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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF BUTANOATE, RETINOATE AND *BIS*(2,2,2-TRICHLOROETHYL)PHOSPHATE DERIVATIVES OF 5-FLUORO-2'-DEOXYURIDINE AND 2',5-DIFLUORO-2'-DEOXYURIDINE

by

Zuping Xia

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

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta

Spring 1998

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

Design, synthesis and biological evaluation of butanoate, retinoate and bis(2,2,2-trichloroethyl)phosphate derivatives of 5-fluoro-2'-deoxyuridine and 2',5-difluoro-2'-deoxyuridine

submitted by Zuping Xia in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences.

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Data: April 16, 1998

DEDICATION

For my wife, son and my parents, whose love and support made the fulfillment of this thesis possible.

ABSTRACT

Advances in molecular biology pertaining to cell growth, differentiation and apoptosis, together with the correlation between drug pharmacokinetics and pharmacodynamics, offer the potential to design better chemotherapeutic agents for cancer therapy. Based on our current understanding of neoplasms and the chemical properties and pharmacological actions of anticancer agents, a group of 3'-*O*-butanoyl, 5'-*O*-butanoyl and 3',5'-di-*O*-butanoyl esters (**2, 3, 6, 9-11**) of 5-fluoro-2'-deoxyuridine (FDU) and 2',5-difluoro-2'-deoxyuridine (DFDU), 3'-*O*-retinoyl and 3',5'-di-*O*-retinoyl esters (**17, 18**) of FDU, and 5'-*O*-*bis*(2,2,2-trichloroethyl)phosphoryl-FDU (**19**) and its 3'-*O*-butanoyl ester (**20**) were designed and synthesized. It was hoped that: *1.* (**19**) and (**20**) could potentially act as a source of FdUMP after entering cells; *2.* by enhancing resistance to phosphorolytic cleavage by 2'-F substitution, the anticancer efficacy of DFDU would be enhanced; *3.* the ester prodrugs would act as a depot to release two active drugs acting via different mechanisms, cytotoxicity and differentiation which have been reported to provide a synergistic effect, resulting in improved drug pharmacokinetics and increased therapeutic index.

The *in vitro* anticancer screening data for a diverse panel of 60 human cancer cell lines derived from nine neoplastic types (breast, CNS, colon, leukemia, lung, melanoma, ovarian, prostate, and renal cancers) showed that (**17**), (**18**) and (**20**) are more potent than FDU. There is a synergistic action produced by the two moieties from these prodrugs. Effects of (**17**) and (**20**) on induction of cell differentiation and cell death in HL-60 APL cells showed that these prodrugs could induce malignant cell differentiation. The ongoing differentiation cells immediately underwent enhanced apoptosis rather than terminal

differentiation. Apoptosis was the major pathway for HL-60 APL cell death caused by (**17**), which is similar to all-*trans*-retinoic acid (RA). However, necrosis played an important role in HL-60 APL cell death upon treatment with (**20**), which is similar to FDU.

Tumor growth delay studies showed: *1.* cytotoxic-differentiation treatment can be synergistic *in vivo*; *2.* (**17**) and (**20**) were better than a mixture of FDU + RA or FDU + sodium butanoate. Another advantage of (**17**) and (**20**) is that they showed low toxicity to normal mice in dose escalation experiments and examinations of clinical chemistry for serum and histopathology.

The plasma pharmacokinetics for (**17**) in mice bearing EMT-6 tumors was best described by a three compartment model. The primary metabolites (FDU and RA) of (**17**) in plasma fit a one compartment model. (**17**) was shown to be a bioactivable prodrug that releases two active drugs (FDU and RA). (**17**) had a longer plasma half-life compared to either FDU or RA and had high bioavailability (90%). Tissue analyses showed that (**17**) could accumulate in tumor. The rapid elimination of (**17**) by liver and kidney may be relevant to its low toxicity observed in the dose escalation experiments, clinical chemistry and histopathology.

In conclusion, both *in vitro* and *in vivo* experiments indicated that, among the compounds evaluated in this study, (**17**) is the most promising prodrug candidate for further biological evaluation in view of its broad spectrum of anticancer activity, greater efficacy and low toxicity.

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LIST OF ABBREVIATIONS

Å	Angstrom
μM	Micro molar concentration
μmol/kg	Micromole(s) per kilogram
μmol/L	Micromole(s) per litre
[S]	Concentration of the prodrug
13-*cis*-RA	13-*Cis*-retinoic acid
3',5'-di-*O*-BuDFDU	3',5'-Di-*O*-butanoyl-2',5-difluoro-2'-deoxyuridine
3',5'-di-*O*-BuFDU	3',5'-Di-*O*-butanoyl-5-fluoro-2'-deoxyuridine
3',5'-di-*O*-RFDU	3',5'-Di-*O*-retinoyl-5-fluoro-2'-deoxyuridine
5,10-CH_2FH_4	N^5,N^{10}-methylenetetrahydrofolate
3'-*O*-Bu-5'-PFDU	3'-*O*-butanoyl-5'-*O*-*bis*(2,2,2-trichloroethyl)phosphoryl-5-fluoro-2'-deoxyuridine
3'-*O*-BuDFDU	3'-*O*-Butanoyl-2',5-difluoro-2'-deoxyuridine
3'-*O*-BuFDU	3'-*O*-Butanoyl-5-fluoro-2'-deoxyuridine
3'-*O*-RFDU	3'-*O*-Retinoyl-5-fluoro-2'-deoxyuridine
5'-*O*-BuDFDU	5'-*O*-Butanoyl-2',5-difluoro-2'-deoxyuridine
5'-*O*-BuFDU	5'-*O*-Butanoyl-5-fluoro-2'-deoxyuridine
5'-PFDU	5'-*O*-*bis*(2,2,2-trichloroethyl)phosphoryl-5-fluoro-2'-deoxyuridine
9-*cis*-RA	9-*Cis*-retinoic acid
Alb	Albumin
ALT	Alanine aminotransferase
Anal. Calcd.	Analysis calculated
AP	Alkaline phosphatase
AP1	Activator protein 1, transcription factor
APL	Acute promyelocytic leukemia
ATP	Adenosine triphosphate
AUC	Area under the curve

BA	Butyric acid
bcl-2	Oncogene, prevents cell death by apoptosis
BUN	Blood urea nitrogen
C_{17}	Concentration of the prodrug (17) in plasma
Ca	Calcium
Ca^{++}	Calcium ion
C_{FDU}	Concentration of FDU in plasma
c-fos	Proto-oncogene
c-jun	Proto-oncogene
CK	Creatine kinase
Cl	Chloride
CL	Total body clearance
C_{max}	Maximum concentration
c-myb	Proto-oncogene
c-myc	Proto-oncogene
CR	Complete regression of tumor
C_{RA}	Concentration of RA in plasma
CRABP	Cellular retinoic acid binding protein
CRABP-I	Cellular retinoic acid binding protein I
CRABP-II	Cellular retinoic acid binding protein II
Creat	Creatinine
D	Dose
d	Doublet
dd	Doublet doublet
ddd	Doublet doublet doublet
DFDU	2',5-Difluoro-2'-deoxyuridine
DHFU	5,6-Dihydro-5-fluorouracil
DMAP	4-Dimethylaminopyridine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

dR-1-P	Deoxyribose-1-phosphate
dTMP	Deoxythymidine-5'-monophosphate
dTTP	Deoxythymidine-5'-triphosphate
dUDP	Deoxyuridine-5'-diphosphate
dUMP	Deoxyuridine-5'-monophosphate
dUTP	Deoxyuridine-5'-triphosphate
ED_{50}	Dose required for 50% of effectiveness
F	Fraction of the tumor cells surviving after drug treatment or fluorine depending on the context
FAD	Flavine adenine dinucleotide
FBS	Fetal bovine serum
FDU	5-Fluoro-2'-deoxyuridine
FdUDP	5-Fluoro-2'-deoxyuridine-5'-diphosphate
FdUMP	5-Fluoro-2'-deoxyuridine-5'-monophosphate
FdUTP	5-Fluoro-2'-deoxyuridine-5'-triphosphate
FU	5-Fluorouracil
FUDP	5-Fluorouridine-5'-diphosphate
FUMP	5-Fluorouridine-5'-monophosphate
FUPA	α-Fluoro-β-ureidopropionic acid
FUrd	5-Fluorouridine
FUTP	5-Fluorouridine-5'-triphosphate
g/L	Gram(s) per litre
G_0	Resting phase during the cell cycle
G_1	Phase of gap 1 during the cell cycle
G_2	Phase of gap 2 during the cell cycle
Glob	Globulin
GLUC	Glucose
h	Hour(s)
HCO_3^-	Bicarbonate
His	Histidine

HO342	Hoechest 33342
HPLC	High performance liquid chromatography
HSLAS	Health sciences laboratory animal services, University of Alberta
Hz	Hertz
i.p.	Intraperitoneal
i.v.	Intravenous
ID_{50}	Dose required for 50% inhibition of capillary formation
iu	International unit(s)
iu/L	International unit(s) per litre
J	Coupling constant
K	Potassium
K_{12}	First order transfer constant for the movement of prodrug from the central compartment to tissue compartment
K_{13}	First order transfer constant for the movement of prodrug from the central compartment to deep tissue compartment
K_{14}	First order transfer constant for the movement of drugs released from the prodrug from the central compartment to tissue compartment
K_{21}	First order transfer constant for the movement of prodrug from the tissue compartment to central compartment
K_{31}	First order transfer constant for the movement of prodrug from the deep tissue compartment to central compartment
K_{41}	First order transfer constant for the movement of drugs released from the prodrug from the tissue compartment to central compartment
kcal	Kilocalorie
kDa	Kilo Dalton
Kf	Formation rate constant of RA or FDU
K_{FDU}	Elimination rate constant of FDU
kg	Kilogram(s)
K_M	Michealis constant
K_{RA}	Elimination rate constant of RA

LXR	Receptor specially dimerizes with RXR.
LXRE	DNA response element of LXR receptor
m	Multiplet
M	Phase of mitosis during the cell cycle or molar concentration depending on the context
Me	Methyl
Mg^{++}	Magnesium ion
min	Minute(s)
mL	Milliliter(s)
mM	Millimolar concentration
mmol	Millimole(s)
MMoL	Myelomonocytic leukemia
mmol/L	Millimole(s) per litre
mol	Mole(s)
mosmol/kg	Milliosmole(s) per kilogram
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
N/A	Not available
Na	Sodium
NaBu	Sodium butanoate
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCI	United States National Cancer Institute
ND	Not detectable
nm	Nanometer
NMR	Nuclear magnetic resonance
ODctrl	The average of optical density measured after 48 hours without exposure of the cells to the drugs
ODdest	The average of optical density measured after 48 hours exposure of the cells to the drugs
ODtzero	The average of optical density measured just before exposure of the

	cells to the drugs
OSM	Osmolality
P	Phosphorus
p53	Tumor suppressor gene
p.o.	Per os
PBS	Phosphate buffer
PG	Percentage growth
PI	Propidium iodide
pmol	Picomole(s)
PPi	Inorganic pyrophosphate
PRPP	5-Phosphoribosyl-1-pyrophosphate
PYLL	Potential years life lost
RA	All-*trans*-retinoic acid
RAR	Retinoic acid nuclear receptor which specifically binds to RARE. It can form heterodimers with RXR.
RARE	DNA response element of RAR receptors in the promotor region of the specific RAR target genes.
ReAc	Retinyl acetate.
RNA	Ribonucleic acid
RXR	Retinoic acid nuclear receptor which specifically binds to RXRE. It can form heterodimers with RAR or homodimer with itself.
RXRE	DNA response element of RXR receptors in the promotor region of the specific RXR target genes
S	Phase of DNA synthesis during the cell cycle
s	Singlet
s.c.	Subcutaneous
SD	Standard deviation
SE	Standard error
Ser	Serine
t	Triplet

$T_{1/2}$	Half-life
$T_{1/2\alpha}$	Half-life of distribution phase
$T_{1/2\beta}$	Half-life of elimination phase
$T_{1/2\gamma}$	Half-life of terminal elimination phase
Td	Average doubling time of the tumor
TGase	Transglutaminase
TGF-β	Transforming growth factor β
T_{max}	Time to reach C_{max}
TOT BILI	Total bilirubin
TP	Total protein
TS	Thymidylate synthase
V	Enzymatic reaction rate
V_d	Biodistribution
V_{FDU}	Biodistribution of FDU
V_{max}	Maximum enzymatic reaction rate
V_{RA}	Biodistribution of RA

1. INTRODUCTION

1-1. Background Information Relevant to the Treatment of Cancer

1-1-1. Incidence of Cancer

Cancer has been a recognized disease entity for thousands of years and it continues to be a dreadful disease (Rasther 1989). Examination of the twelve leading causes of potential years life lost (PYLL) for the year 1993 in Canada showed that cancer ranked number one in both men and women (Figure 1-1-1) (Gaudette et al. 1996).

