

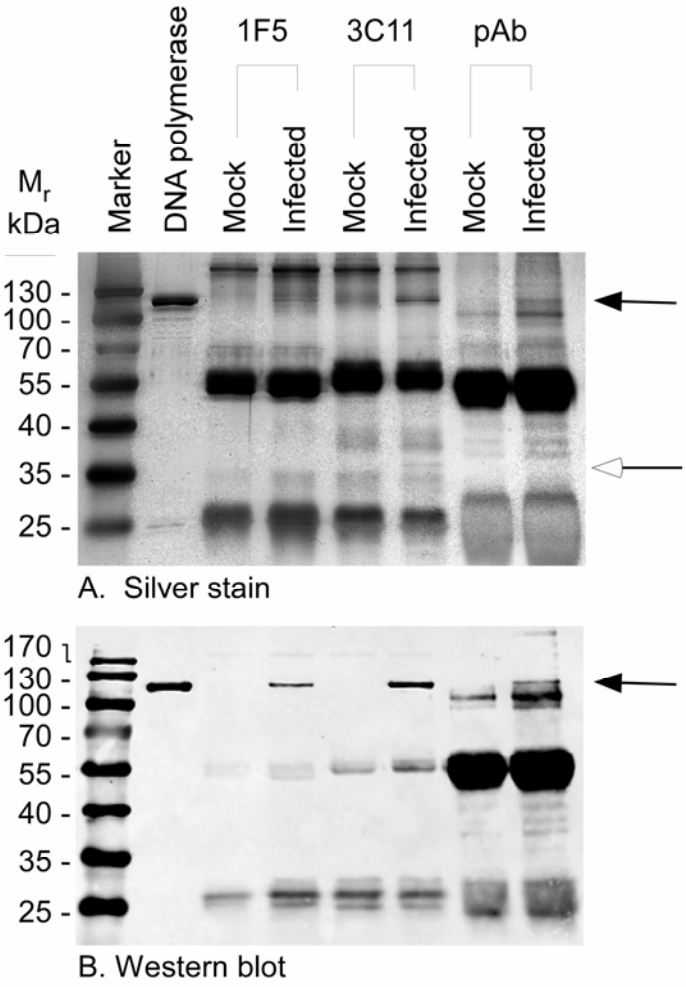

Figure A.9 Immunoprecipitation analysis. BSC-40 cells were infected (or mock infected) with vaccinia virus and then cell-free extracts were prepared 4 h post-infection. These extracts were incubated overnight at 4°C with the indicated antibodies, and the immune complexes collected using protein A and protein G sepharose beads. The beads were recovered, washed, mixed with SDS-PAGE loading buffer, and the proteins separated using 10% SDS-PAGE gels. Purified vaccinia DNA polymerase was also included as a reference standard. **Panel A.** A silver stained gel. The vaccinia virus DNA polymerase and the ~35 kDa band that co-immunoprecipitates with this protein when using the 3C11 antibody are indicated by the closed and open arrows, respectively. **Panel B.** Western blot. The proteins were transferred to nitrocellulose and the DNA polymerase detected using a rabbit polyclonal antibody and goat anti-rabbit IRDye® 680 as primary and secondary antibodies, respectively. The membrane was scanned using an Odyssey® infrared imaging system. The vaccinia virus DNA polymerase is indicated by the arrow.

Domi and Beaud, 2000; Mallardo *et al.*, 2002; Schramm *et al.*, 2006; Tolonen *et al.*, 2001. Nevertheless the mechanism of poxvirus replication is still surprisingly poorly understood. The origin(s) of replication have never been identified and it is not yet clear whether poxvirus replication involves leading and lagging strand DNA synthesis. Further progress requires new tools that can be used to purify poxvirus replication complexes, and to map the arrangement of different proteins with respect to other viral proteins and within infected cells. In this communication the properties of two new monoclonal antibodies and one polyclonal antibody that may help serve this purpose are described.