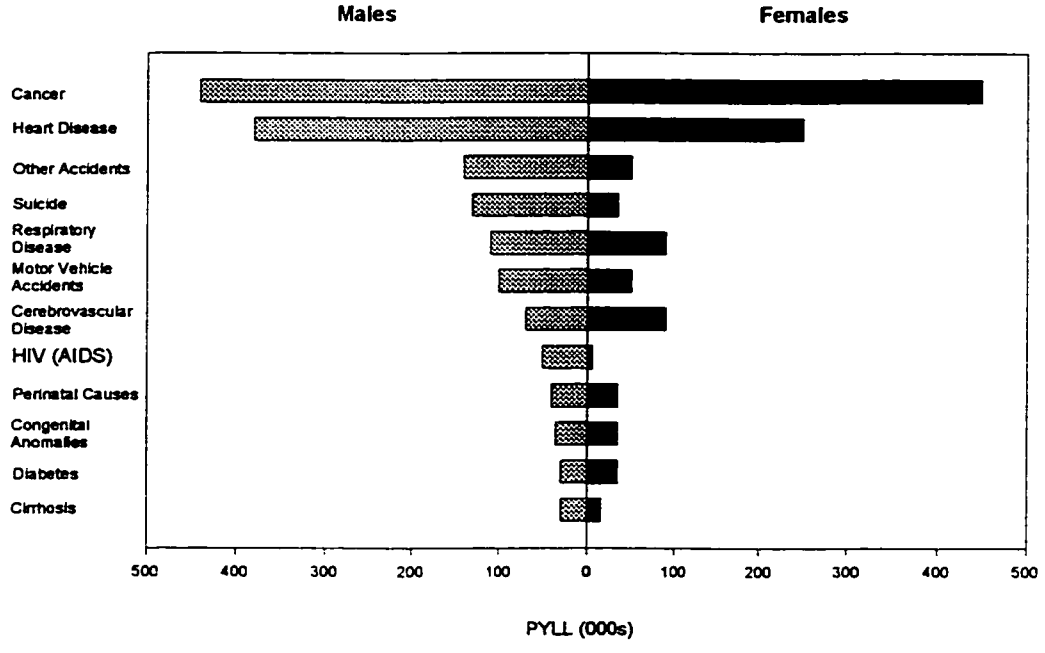


Figure 1-1-1. Twelve leading causes of potential years of life lost (PYLL) in Canada during 1993 (Gaudette et al. 1996).

The PYLL is the total number of years of life lost obtained by multiplying, for each age group, the number of deaths by the life expectancy of survivors. This data indicated that cancer was the first cause of potential years of life lost in Canada for the year 1993. In other words, 28% of the PYLL was due to cancer. In 1996, an estimated 129,200 new cases of cancer and 61,800 deaths from cancer occurred in Canada as shown in Figure 1-1-2. These statistics are similar in the USA (Murphy et al. 1997).

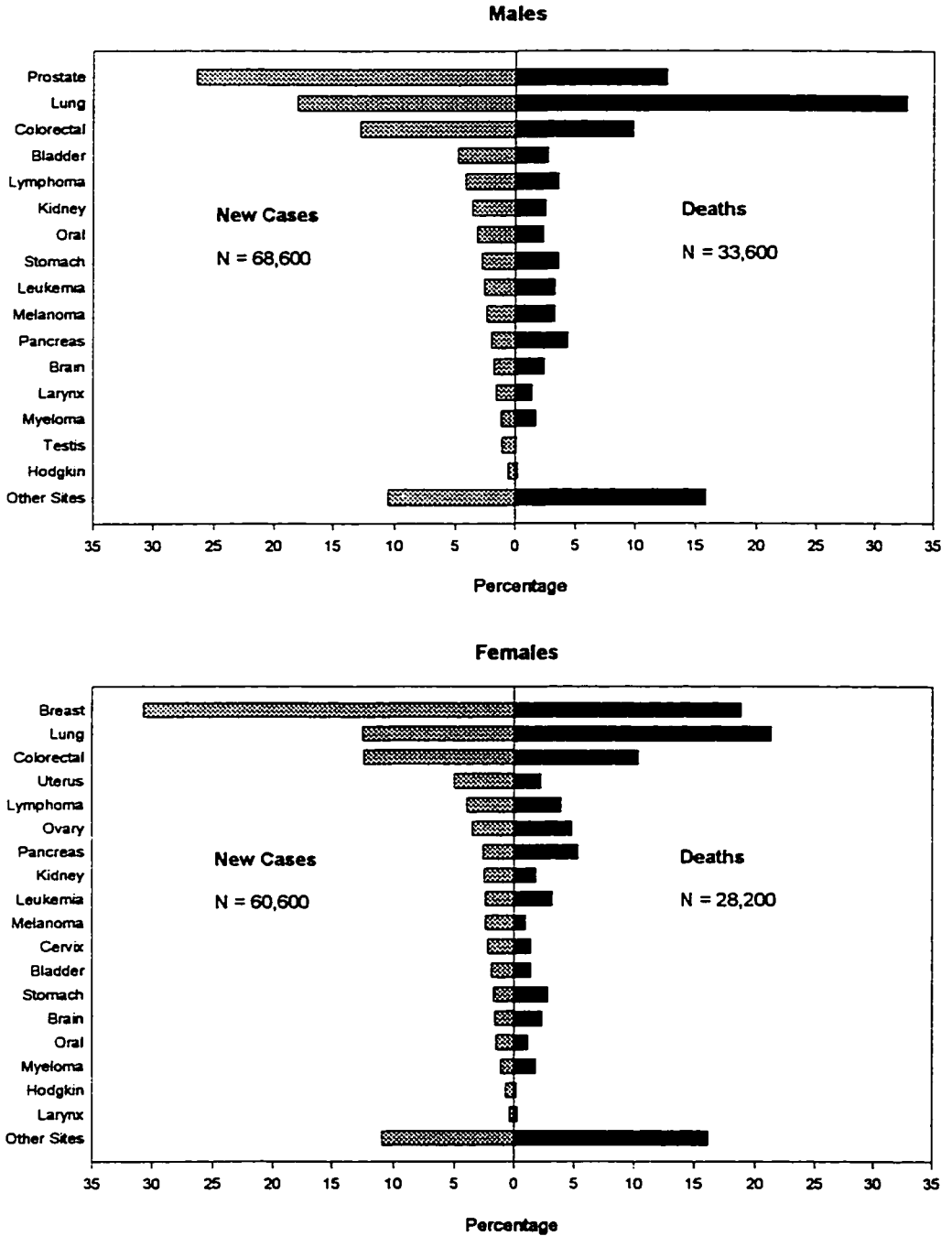


Figure 1-1-2. Percentage distribution of estimated new cases and deaths for selected cancer sites for males and females in Canada during 1996 (Gaudette et al. 1996).

1-1-2. Cancer: Characteristics, Causes and Complications

Cancer is a disease of cells which is associated with the loss of normal self-controlled homeostasis involving cell proliferation, differentiation, and cell death. Some differences between cancer and the normal tissues include: (1) decreased cellular differentiation; (2) uncontrolled cell proliferation and cell apoptosis (programmed cell death); (3) invasion of surrounding tissue; (4) cellular heterogeneity; (5) metastasis and establishment of new malignant growth at ectopic sites.

Cancer is many diseases (Montgomery 1988) that may be induced by numerous initiators. There are several possible initiating extrinsic causes which can divided into three categories: (1) chemical agents (Earle 1943; Miller et al. 1981; Levin et al. 1982; Strickland et al. 1995); (2) physical agents (Guerrero et al. 1984; Weichselbaum et al. 1989); (3) infectious agents (Hayward et al. 1981; Mann et al. 1987; Nerenberg et al. 1987).

The intrinsic reason for the development of cancer is attributed to a debilitated immune system which can be due to a variety of factors, such as age, living environment, familial inheritance, etc. Furthermore, cancer is not like a bacterial infection that the host body readily recognizes as a foreign invader. The host immune system, especially a weakened immune system, is often not sensitive enough to respond to the self-cellular malignancy process, or it is overwhelmed by the cancer growth since cancerous cells are marginally different at first from the cells in normal tissue from which they arise. The subtle difference between cancerous and normal cells complicates cancer chemotherapy, since current anticancer drugs are not selective in their ability to distinguish whether a cell is malignant or normal.

Cancer heterogeneity, among a variety of neoplasms as well as heterogeneous cells within a single malignant neoplasm, further complicates cancer chemotherapy. Since most human cancers, if not all, originate from a single transformed cell (Wainscoat et al. 1990; Sidransky et al. 1992), the differences among various cancers or among cells within a single neoplasm must reflect a complex pattern of clonal diversification during cancer progression (Nowell 1986). At least three mechanisms contribute to the generation of this diversity as a neoplasm develops from a single cell to a detectable cancer: (1)

differentiation-related heterogeneity within a clone; (2) nutritional heterogeneity; (3) generation of new subclones during cancer progression (Buick et al. 1992). The heterogeneity is exhibited in a wide range of genetic, biochemical, immunologic, and biologic differences (Heppner 1984). Therefore, it is not surprising that heterogeneous subpopulations of neoplastic cells even within a single neoplasm respond differentially to various therapeutic agents. Furthermore, a major challenge in the treatment of malignant metastases is the heterogeneity of cancer cells (Poste et al. 1979; Fidler et al. 1982; Fidler et al. 1985), which are often resistant to conventional therapy. These malignant metastases, which are ultimately responsible for the majority of deaths caused by cancer, constitute the most difficult aspect of cancer treatment.

In conclusion, both intrinsic and extrinsic conditions contribute to the induction of cancer. Human malignant neoplasia is the outcome of comprehensive interactions between internal and external signals. It is now evident that the balance among cellular proliferation, differentiation and apoptosis (programmed cell death) plays a vital role in maintenance of normal tissue homeostasis, and that derangement of one of these processes can lead to neoplastic disease (Lyons et al. 1997). Intracellular signaling pathways mediate the main events in a cell's life cycle, such as growth, proliferation, differentiation, and apoptosis. The process of carcinogenesis involves the disruption of proliferation, differentiation, and apoptosis in the transformed cells. Intracellular signaling is initiated either by extracellular signals or by intracellular gene products, both of which can trigger intracellular signaling and cell functional responses. The final targets of carcinogenesis are related to cellular gene regulation and mutation. Mutation may permanently change the structures of DNA following activation of abnormal expression of oncogenes or restraint of normal expression of tumor suppressor genes. This kind of carcinogenesis is virtually irreversible. On the other hand, carcinogenesis may be due to abnormal expression of the gene, with no permanent change of DNA structure, so that it may be reversible. Only the latter kind of neoplasm, such as acute promyelocytic leukemia (APL), may respond to differentiation inducing therapy.

1-1-3. Cancer Treatment and Chemotherapy

Numerous approaches have been developed to treat cancer. Most cancer treatments currently include surgery or radiation therapy to remove the primary tumor, combined with chemotherapy that may eradicate micrometastasis. Although biological approaches, such as immunotherapy (Gaynor et al. 1995) and gene therapy (Schrier et al. 1997), recently have received a lot of attention for the treatment of cancer, they do not replace earlier methods since cancer is a multiplicity of diseases. At the 1997 Western Canada Cancer Research Meeting, one clinical oncologist (Venner 1997) indicated that at least 90% of cancer patients are currently treated by chemotherapy. Chemotherapy has played, and no doubt will continue to play, an important role in cancer therapies, whether used alone or in concert with other treatment modalities. Perhaps more than anyone, clinical oncologists appreciate the need for new and better drugs than those now available. Moreover, recent advances in understanding the mechanisms of cell growth regulation, differentiation and apoptosis together with the mechanism of action of anticancer drugs allow us to pursue cancer chemotherapy at cellular and molecular levels. Rational chemotherapeutic targets of cancer are more and more directed at the molecular components of the cellular signaling pathway, the oncogenes or suppressor genes or their products (Jacson 1997; Weiss 1997). This results in a crossover of biological treatments with chemotherapy, and sometimes it is very difficult to distinguish between them. For example, it has recently been found that 5-fluoro-2'-deoxyuridine (FDU), a cytotoxic antimetabolite used for 40 years in cancer chemotherapy, may regulate *c-fos* and *c-jun* gene expression as well as the protease level, which are associated with cell apoptosis (Kakutani et al. 1996). Therefore clinical use of FDU could be interpreted as a kind of gene therapy.