Vaccinia DNA polymerase has proven very difficult to produce in a recombinant form using heterologous expression systems. Codon-optimization of this gene for expression in *E. coli* was employed; however, expression of full-length vaccinia virus DNA polymerase could not be achieved. As a result, a subdomain of the protein encoding the amino-terminal 3′-to-5′ proofreading exonuclease domain was produced. Although the His_6-tagged $E9_{1\text{-}N450}$ protein expressed well in *E. coli*, it formed inclusion bodies. Nevertheless, these inclusion bodies served as a useful primary antigen in mice and, by using two final boosts with full-length protein, serum antibody titers were generated that better recognized native vaccinia DNA polymerase rather than the $E9_{1\text{-}N450}$ antigen in ELISAs. The fusion and cloning of hybridomas generated five monoclonal cell lines that all produced DNA polymerase specific antibodies as judged by ELISA. All of these hybridoma cell lines were tested for functionality, but only lines 1F5 and 3C11 eventually proved useful.

The monoclonal antibodies were first tested in Western blot applications and unfortunately found wanting. This finding may not be too surprising considering that the immunization schedule was designed to shift the immune response towards one favoring native protein epitopes. Since none of the monoclonal antibodies seemed well suited to this application, rabbits were also immunized with affinity purified $E9_{1\text{-}N450}$ protein. This antibody worked very well in Western blotting applications. Based on these analyses, it is estimated that when harvested 4 h post-infection, vaccinia DNA polymerase comprises ~0.02% of the total cell protein (i.e. 40 µg of infected cell extract contains 5-10 ng of polymerase).

The opposite situation was seen when these antibodies were used for immunofluorescence microscopy. The polyclonal antibody did not show sufficient specificity whereas the 1F5 and 3C11 monoclonal antibodies specifically stained viral factories in vaccinia-infected cells. The two monoclonal antibodies stained cells differently. The 1F5 antibody stained only the factories, whereas the 3C11 antibody produced bright staining in the factories and more diffuse cytoplasmic staining. These differences in staining pattern likely reflect differences in binding affinity. Vaccinia DNA polymerase is expected to concentrate in factories because the virosomes represent sites of viral DNA synthesis. However, smaller amounts of protein would also still likely reside in the cytoplasm, if perhaps only transiently. If the 1F5 antibody has a lower affinity for vaccinia DNA polymerase relative to the 3C11 antibody, it might detect only the more highly concentrated virosomal pool of enzyme. Alternatively, these antibodies may detect different isoforms of polymerase in cytoplasm and factory, forms that perhaps vary depending upon how E9 associates with other macromolecules. Further work (such as surface plasmon resonance analysis) is needed to determine the cause of these different staining patterns.

The specificity of the two monoclonal antibodies was also tested using cells infected with other selected poxviruses. The 3C11 antibody recognized all of the orthopoxviruses in the panel of viruses, as judged by immunofluorescence microscopy, whereas 1F5 seemed to be highly specific for vaccinia virus DNA polymerase. Amongst these different DNA polymerases, the vaccinia enzyme shares 99% and 98% amino acid sequence identity with cowpox and ectromelia virus DNA polymerases, respectively, whereas the other poxvirus proteins tested share less than 70% identity with E9. Ectromelia virus DNA polymerase is one of the more divergent Orthopoxvirus enzymes and the fact it is still recognized by 3C11 suggests that this antibody might be a pan-orthopoxvirus specific reagent. The specificity of the 1F5 antibody towards vaccinia virus E9 is also rather remarkable and could help determine the epitope recognized by this antibody. There are only 11 amino acid substitutions that differentiate the vaccinia virus and cowpox virus DNA polymerases over the 450 amino acid span of the antigen.

Finally, all three antibodies can be used to immunoprecipitate vaccinia DNA polymerase from infected cell extracts. Although an exhaustive analysis of the proteins that can be co-immunoprecipitated with the DNA polymerase has not been performed, at least one additional protein found only in infected cell lysates is observed associating with the polymerase (Figure A.9). It does not match the size of the A20 and D4 proteins that have been shown previously to bind vaccinia DNA polymerase (Ishii and Moss, 2002; McCraith *et al.*, 2000; Stanitsa *et al.*, 2006) and it suggests that additional interactions between various replication proteins remain to be discovered.