There are many success stories in cancer chemotherapy (see Table 1), in which anticancer drugs have played a major role (Verweij et al. 1997). Accordingly, combination treatment using multi therapies, including chemoprevention and chemotherapy, will be likely of most success in the future treatment of cancer.

Table 1. The probability of cancer cure using chemotherapy in advanced stage disease (Verweij et al. 1997)

Disease	Approximate cure rate (%)
Children	
Acute lymphocytic leukemia	>50
Non-Hodgkin's lymphoma	>50
Burkitt's lymphoma	>50
Wilms' tumor	>50
Ewing's sarcoma	>50
Embryonic rhabdomyosarcoma	>50
Adults	
Gestational choriocarcinoma	90
Testicular cancer	>75
Hodgkin's lymphoma	>50
Aggressive non-Hodgkin's lymphoma	>50
Acute myeloid leukemia	25-50
Ovarian cancer	10-20

Unfortunately, the cancers cured by chemotherapy have a low incidence compared to lung, breast, prostate and colon cancers. In addition, the tumor metastases, tumor heterogeneity, drug toxicity and drug resistance are still major obstacles in cancer chemotherapy. Therefore, accompanied with the advancements in molecular biology pertaining to cell growth, differentiation and death, and the correlation between drug pharmacokinetics and pharmacodynamics, there is an urgent need to design and develop new chemotherapeutic agents to improve the cancer therapy.

1-2. 5-Fluoro-2'-deoxyuridine (FDU)

Nucleosides are fundamental components in nucleic acid synthesis. Therefore, it is reasonable that nucleoside analogues exhibit various activities via their interactions in many enzymatic pathways involving nucleoside and nucleotide synthesis and metabolism (Daher et al. 1990) or act as a chain disturber in the DNA polymerization process

(Houghton et al. 1996). Thus, nucleoside analogues are major candidates for cancer chemotherapy (Horwitz 1989; Perigaud et al. 1992).

FDU is an analogue of the physiological 2'-deoxyuridine that has a fluorine substituted at the 5-position in place of a hydrogen. FDU shares the same metabolic pathway with 2'-deoxyuridine. FDU can function as a competent substrate in enzymatic reactions used by vital cells in their biological processes, such as DNA synthesis. The chemical structure of FDU is shown below in Figure 1-2-1.

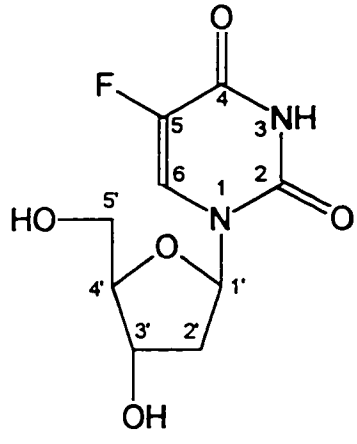


Figure 1-2-1. Chemical structure of FDU.

FDU has been used extensively in the clinic for anticancer chemotherapy (Heidelberger 1975), involving the treatment of advanced adenocarcinoma of the gastrointestinal tract (Moore et al. 1960), oral cancer (Couture 1968), colon cancer (Moertel 1978; Bajetta et al. 1997), pancreatic cancer (Barone 1975), liver cancer (Ramming 1983), biliary tract cancer (Davis et al. 1974), breast cancer (Papac et al. 1966) and metastatic renal cancer (Aveta et al. 1997).

1-2-1. Mechanisms of FDU Anticancer Activity

Generally, anticancer drugs produce their effect by interfering in one way or another in DNA replication, expression, or inhibiting nucleic acid synthesis. Thus, their ability to influence protein synthesis is a common pharmacological effect of all anticancer drugs. FDU has at least three mechanisms by which it interferes in DNA synthesis: (1) inhibition of thymidylate synthase (TS) (Langenbach et al. 1972; Santi et al. 1974; Pratt et al. 1994); (2) incorporation into DNA (Danenberg et al. 1981; Kufe et al. 1983; Schuetz et al. 1984; Schuetz et al. 1986); (3) dUTP misincorporation into DNA (Canman et al. 1994) (see Figure 1-2-2). The outcome of these interactions of FDU on the primary targets may disrupt the cell cycle (Huschtscha et al. 1996). This perturbation of the cell cycle may act as a trigger to initiate the apoptotic cascade due to the misinterpretations or imbalances of regulatory protein molecules (Kung et al. 1990; Askew et al. 1991; Stewart 1997). Induction of cellular apoptosis by FDU (Furuya et al. 1996) may be in part due to the expression of a 40 kDa endonuclease that causes DNA double strand cleavage (Hwang et al. 1995). Indeed, it has been observed that the DNA break is relevant to FDU cytotoxicity (Yin et al. 1991). Cell death induced by FDU may also be associated with an increased expression of *c-fos*, *c-jun*, proteases (Kakutani et al. 1996) and a down-regulation of *bcl-2* (Fisher et al. 1993).

Another recognized cytotoxic mechanism for FDU is the incorporation of fluorouridine triphosphate (FdUTP), a metabolite of FDU, into RNA (see Figure 1-2-2). However, based on literature reports (Van Laar et al. 1996; Inada et al. 1997) and new data (see section **3-2**, ***in vitro*** tumor cell growth inhibition screen results presented in Figures 3-2-1 to 3-2-9 which have shown that FDU is more potent than 5-fluorouracil (FU)), the incorporation of FDU into RNA does not show a significant correlation to anticancer cytotoxicity.

1-2-2. Clinical Limitations of FDU in Cancer Chemotherapy

After administration, FDU is metabolized to the 5'-monophosphate ester of FDU (FdUMP). FdUMP will either undergo strong binding to TS together with reduced folate cofactor, N^5,N^{10}-methylenetetrahydrofolate (5,10-CH_2FH_4, see Figure 1-2-3), or be

further phosphorylated to the fraudulent ribonucleotide triphosphate (FdUTP) which will be incorporated into DNA. Unfortunately, the cellular anabolism of FDU to FdUMP by pyrimidine nucleoside kinase is offset by the catabolic effect of pyrimidine phosphorylases, such as thymidine phosphorylase (Niedzwicki et al. 1983), which cleave the glycosyl bond to yield FU (Chaudhuri et al. 1959; Birnie et al. 1963; Woodman et al. 1980; Uchida et al. 1990) (see Figure 1-2-2). The susceptible catabolism of FDU is responsible, at least in part, for the observation that FDU is more potent than FU *in vitro* (Bosch et al. 1958; Rich et al. 1958; Laskin et al. 1976; Yin et al. 1991) but not *in vivo* (Heidelberger et al. 1958; Reitmeier et al. 1965). Moreover, development of resistance to FDU can result from the cancer cell heterogeneity, the depletion of activating pyrimidine nucleoside kinases (Reichard et al. 1959; Bapat et al. 1983), high activity of pyrimidine phosphorylase (Uchida et al. 1990) or TS overproduction (Berger et al. 1985) and TS structural variation (Bapat et al. 1983; Berger et al. 1988; Davis et al. 1993; Hughey et al. 1993; Reilly et al. 1997). In addition, 5'-mononucleotides are also prone to rapid degradation to the corresponding nucleosides by nonspecific phosphohydrolases (Hampton et al. 1962; Holmsen et al. 1968).

Due to its poor bioavailability (Clarkson et al. 1964), FDU is not suitable for oral administration. Following intravenous dosing, about 29% of the dose is excreted by the kidney as inactive metabolites. Owing to its rapid plasma elimination ($T_{1/2}$ = 7 min) (LaCreta 1987), FDU is most frequently administered by intraarterial infusion (Ensminger et al. 1978; Cao et al. 1993; Anon 1996; Durand-Zaleski et al. 1997). However, toxicity and infection relating to the prolonged use of an arterial catheter has been observed (Oberfield et al. 1979; Sterchi et al. 1989). In addition, significant myelosuppression (Curreri et al. 1959; Ansfield et al. 1960), gastrointestinal toxic effects (Kemeny et al. 1987) and even adverse central nervous system toxicity (Koenig et al. 1970) may be associated with FDU administration.

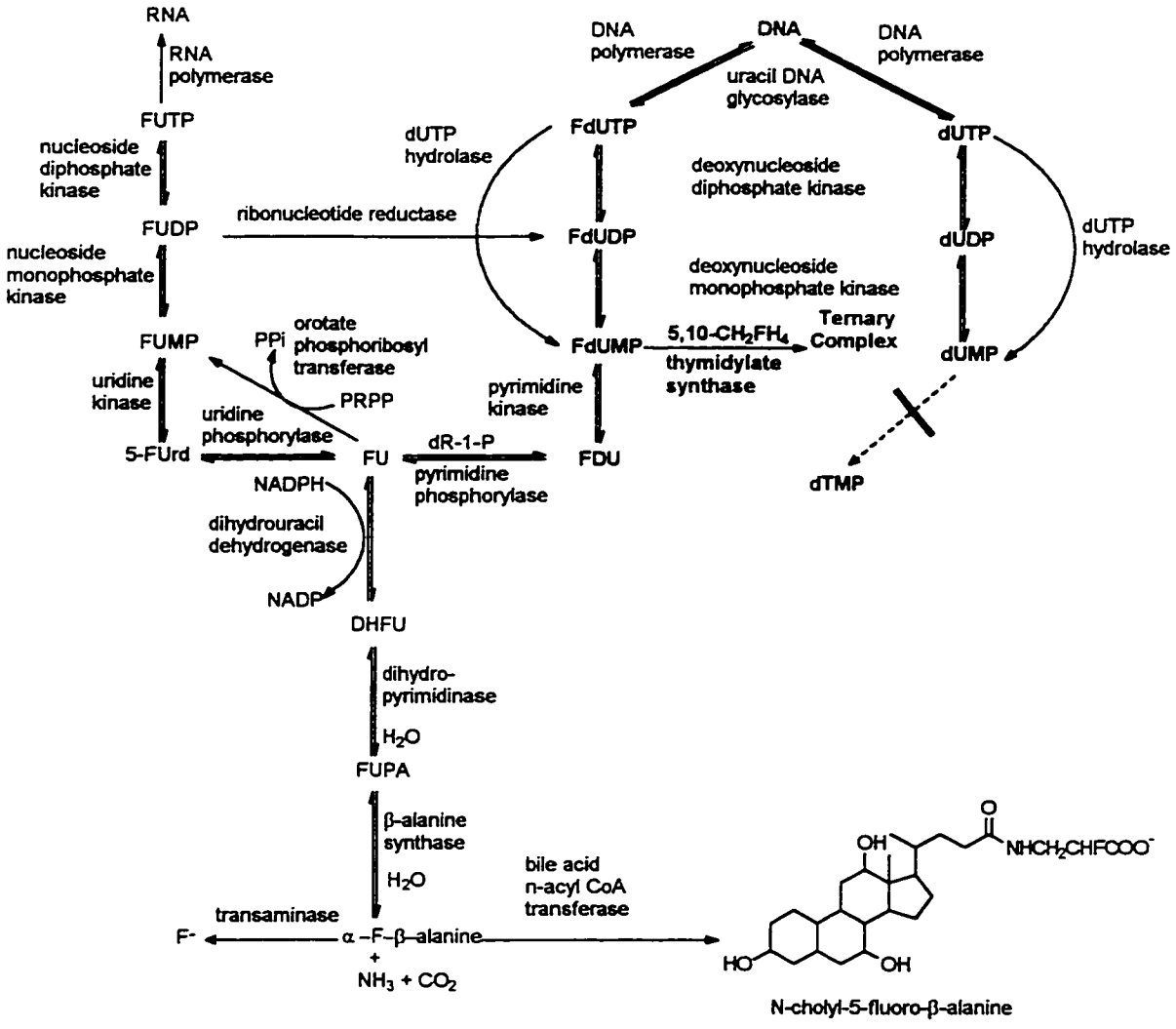
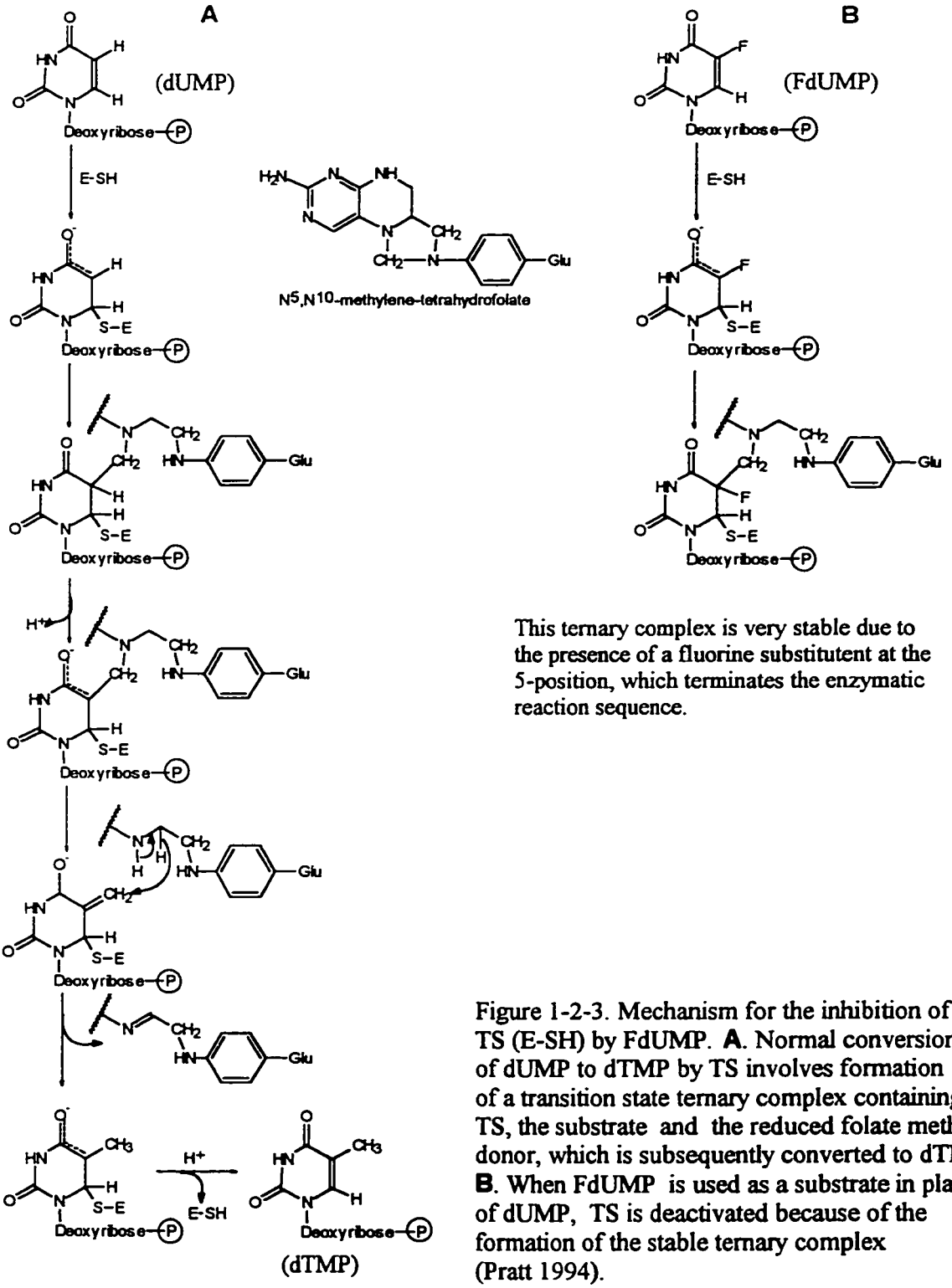


Figure 1-2-2. FDU metabolic pathway (Sweeny et al. 1987; Hull et al. 1988; Canman et al. 1994; Houghton et al. 1996).

FUTP is 5-fluorouridine-5'-triphosphate; FUDP is 5-fluorouridine-5'-diphosphate; FUMP is 5-fluorouridine-5'-monophosphate; 5-FUrd is 5-fluorouridine; PPi is inorganic pyrophosphate; PRPP is 5-phosphoribosyl-1-pyrophosphate; dR-1-P is 2-deoxyribose-1-phosphate; FdUTP is 5-fluoro-2'-deoxyuridine-5'-triphosphate; FdUDP is 5-fluoro-2'-deoxyuridine-5'-diphosphate; dUTP is 2'-deoxyuridine-5'-triphosphate; dUDP is 2'-deoxyuridine-5'-diphosphate; dUMP is 2'-deoxyuridine-5'-monophosphate; dTMP is 2'-deoxythymidine-5'-monophosphate; DHFU is 5,6-dihydro-5-fluorouracil; FUPA is α-fluoro-β-ureidopropionic acid.



This ternary complex is very stable due to the presence of a fluorine substitutent at the 5-position, which terminates the enzymatic reaction sequence.

Figure 1-2-3. Mechanism for the inhibition of TS (E-SH) by FdUMP. **A**. Normal conversion of dUMP to dTMP by TS involves formation of a transition state ternary complex containing TS, the substrate and the reduced folate methyl donor, which is subsequently converted to dTMP. **B**. When FdUMP is used as a substrate in place of dUMP, TS is deactivated because of the formation of the stable ternary complex (Pratt 1994).

1-3. Induction of Cell Differentiation and Cell Apoptosis (Programmed Cell Death): Another Approach for Cancer Chemotherapy

Inducing cell differentiation in the neoplasm has been attractive and important, since this approach to cancer chemotherapy offers potential advantages such as reduced toxic side effects and more selective action on cancer cells compared to conventional chemotherapy (Sachs 1987; Scott et al. 1988). A fundamental characteristic of many cancer cells is their incomplete differentiated state (Reiss et al. 1986; Pardee et al. 1988; Lynch 1995). Differentiation therapy follows the concept that the abnormality of immature neoplastic cells can be circumvented by differentiation inducers based upon the ability of differentiating agents to interact with the intracellular signaling systems used by growth factors and growth factor receptors (Ebert et al. 1994). Studies conducted over the last few decades discovered a number of compounds that are able to induce *in vitro* malignant cell differentiation. However, it was the *in vivo* use of all-*trans*-retinoic acid (RA) for the treatment of acute promyelocytic leukemia (APL) that pioneered the movement of this differentiation therapy from laboratory research to clinical application (Huang et al. 1988).

Recently, induction of cell apoptosis, an alternative mode of cell death to necrosis, has been the focus of considerable attention in cancer chemotherapy (Chresta et al. 1996; Lutzker et al. 1996; Hannun 1997; Stewart 1997) and in cancer chemotherapy resistance (Hickman 1996). It has been recognized that: (1) apoptosis is an essential component and part of the cell self-defense responses to development and homeostasis (Fotedar et al. 1996; Lockshin et al. 1996; Milligan et al. 1996; Milligan et al. 1997; Thatte et al. 1997); (2) most, if not all, anticancer agents including both traditional cytotoxic drugs and differentiation inducers have been found to effect tumor cell killing via apoptosis (Hickman 1992; Hannun 1997; Hickman et al. 1997; Stewart 1997; Thatte et al. 1997). The recognition that cancer cell death results from the apoptotic process occurring subsequent to interaction of the drugs on the primary targets offers a new perspective of cancer chemotherapy. Apoptosis is a mode of death common to differentiated cells at the

end of their life span, that is, apoptosis may be closely linked to the normal differentiation pathway (Darzynkiewicz 1995; Yamamoto 1995; Bosman et al. 1996; Milligan et al. 1996; Ross 1996; Williams et al. 1996). On the other hand, induction of malignant cell differentiation may potentiate apoptosis (Bhatia et al. 1995; Kalemkerian et al. 1996). A brief illustration of the relationship among cell proliferation, cell differentiation, and apoptosis is presented in Figure 1-3 (Milligan et al. 1996).

Cells die by one of two modes: necrosis or apoptosis. Cell death by necrosis occurs as a result of a marked toxic or physical insult. Necrosis can be described as cellular metabolic collapse and occurs when a cell no longer maintains homeostasis. It is characterized by the random degradation of cellular DNA and cell membrane permeance at an early stage of cell death. As ATP becomes exhausted and consequently transmembrane ion gradients disappear, the cell swells and the membrane ruptures, spilling out lysosomal enzymes which mediate non-specific inflammation in surrounding tissue (Dive et al. 1992). Necrosis is the passive death of the cell without genetic influence. In contrast to necrosis, apoptosis is an active cascade pathway for cell death activated by certain signals, therefore sometimes it is called "suicide". Apoptosis often occurs as a component of normal development or homeostasis. From current understanding, apoptosis has three basic processes that must occur during apoptosis: (1) the cell must receive a signal to die; (2) the cell must then activate itself death machinery; (3) the debris from the dead cell must be removed. The signals that can trigger this type of death are many, including cytotoxic drugs, such as FDU, and differentiation inducers, such as RA.

Once a cell is signaled to die, specific genes and enzymes within the condemned cell are subsequently either activated or repressed depending on cellular development context or cell type; such agents include *bcl-2*, *c-myc*, *c-fos*, *c-fun*, *p53*, protease, transglutaminase, and endonuclease (Colotta et al. 1992; Evan et al. 1992; Driscoll 1995; Fotedar et al. 1996; Vaux et al. 1996; Bellamy 1997; Brown 1997; Hannun 1997; Jehn et al. 1997; Thornberry 1997).

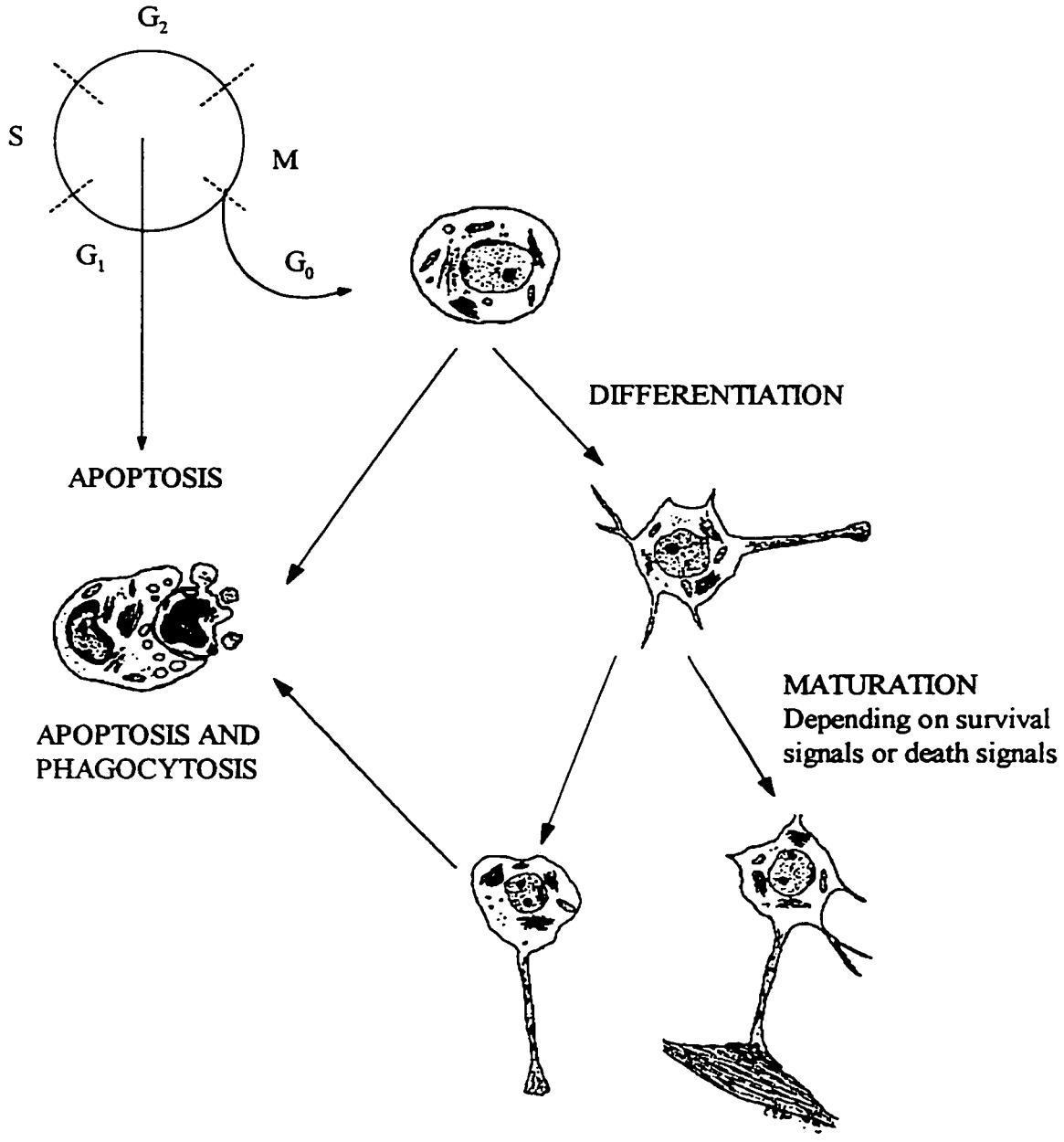


Figure 1-3. Development of a cell during its life span: proliferation, differentiation and apoptosis (Milligan et al. 1996).