In conclusion, two monoclonal antibodies and one polyclonal antibody against vaccinia DNA polymerase have been developed. Although no one antibody is suitable for all applications, collectively this set of reagents can be used for Western blots, immunofluorescence microscopy, and immunoprecipitation analyses. These antibodies will be useful tools for studying the role played by DNA polymerases in poxvirus DNA replication as well as the mechanics of replication.

A.5 AUTHOR CONTRIBUTION TO DATA

Wendy Magee performed the DNA cloning and *E. coli* protein expression experiments, recloned the hybridoma cell lines, performed ELISAs and isoptying, performed the Western blot shown on Figure A.4, and conducted all of the immunofluorescence and immunoprecipitation experiments described in this study. Wendy Magee and Dr. Soraya Shahhosseini performed the hybridoma fusion. Dr. James Lin purified the full-length vaccinia DNA polymerase, performed the Western blot shown in Figure A.5, and assisted with the affinity purification of $E9_{1\text{-}N450}$ and the 1F5 and 3C11 monoclonal antibodies.

A.6 REFERENCES

Andrei, G., D. B. Gammon, P. Fiten, E. De Clercq, G. Opdenakker, R. Snoeck, and D. H. Evans. 2006. Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice. J. Virol. **80:** 9391-9401.

Baroudy, B. M., S. Venkatesan, and B. Moss. 1982. Incompletely base-paired flip-flop

terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. Cell **28:** 315-324.

Beaud, G. 1995. Vaccinia virus DNA replication: a short review. Biochimie **77:** 774-779.

Bickar, D., and P. D. Reid. 1992. A high-affinity protein stain for western blots, tissue prints, and electrophoretic gels. Anal. Biochem. **203:** 109-115.

Black, E. P., N. Moussatche, and R. C. Condit. 1998. Characterization of the interactions among vaccinia virus transcription factors G2R, A18R, and H5R. Virology **245:** 313-322.

Blanco, L., A. Bernad, and M. Salas. 1992. Evidence favouring the hypothesis of a conserved 3'-5' exonuclease active site in DNA-dependent DNA polymerases. Gene **112:** 139-144.

Cairns, J. 1960. The initiation of vaccinia infection. Virology **11:** 603-623.

Challberg, M. D., and P. T. Englund. 1979. Purification and properties of the deoxyribonucleic acid polymerase induced by vaccinia virus. J. Biol. Chem. **254:** 7812-7819.

Colinas, R. J., R. C. Condit, and E. Paoletti. 1990. Extrachromosomal recombination in vaccinia-infected cells requires a functional DNA polymerase participating at a level other than DNA replication. Virus Res. **18:** 49-70.

De Silva, F. S., W. Lewis, P. Berglund, E. V. Koonin, and B. Moss. 2007. Poxvirus DNA primase. Proc. Natl. Acad. Sci. USA **104:** 18724-18729.

Domi, A., and G. Beaud. 2000. The punctate sites of accumulation of vaccinia virus early proteins are precursors of sites of viral DNA synthesis. J. Gen. Virol. **81:** 1231-1235.

Garcia, A. D., L. Aravind, E. V. Koonin, and B. Moss. 2000. Bacterial-type DNA Holliday junction resolvases in eukaryotic viruses. Proc. Natl. Acad. Sci. USA **97:** 8926-8931.

Harlow, E., and D. Lane. 1999. Using antibodies: a laboratory manual, 1st ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Ishii, K., and B. Moss. 2002. Mapping interaction sites of the A20R protein component of the vaccinia virus DNA replication complex. Virology **303:** 232-239.

Ito, J., and D. K. Braithwaite. 1991. Compilation and alignment of DNA polymerase

sequences. Nucleic Acids Res. **19:** 4045-4057.

Knight, J. C., F. J. Novembre, D. R. Brown, C. S. Goldsmith, and J. J. Esposito. 1989. Studies on tanapox virus. Virology **172:** 116-124.

Köhler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature **256:** 495-497.

Lin, Y.-C. J., J. Li, C. R. Irwin, H. Jenkins, L. DeLange, and D. H. Evans. 2008. Vaccinia virus DNA ligase recruits cellular topoisomerase II to sites of viral replication and assembly. J. Virol. **82:** 5922-5932.