It is worthy of notice that several of these genes are known to be regulators of the cell cycle, and therefore they may play an important role in a cellular decision to undergo cell proliferation, differentiation, or apoptosis. Apoptosis is characterized by morphological changes and biological changes (Bosman et al. 1996; Leithoff 1996). Distinct morphological changes are cell shrinkage and condensation of the chromatin. The nucleus may also disintegrate into fragments due to the activation of endonuclease which results in what is commonly referred to as the DNA ladder, as demonstrated by gel electrophoresis. When these fragments separate from the cells by transglutaminase (Fesus et al. 1991), apoptotic bodies are formed, which are then phagocytosed (Savill 1997). Because of rapid phagocytosis and no release of proteolytic enzymes, removal of apoptotic bodies does not produce an inflammatory response.

1-4. All-*trans*-retinoic Acid (RA)

RA is a hydrophobic molecule composed of a cyclohexene ring conjugated to an unsaturated hydrocarbon chain (Figure 1-4-1). RA is required for normal cell growth, differentiation and metabolism in the adult and in embryogenesis.

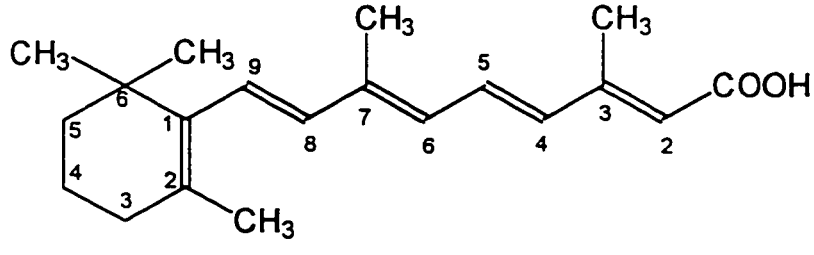


Figure 1-4-1. Chemical structure of all-*trans*-retinoic acid (RA).

Although RA has been reported to induce *in vitro* malignant cell differentiation or to suppress cell proliferation in numerous cancer cell lines such as acute promyelocytic leukemia (APL) (Breitman et al. 1980), teratocarcinoma (Strickland et al. 1978), endometrial adenocarcinoma (Carter et al. 1996), thyroid carcinoma (Schmutzler et al. 1996), breast cancer (Toma et al. 1997), lung cancer (Ou et al. 1996), melanoma (Edward et al. 1997) and papillomavirus-induced cancer (Khan et al. 1993), its greatest success in the clinic has been for the treatment of APL (Chomienne et al. 1996) with a mean complete cure response rate of 84% (Hong et al. 1994).

1-4-1. RA Signaling Pathways: Molecular and Cellular Mechanisms of RA Action

A simplified model of RA signaling pathways in cells is depicted in Figure 1-4-2. Fat-soluble RA may directly pass through the lipid bilayer of the cell membrane by simple diffusion, after which it may interact with the intracellular RA binding proteins, CRABP-I and CRABP-II (Warrell 1994a). It is this RA-protein binding that initiates a cascade of events such as metabolism (Fiorella et al. 1991; Boylan et al. 1992), crossing the nuclear membrane and forming RA-nuclear receptor complex (Blomhoff et al. 1990). Although the enzymes that are specifically involved in the metabolism of RA have not yet been identified (Blaner et al. 1994), the cytochrome P450 enzyme system has been reported to be active in metabolizing RA (Napoli 1996). CRABPs may play a direct role in RA metabolism (Fiorella et al. 1991). RA-CRABP complex itself may serve as a substrate for the metabolic enzymes. The major known metabolic pathways for RA are shown in Figure 1-4-3.

To date two types of retinoic acid nuclear receptors including 14 subtypes have been identified (Giguere et al. 1987; Petkovich et al. 1987; Mangelsdorf et al. 1990; Chambon 1996). They are retinoic acid receptors RAR, including $RAR_{\alpha 1}$, $RAR_{\alpha 2}$, $RAR_{\beta 1}$, $RAR_{\beta 2}$, $RAR_{\beta 3}$, $RAR_{\beta 4}$, $RAR_{\gamma 1}$ and $RAR_{\gamma 2}$, and retinoid X receptors RXR, including $RXR_{\alpha 1}$, $RXR_{\alpha 2}$, $RXR_{\beta 1}$, $RXR_{\beta 2}$, $RXR_{\gamma 1}$ and $RXR_{\gamma 2}$. Both RARs and RXRs act as ligand-dependent transcriptional regulators by binding specific DNA sequences, or response elements RARE and RXRE, in the promotor regions to regulate the expression of the target genes. RARs and RXRs may also down regulate the expression of genes by

antagonizing certain other transcriptional factors, such as AP1 (Nagpal et al. 1995). The pleiotropic effects of RA have been explained by its ability to regulate expression of many target genes (Nagpal et al. 1996).

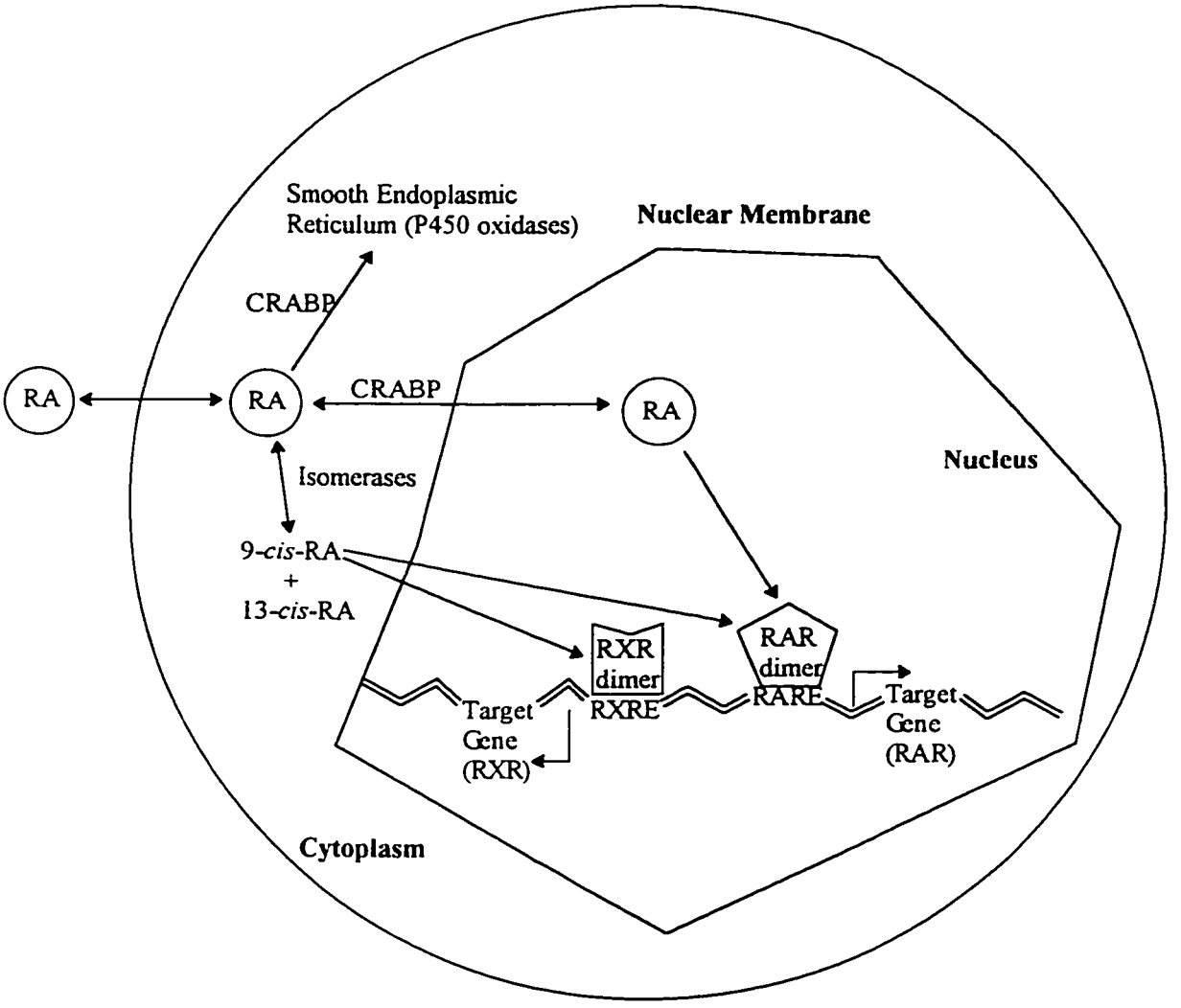


Figure 1-4-2. Simplified RA signaling pathway in a cell (Mangelsdorf et al. 1994). CRABP is cellular retinoic acid binding protein. RAR and RXR represent retinoic acid nuclear receptor and retinoid X receptor, respectively. RARE and RXRE are the DNA response elements in the promotor regions of the specific RAR and RXR target genes, which specially respond to the RAR dimer and RXR dimer, respectively.

The presence of two receptor systems implies the existence of corresponding responsive target genes to each receptor type. It has been suggested that RARs are implicated in the regulation of cellular proliferation and differentiation, whereas the RXRs function as modulators of apoptosis (Mehta et al. 1996; Boehm et al. 1997).

RARs and RXRs may form either heterodimers, RXR-RAR and RAR-RXR, or a homodimer, RXR-RXR, during their functional interaction (Kleiwer et al. 1992). The biological activity of RA appears to be mediated via activation of heterodimers which regulate target gene expression by specific interaction with RARE. In this case, RXR serves as a ligand-independent cofactor for RAR-mediated trans-activation (Kalemkerian et al. 1996) or allows agonist RXR to act after the heterodimer binds to DNA (Chen et al. 1996). RA may induce allosteric change in the receptor such that the complex facilitates binding to high-affinity sites in chromatin to modulate transcription. Although RA is a ligand for both RAR and RXR receptors, 9-*cis*-retinoic acid (9-*cis*-RA), one of the reversible cellular metabolites of RA, was found to be 40-fold more potent than RA in binding to RXR nuclear receptors (Mangelsdorf et al. 1992). The activity of 9-*cis*-RA appears to be mediated primarily through binding of RXR homodimer to RXRE. In addition, a recently discovered nuclear receptor, LXR, which dimerizes with ligand-activated RXR and binds a specific LXRE, has implied another possible 9-*cis*-RA signaling pathway (Willy et al. 1995). Therefore, interconversion of RA and 9-*cis*-RA within the cell may provide a means to regulate the activity of different retinoic acid signaling pathways, either by RAR, RXR or LXR. By regulating the production of RA and 9-*cis*-RA, the cell may be able to control which kind of receptor and when it would be activated. Thus, the formation of 9-*cis*-RA by isomerization of RA may be a very important path in mediating RA function. Moreover, 9-*cis*-RA has been found to bind strongly to and transactivate not only RXR receptors, but also RAR receptors (Mangelsdorf et al. 1994). Therefore, 9-*cis*-RA may serve as a bifunctional ligand. Indeed, it has been reported that RA and 9-*cis*-RA have a synergetic action *in vitro* to inhibit cell growth and induce differentiation of RA-resistant HL-60 cells into mature granulocytes (Kizaki et al. 1994). The differentiation properties affected by RA involve changes in the

growth properties of cells, the morphology of cells, and the regulation of a variety of genes, such as *c-myc* (Westin et al. 1982; Bentley et al. 1986), *c-myb* (Boise et al. 1992), *bcl-2* (Nagy et al. 1996) and *c-jun* (De Groot et al. 1990). These regulations may affect the metastatic behavior of cancer cells. For example, when metastatic B16 melanoma cells are pretreated with RA and then injected into the tail vein of mice, RA causes a significant inhibition of lung colonization by these cells (Edward et al. 1992).

In addition, the growth of solid tumors and their metastases requires the formation of a blood capillary network to transport the nutrients for the tumor growth. RA has been reported to be a strong inhibitor of embryonic angiogenesis, with an ID_{50} (dose required for 50% inhibition of capillary formation) of 330 pmol/egg (Nagpal et al. 1996). It has been found that a binding protein, an angiogenesis modulator, for fibroblast growth factors was down regulated in squamous cell carcinoma after treatment with RA, which may be responsible for the reduction of angiogenesis (Liaudet-Coopman et al. 1997).

Another intriguing point is that the RA-associated increase in the proportion of differentiated cells is quickly followed by an increase in the proportion of cells displaying morphologic characteristics of apoptosis (Martin et al. 1990). It has been demonstrated that RA can induce apoptosis in a variety of cell lines (Glozak et al. 1996; Taetle et al. 1996; Taimi et al. 1997; Toma et al. 1997; Zheng et al. 1997). In some cases, RA-mediated differentiation is a prerequisite for the induction of apoptosis (Kalemkerian et al. 1996), such as APL (Martin et al. 1990; Solary et al. 1994), MMoL (myelomonocytic leukemia) (Anzai et al. 1994), neuroblastoma (Thiele et al. 1985), and embryonal carcinoma (Atencia et al. 1994), although this is not always the case.

It has been recognized that RA is a biologically multifunctional molecule (Allen et al. 1989; Smith et al. 1992). To account for this diversity and complexity, the biological activity of RA must be integrated with the other biologically active molecules such as growth factors, enzymes, hormones, and gene expression. In other words, the function of growth factors, enzymes and hormones, as well as RA, must not be interpreted in isolation. These molecules are all part of a unifying regulatory system used by cells and tissues relating to their proliferation, differentiation and death. For instance, RA-receptors may induce transforming growth factor-β (TGF-β) activity (Robert et al. 1992). It has

been reported that secreted TGF-β2 mediates at least 30% of the inhibition of keratinocyte DNA synthesis resulting from treatment with RA (Glick et al. 1989). TGF-β2 is thought to mediate the inhibition of DNA synthesis associated with the differentiation process (Glick et al. 1990). Another example is transglutaminases (TGases). Type II TGase accumulates to a high level after exposure of human monocytic (Mehta et al. 1986) and promyelocytic (Maddox et al. 1988; Benedetti et al. 1996) leukemia cells to RA. Since it has been suggested that the induction and activation of Type II TGase, with resultant intracellular protein cross-linking, may be required for programmed cell death, or apoptosis, it follows that RA may play a role in apoptosis. Finally, the action of RA receptors as transcription factors for specific genes may depend on a specific cellular system (Nagpal et al. 1992).

1-4-2. Clinical Limitations of RA in Cancer Chemotherapy

While differentiation therapy using RA in APL patients is encouraging, several limitations prevent better clinical outcomes. It has been found that there were abnormalities in RAR_{α} in RA resistant cell lines (Miller et al. 1993). In addition, it has been observed that prolonged use of RA alone may result in the development of drug resistance (Muindi et al. 1992). For example, a pharmacokinetic study has shown, following a protocol in which four Rhesus monkeys received 50 mg/m^2 RA daily by intravenous bolus injection for 8 consecutive days, that the drug maximum elimination velocity (V_{max}) of the capacity-limited elimination increased approximately 3-fold from day 1 to day 8 (Adamson et al. 1993a). This increase in rate of elimination may be the reason that, after a few months of complete remission under maintenance RA therapy, almost all of the patients suffering from APL relapsed (Degos et al. 1995). Additionally, the absorption of RA is highly variable following oral administration (Adamson et al. 1993b).

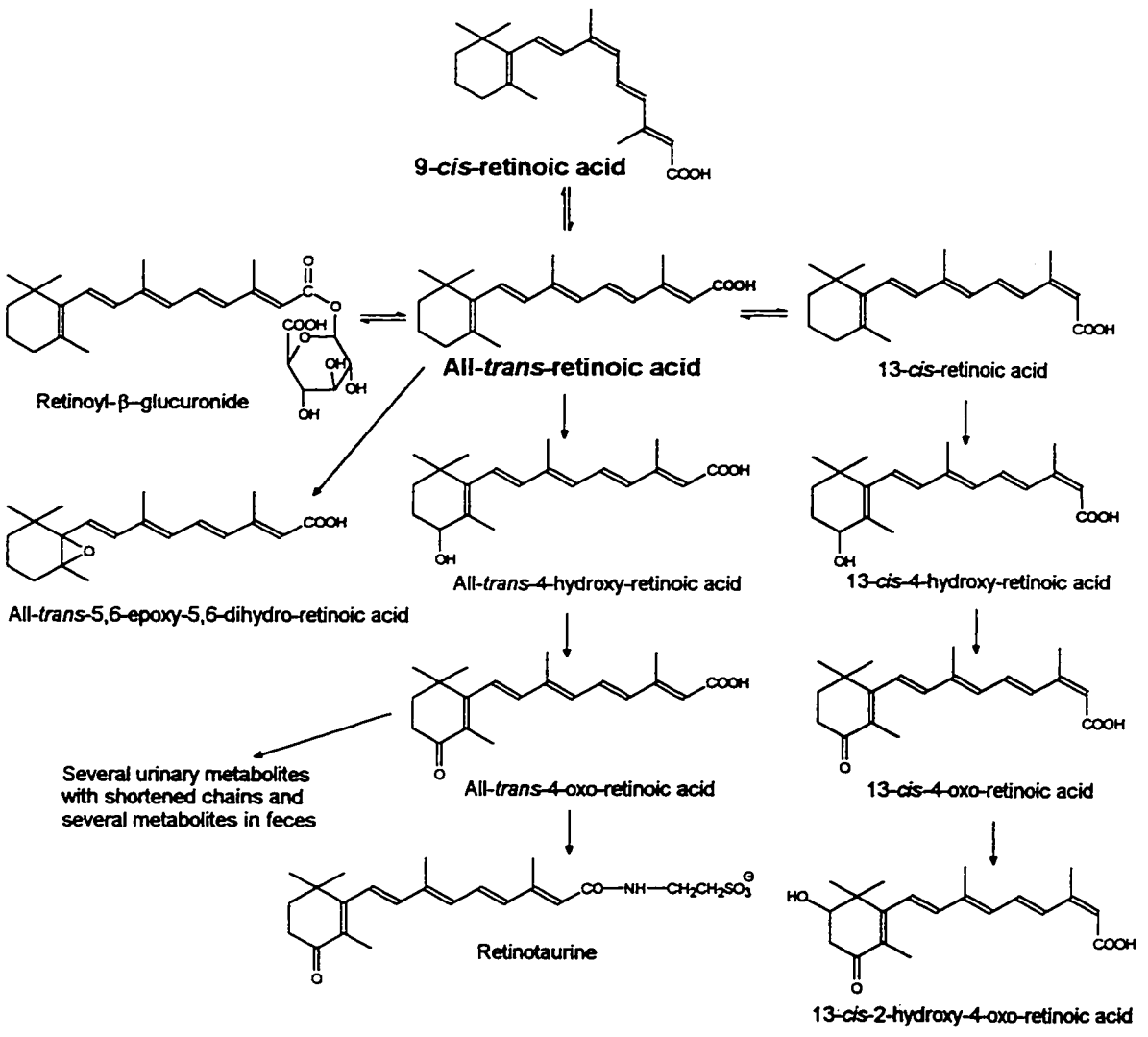


Figure 1-4-3. RA metabolic pathway (Allen et al. 1989; Blaner et al. 1994).

There are two possible explanations for this pharmacokinetic phenomenon, which are both based on the knowledge that RA can transcriptionaly increase CRABP-II and P450 gene expression (Degos et al. 1995). One reason is increased levels of cellular RA-binding protein (Cornic et al. 1992; Delva et al. 1993), which is associated with alterations of RA metabolism (Fiorella et al. 1991; Boylan et al. 1992). The second reason is increased RA metabolism by two metabolic pathways: cytochrome P450-mediated (Van Wauwe et al. 1988) and glucuronide conjugate metabolism (Kraft et al. 1991). It has been reported that no response to RA was observed in patients who had already received RA and relapsed shortly after its discontinuation (Delva et al. 1993; Warrell et al. 1994b).

On the other hand, patients treated with RA often exhibit side effects related to vitamin A toxicity (Silverman et al. 1987; Levin 1995) that can be dose-limiting (Warrell et al. 1991). In addition to common retinoid side effects, approximately 25-30% of APL patients who received RA treatment have suffered a unique combination of adverse reactions called retinoic acid syndrome which, once established in a patient, has proved very difficult to manage. Several patients have developed progressive hypoxemia and died from multiple organ failure (Frankel et al. 1992).

1-5. Butyric Acid (BA)

BA is a natural short chain fatty acid in the body. Its structure is shown in Figure 1-5-1. BA is present in some foods, such as butter where it constitutes up to 5% (w/w), and BA is produced in the mammalian digestive tract by microbial fermentation (Bergman 1990).

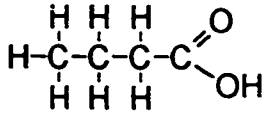


Figure 1-5-1. Chemical structure of butyric acid (BA).

Since the 1970's, when BA was found to induce malignant cell differentiation and inhibit neoplastic cell proliferation (Leder et al. 1975; Prasad et al. 1976; Leavitt et al. 1978), BA has received widespread attention as a potential differentiation inducer for cancer chemotherapy. BA induces *in vitro* cytodifferentiation and growth inhibition in a broad spectrum of neoplastic cells, including human leukemia (Leder et al. 1975; Novogrodsky et al. 1983; Breitman et al. 1990), human neuroblastoma (Prasad et al. 1976), human breast cancer (Guilbaud et al. 1990), human pancreatic cancer (McIntyre et al. 1984), human colon cancer (Augeron et al. 1984; Gum et al. 1987; Gamet et al. 1992), and human ovarian cancer (Langdon et al. 1988; Brooks et al. 1991). The effects (ED_{50}) of BA on malignant cells *in vitro* have been demonstrated to fall into the "mM" range (Breitman et al. 1994). In addition, it has been reported that a low fibre diet increases the risk of colorectal cancer. This may be directly connected with BA produced by bacterial fermentation of dietary fibre in large intestine (Williams et al. 1996).

1-5-1. Mechanisms of Butyric Acid (BA) Anticancer Activity

Early studies have shown that BA strongly increases the acetylation of histones H3 and H4 by inhibiting histone deacetylase (Riggs et al. 1977; Candido et al. 1978; Sealy et al. 1978). Histones are a group of heterogeneous basic proteins in cells, which complex with DNA via their negative charged backbone chain to generate highly compact structures of nucleoprotein called chromatin. It is commonly acknowledged that histone hyperacetylation may cause relaxation of the chromatin structure to make DNA accessible to a variety of molecules (Lee et al. 1993) and consequently regulate gene expression. Stimulation of DNA transcription was observed with an increase in histone acetylation (Kruh 1982). It has also been reported that histone acetylation correlated with butyrate-induced cell apoptosis (McBain et al. 1997). BA induces the expression of *c-jun* and *c-fos* (Toscani et al. 1988), perturbs the cell cycle (Heerdt et al. 1997), increases the level of cellular enzymes, such as alkaline phosphatase (Lupton 1995) and TGase (Kruh et al. 1991), and suppresses the expression of *c-myc* (Rabizadeh et al. 1993). BA can cause permanent down-regulation of *c-myc* in Burkitt's lymphoma cells (Rottleb et al. 1995). Cell differentiation was accompanied by an early reduction of *c-myc* expression (Westin et

al. 1982; Lachman et al. 1985). It is known that, in resting cells or in terminally differentiated cells, *c-myc* expression is shut off. BA also down regulates *bcl-2* gene expression in MCF-7 human breast cancer cells, which is involved in the resistance of the cell to apoptosis (Mandal et al. 1996).

On the basis of the fatty acid structure of BA and the relatively high concentration needed for inducing cell differentiation and growth inhibition, BA may be incorporated into cell membrane lipids and alter the membrane biology (Rottleb et al. 1995). Another possible mechanism suggested is that BA could induce the synthesis of some hormone-like compounds, such as RA, that could induce cell differentiation and growth inhibition (Kruh et al. 1991).