Magee, W. C., K. A. Aldern, K. Y. Hostetler, and D. H. Evans. 2008. Cidofovir and *(S)*-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine are highly effective inhibitors of vaccinia virus DNA polymerase when incorporated into the template strand. Antimicrob. Agents Chemother. **52:** 586-597.

Magee, W. C., K. Y. Hostetler, and D. H. Evans. 2005. Mechanism of inhibition of vaccinia virus DNA polymerase by cidofovir diphosphate. Antimicrob. Agents Chemother. **49:** 3153-3162.

Mallardo, M., E. Leithe, S. Schleich, N. Roos, L. Doglio, and J. Krijnse Locker. 2002. Relationship between vaccinia virus intracellular cores, early mRNAs, and DNA replication sites. J. Virol. **76:** 5167-5183.

McCraith, S., T. Holtzman, B. Moss, and S. Fields. 2000. Genome-wide analysis of vaccinia virus protein-protein interactions. Proc. Natl. Acad. Sci. USA **97:** 4879-4884.

McDonald, W. F., V. Crozel-Goudot, and P. Traktman. 1992. Transient expression of the vaccinia virus DNA polymerase is an intrinsic feature of the early phase of infection and is unlinked to DNA replication and late gene expression. J. Virol. **66:** 534-547.

McDonald, W. F., and P. Traktman. 1994. Overexpression and purification of the vaccinia virus DNA polymerase. Protein Expr. Purif. **5:** 409-421.

Mediratta, S., and K. Essani. 1999. The replication cycle of tanapox virus in owl monkey kidney cells. Can. J. Microbiol. **45:** 92-96.

Moss, B. 2001. *Poxviridae:* The viruses and their replication, p. 1249-1283. *In* D. M. Knipe,P. M. Howley (eds.), Fundamental Virology. Lippincott Williams & Wilkins, Philadelphia.

Moyer, R. W., and R. L. Graves. 1981. The mechanism of cytoplasmic orthopoxvirus DNA replication. Cell **27:** 391-401.

Schramm, B., C. A. M. de Haan, J. Young, L. Doglio, S. Schleich, C. Reese, A. V. Popov, W. Steffen, T. Schroer, and J. Krijnse Locker. 2006. Vaccinia -virus-induced cellular contractility facilitates the subcellular localization of the viral replication sites. Traffic **7:** 1352-1367.

Shahhosseini, S., D. Das, X. Qiu, H. Feldmann, S. M. Jones, and M. R. Suresh. 2007. Production and characterization of monoclonal antibodies against different epitopes of Ebola virus antigens. J. Virol. Methods **143:** 29-37.

Shahhosseini, S., S. Guttikonda, P. Bhatnagar, and M. R. Suresh. 2006. Production and characterization of monoclonal antibodies against shope fibroma virus superoxide dismutase and glutathione-s-transferase. J. Pharm. Pharmaceut. Sci. **9:** 165-168.

Stanitsa, E. S., L. Arps, and P. Traktman. 2006. Vaccinia virus uracil DNA glycosylase interacts with the A20 protein to form a heterodimeric processivity factor for the viral DNA polymerase. J. Biol. Chem. **281:** 3439-3451.

Tolonen, N., L. Doglio, S. Schleich, and J. Krijnse Locker. 2001. Vaccinia virus DNA replication occurs in endoplasmic reticulum-enclosed cytoplasmic mini-nuclei. Molecular Biology of the Cell **12:** 2031-2046.

Traktman, P. 1996. Poxvirus DNA replication, p. 775-798. *In* D. L. DePamphilis (ed.), DNA Replication in Eukaryotic Cells. Cold Spring Harbor Laboratory Press, Plainview, NY.

Willer, D. O., M. J. Mann, W. Zhang, and D. H. Evans. 1999. Vaccinia virus DNA polymerase promotes DNA pairing and strand-transfer reactions. Virology **257:** 511-523.

Yao, X.-D., and D. H. Evans. 2001. Effects of DNA structure and homology length on vaccinia virus recombination. J. Virol. **75:** 6923-6932.

Zhang, W., and D. H. Evans. 1995. DNA strand transfer catalyzed by the 5'-3' exonuclease domian of *Escherichia coli* DNA polymerase I. Nucleic Acids Res. **23:** 4620-4627.