The comprehensive outcome of BA treatment is not only induction of cell differentiation but also induction of cell apoptosis (Heerdt et al. 1994; Mandal et al. 1996; McBain et al. 1996; Aukema et al. 1997; Heerdt et al. 1997; Kurita-Ochiai et al. 1997; Mandal et al. 1997; McBain et al. 1997; Zimra et al. 1997).

1-5-2. Clinical Limitations of BA in Cancer Chemotherapy

Clinical trials in cancer patients, using BA as the sole therapeutic agent, have been reported to be unsuccessful (Miller et al. 1987), although no side effects were observed. Pharmacokinetic studies showed, during infusion of 500 mg/kg/day of sodium butyrate (NaBu) continuously over 10 days, that the plasma concentration of BA increased about 6-fold over the endogenous level (39-59 μM), which is much lower than the "mM" range generally required for *in vitro* efficacy (Miller et al. 1987). The plasma half-life was only 6 min after the end of infusion, mainly due to rapid metabolism (see Figure 1-5-2) and to a lesser extent excretion.

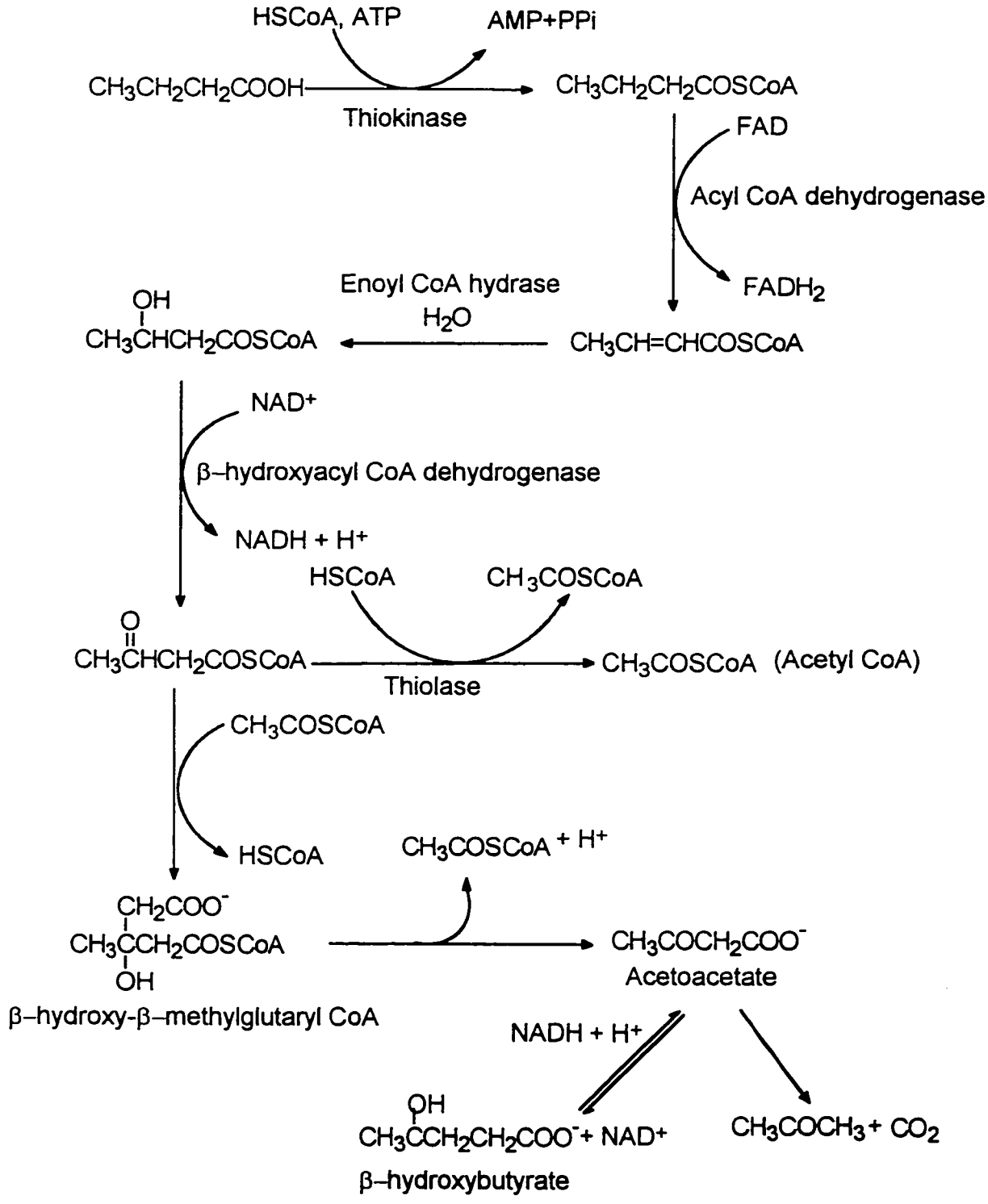


Figure 1-5-2. BA metabolic pathway (Dagley et al. 1970; Rawn 1989).

1-6. Development of Novel Cytotoxic-differentiation Double-barreled Prodrugs for Cancer Chemotherapy Based on Our Current Understanding of Neoplasm and the Chemical Properties and Pharmacological Actions of Anticancer Agents

The term prodrug refers to a pharmacologically inactive compound that can be converted to an active drug by a metabolic biotransformation.

1-6-1. Rationale for Chemical Modification 1: Ester Prodrugs

Using esters as prodrugs to improve the pharmacokinetic and pharmacodynamic properties of the drugs containing a carboxyl or hydroxyl group stems primarily from the fact that the human body is rich in esterases which are capable of hydrolyzing ester prodrugs (Bundgaard 1991) and releasing the prodrug moiety. The distribution of esterases is ubiquitous, so metabolic regeneration of the drug is facile. The active site on the esterase molecule is believed to contain at least two important sites, a basic moiety or proton acceptor, such as a histidine residue, and a binding site, such as a serine residue (Saier 1987). The mechanism by which esters undergo esterase-catalyzed cleavage is illustrated in Figure 1-6.

By appropriate esterification of molecules containing hydroxyl or carboxyl groups, it is possible to prepare ester derivatives with, theoretically, any degree of hydrophilicity or lipophilicity. Therefore it may be possible to control the disposition of the drug *in vivo*. Finally, ester prodrugs having varied stability toward esterases can be designed by appropriate manipulation of electronic and steric factors of the R and/or R' groups in a $R'COOCH_2R$ ester molecule so that the rate of ester cleavage can be controlled (Kawaguchi et al. 1985a; Kawaguchi et al. 1985b; Kawaguchi et al. 1985c; Kawaguchi et al. 1988), which may lead to the desired drug pharmacokinetics.

FDU contains two sugar hydroxyl groups that can be esterified with a carboxylic acid. Esters of FDU, as prodrugs of FDU, have been widely explored (Nishizawa et al. 1965; Kanzawa et al. 1981; Kawaguchi et al. 1988; Yamashita et al. 1988; Nozaki et al. 1990). It has been reported that *O*-butanoyl esters are more effective anticancer agents than FDU following oral administration to mice bearing adenocarcinoma-775 (Nishizawa et al. 1965). Since the cytotoxicity of FDU may depend markedly upon the duration of the

exposure of tumor cells to the drug, greater antitumor activity of the esters may be due in part to their slower rate of FDU regeneration by esterases (Kawaguchi et al. 1985a), and therefore longer duration of the exposure of cancer cells to the drug.

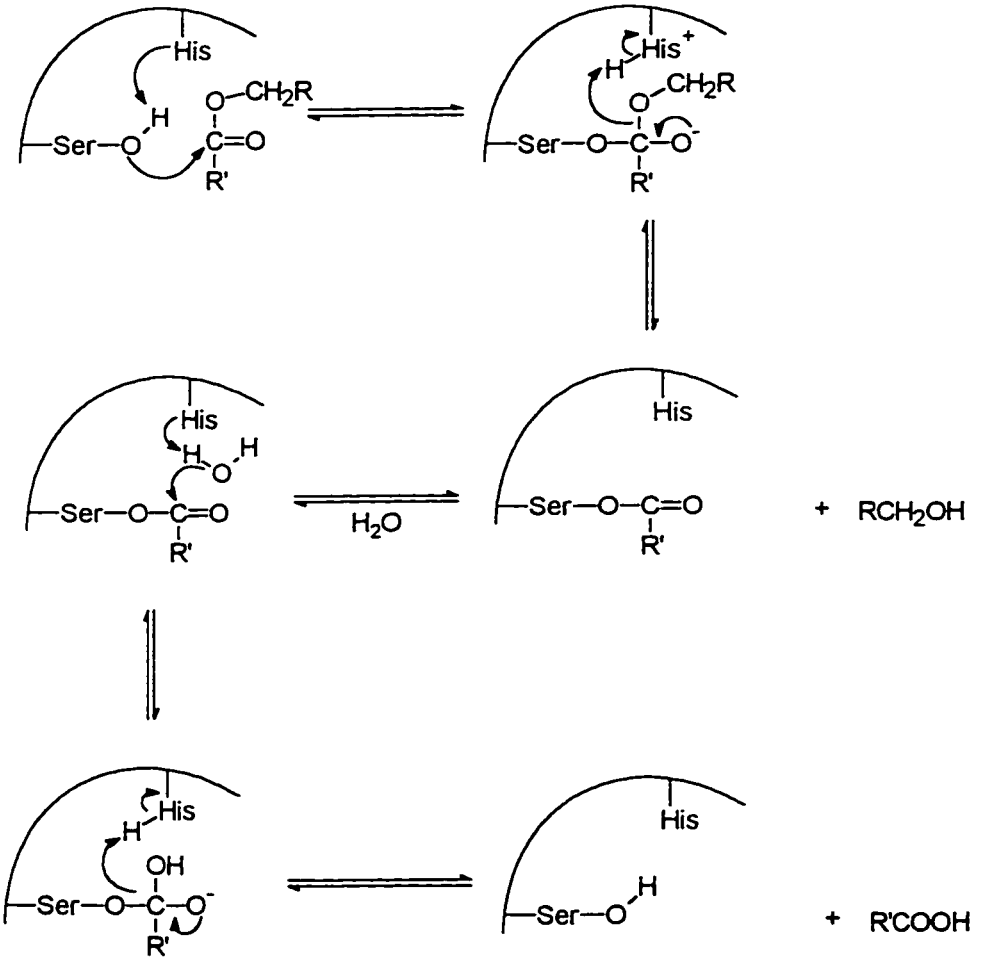


Figure 1-6. Esterase-catalyzed cleavage of ester prodrugs (Walsh 1979; Saier 1987). His: histidine residue; Ser: serine residue; $R'COOCH_2R$: ester prodrug.

Both RA and BA contain carboxyl groups. A variety of ester prodrugs for RA or BA have been recently developed, which may improve their pharmacokinetics and may be more effective inducers of cell differentiation and/or apoptosis in a broad spectrum of neoplasms (Allen et al. 1989; Pouillart et al. 1991; Rephaeli et al. 1991; Nudelman et al. 1992; Chen et al. 1994; Curley et al. 1996; Formelli et al. 1996; Manfredini et al. 1997).

1-6-2. Rationale for Chemical Modification 2: Neutral Masked Nucleotide Prodrugs

The monophosphate nucleotide, FdUMP, could be a superior drug to the nucleoside, FDU, because it may directly bind to TS so that it may avoid cleavage of its glycosyl bond by nucleoside phosphorylase and bypass the necessary phosphorylation by pyrimidine kinase. Unfortunately, ionic nucleotides penetrate cell membranes poorly, and are readily dephosphorylated extracellularly. Therefore, the development of lipophilic neutral masked nucleotide prodrugs that can cross the cell membrane and generate the nucleotide intracellularly has been extensively investigated (Mukherjee et al. 1962; Remy et al. 1962; Imai et al. 1981; McGuigan et al. 1993a; McGuigan et al. 1993b; McGuigan et al. 1993c; Farquahar et al. 1994; Fries et al. 1995; Shuto et al. 1995; Abraham et al. 1996) due to their potential advantages. It was therefore of interest to design nucleotide prodrugs possessing a neutral lipophilic 5'-*O*-*bis*(2,2,2-trichloroethyl)phosphoryl group. It was anticipated that the neutral lipophilic nucleotides would readily cross the cell membrane prior to their biotransformation to the corresponding nucleotide.

1-6-3. Rationale for Chemical Modification 3: 2'-Ribo Fluorine Substitution

It has been shown that 2',5-difluoro-2'-deoxyuridine (DFDU), unlike FDU, was resistant to glycosyl bond cleavage by phosphorylases due to the stabilizing effect of the 2'-ribo F-substituent, and interestingly, DFDU provided a tumor to blood ratio of 10:3 at 4 hours in BDF1 mice bearing Lewis lung tumors (Mercer et al. 1987). A fluorine substituent introduced at the 2'-position of FDU was based on the following properties of fluorine (Goldman 1969):

1. The strong polarization of the C-F bond is due to the high electronegativity of fluorine, which is the strongest of all elements and produces a strong inductive effect. F-substituent can substantially alter the reaction behavior of adjacent centers, such as the 6-position of FDU, which is the binding site for thymidylate synthase (TS), and the 1'-position of DFDU, which is the position susceptible to cleavage by nucleoside phosphorylase.

2. The van der Waals radius of fluorine atom is closest to hydrogen (F = 1.35 Å; H = 1.1 Å). The sizes of hydrogen and fluorine are so similar that replacement of hydrogen by fluorine may have no significant steric influence with respect to certain biologic processes.

3. The strong C-F bond (107 kcal/mol) is likely responsible for *in vivo* competitive enzyme inhibition by FDU.

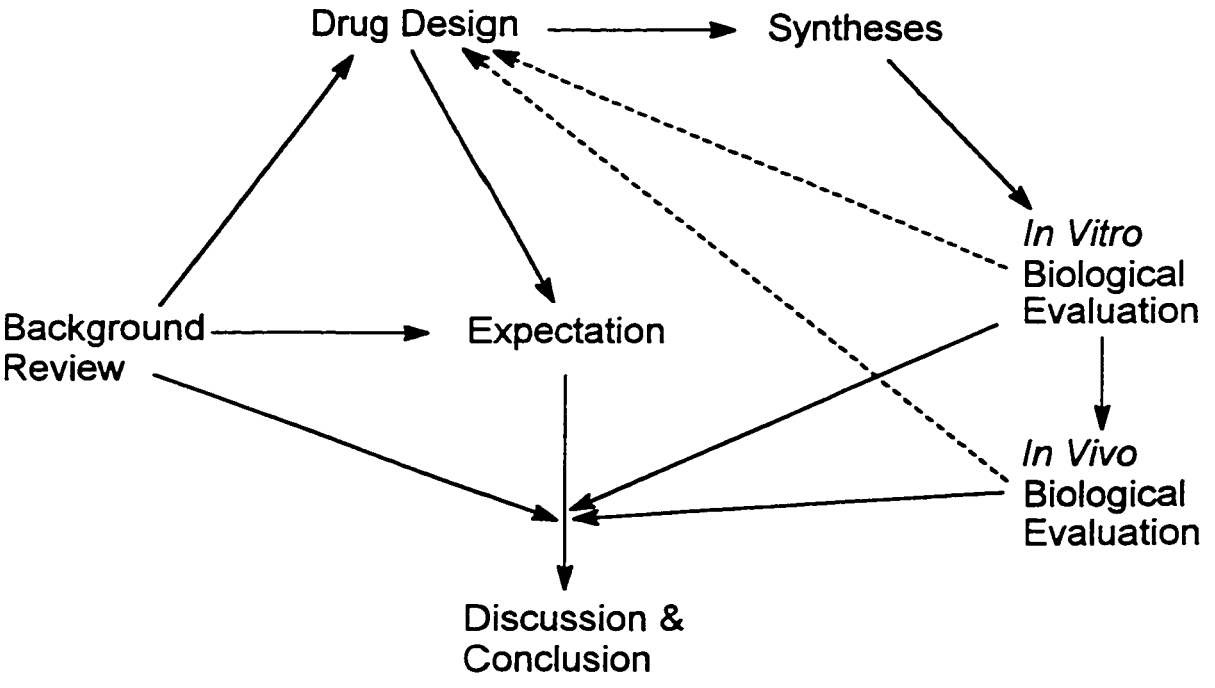
1-6-4. Rationale for Combination of FDU with RA or BA: Cytotoxic-differentiation Double-barreled Prodrugs

One approach which may circumvent the clinical limitations for FDU, RA or BA to some extent is the physical combination of FDU or RA or BA with other agents that give rise to additive or synergistic effect (Breitman et al. 1990b; Smith et al. 1992; Breitman et al. 1994; Dorr et al. 1994; Ashour et al. 1995; Bhatia et al. 1995; Degos et al. 1995; Nagpal et al. 1996). However this approach may not improve drug pharmacokinetics and may not reduce drug toxicity. Moreover, several cell lines have been treated successfully with combined cytotoxic-differentiation therapy. It has been shown that induced differentiation of M1 myeloid leukemic cells by interleukin-6, a differentiation inducer, increased their apoptotic response to 1-β-D-arabinofuranosylcytosine (Lotem et al. 1994). Enhanced cell killing has been observed in human colon carcinoma cells treated sequentially with FU and the differentiation inducer *N*-methylformamide *in vitro* (Zupi et al. 1988; Arancia et al. 1991). It has been reported that the cytotoxicity of FU toward Friend erythroleukemia cells was enhanced by treatment of the cells with the differentiation inducer, hexamethylene bisacetamide (Waxman et al. 1990; Huang et al. 1994). This increased cell death may be due to an increased DNA strand breaks produced

by FU, followed by inhibition of DNA repair by the differentiation inducer. It has also been observed that retinoids increased the therapeutic efficacy of nitrosourea, cyclophosphamide, and FU in mice (Tomita et al. 1982). The treatment of prostatic carcinomas in nude mice by methotrexate was more effective after prior treatment with differentiation inducers (Mickev 1986). There is evidence that synthesis of a Ca^{++}/Mg^{++}-dependent endonuclease, which is involved in apoptosis, is induced (McMahon et al. 1984) and *bcl-2*, an apoptotic resistant gene, expression is rapidly down regulated (Terui et al. 1995) at early stages of cell differentiation. It has also been observed that shortly after induction of cell differentiation, extensive DNA cleavage, a characteristic of apoptosis, occurs (Farzaneh et al. 1982; Gunji et al. 1992). This evidence suggests that differentiated cells may be more sensitive to induction of apoptosis when triggered with anticancer drugs. It is, therefore, of interest to design double-barreled ester prodrugs by chemical combination of anticancer nucleoside analogs with a differentiation inducer such as RA or BA.

2. PROJECT

This thesis research project, which involves the design, synthesis and biological evaluation of cytotoxic-differentiation double-barreled prodrug esters of FDU and DFDU, was based on the foregoing background review. The overall project process is described in Scheme 1 and the types of chemical modifications are shown in Figure 2-1.



Scheme 2-1. Project outline for the development of cytotoxic-differentiation double-barreled anticancer prodrugs.

Since malignant cell subpopulations in a neoplasm are markedly heterogeneous (Trope 1982) and it is known that cytotoxic-differentiation therapy may be synergistic and more effective to induce cell apoptosis (Mickev 1986; Lotem et al. 1994), a combination

of two drugs acting by different mechanisms, such as either FDU with RA or BA, could be expected to reduce the incidence of drug resistance and increase cure probability.

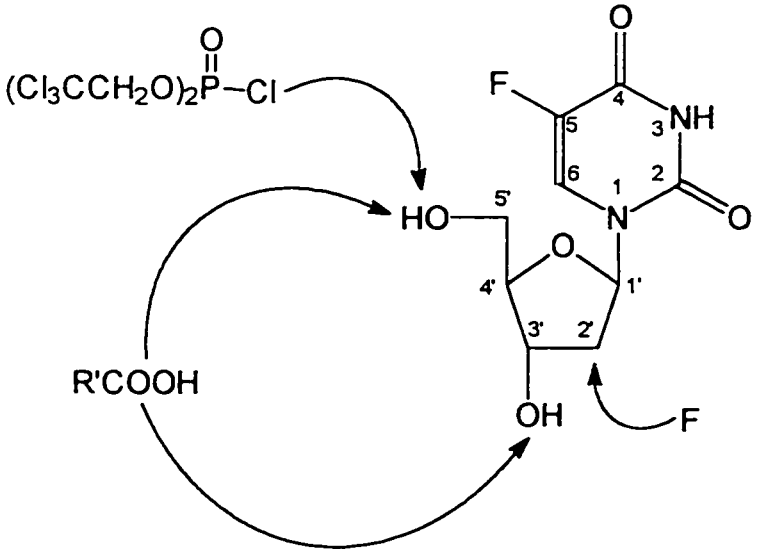


Figure 2-1. Chemical modifications of FDU.
R' is butanoyl or retinoyl moiety.

Due to improved pharmacokinetics, these prodrugs were anticipated to provide superior efficacy compared to the use of a physical combination or concurrent dosing of the individual drugs. It was expected that *O*-retinoyl and *O*-butanoyl ester prodrugs would act as depots to release the active drugs, which could result in an improved drug concentration in plasma, subsequently to enhance the drug concentration at target tissue (intracellular) compared to either single drug. In addition, these lipophilic esters of FDU would improve the bioavailability following oral administration.

The masked neutral 5'-monophosphats of FDU formed by chemical conjugation of FDU with *bis*(2,2,2-trichloroethyl)phosphorolchloridate were designed as cell membrane-permeable prodrugs, which were anticipated to be a source of FdUMP after either

intracellular chemical or enzymatic hydrolysis. Furthermore, the reliance on bioactivation by pyrimidine kinase and rapid catabolism (phosphorolysis) of FDU might be bypassed (Uchida et al. 1990). It was hoped that, by enhancing resistance of FDU to phosphorolytic cleavage by nonspecific phosphohydrolyases (Farquahar et al. 1994) via fluorine substitution at the 2'-position of FDU, the anticancer efficiency would be increased.

The *in vitro* biological evaluation of these synthetic double-barreled prodrugs included:

1. Cell growth inhibition screens against a panel of nine human tumor types that encompassed 60 cell lines (eight cell lines of breast cancer, six cell lines of CNS cancer, seven cell lines of colon cancer, six cell lines of leukemia, nine cell lines of lung cancer, eight cell lines of melanoma, six cell lines of ovarian cancer, two cell lines of prostate cancer, and eight cell lines of renal cancer). This cytotoxic screen was performed by the Developmental Therapeutics Program, Division of Cancer Treatment, United States National Cancer Institute (NCI).
2. Studies of inducing cell differentiation and apoptosis in HL-60 human acute promyelocytic leukemia (APL) cells by selected compounds that showed the most potent anticancer activities in the cell growth inhibition screens.
3. Effects of RA and sodium butanoate (NaBu) on growth inhibition of EMT-6 murine mammary cancer cells, which served as a basis for the selection of EMT-6 tumor as an *in vivo* model.

The *in vivo* biological evaluations of double-barreled prodrugs synthesized in this project included:

1. Dose escalation and toxicity studies of 3'-*O*-retinoyl-5-fluoro-2'-deoxyuridine and 3'-*O*-butanoyl-5'-*O*-*bis*(2,2,2-trichloroethyl)phosphoryl-5-fluoro-2'-deoxyuridine in healthy BDF1 mice.
2. Responses of EMT-6 tumor *in vivo* to 3'-*O*-retinoyl-5-fluoro-2'-deoxyuridine and 3'-*O*-butanoyl-5'-*O*-*bis*(2,2,2-trichloroethyl)phosphoryl-5-fluoro-2'-deoxyuridine compared to RA, NaBu and FDU alone or in combination in Balb/c mice.
3. Pharmacokinetic studies of 3'-*O*-retinoyl-5-fluoro-2'-deoxyuridine, the most potent cytotoxic-differentiation double-barreled prodrug synthesized in this study.

3. RESULTS AND DISCUSSION

3-1. Chemical Syntheses

The compounds synthesized in this project are listed in Table 3-1-1.

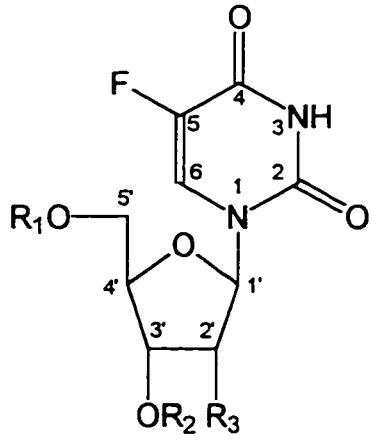
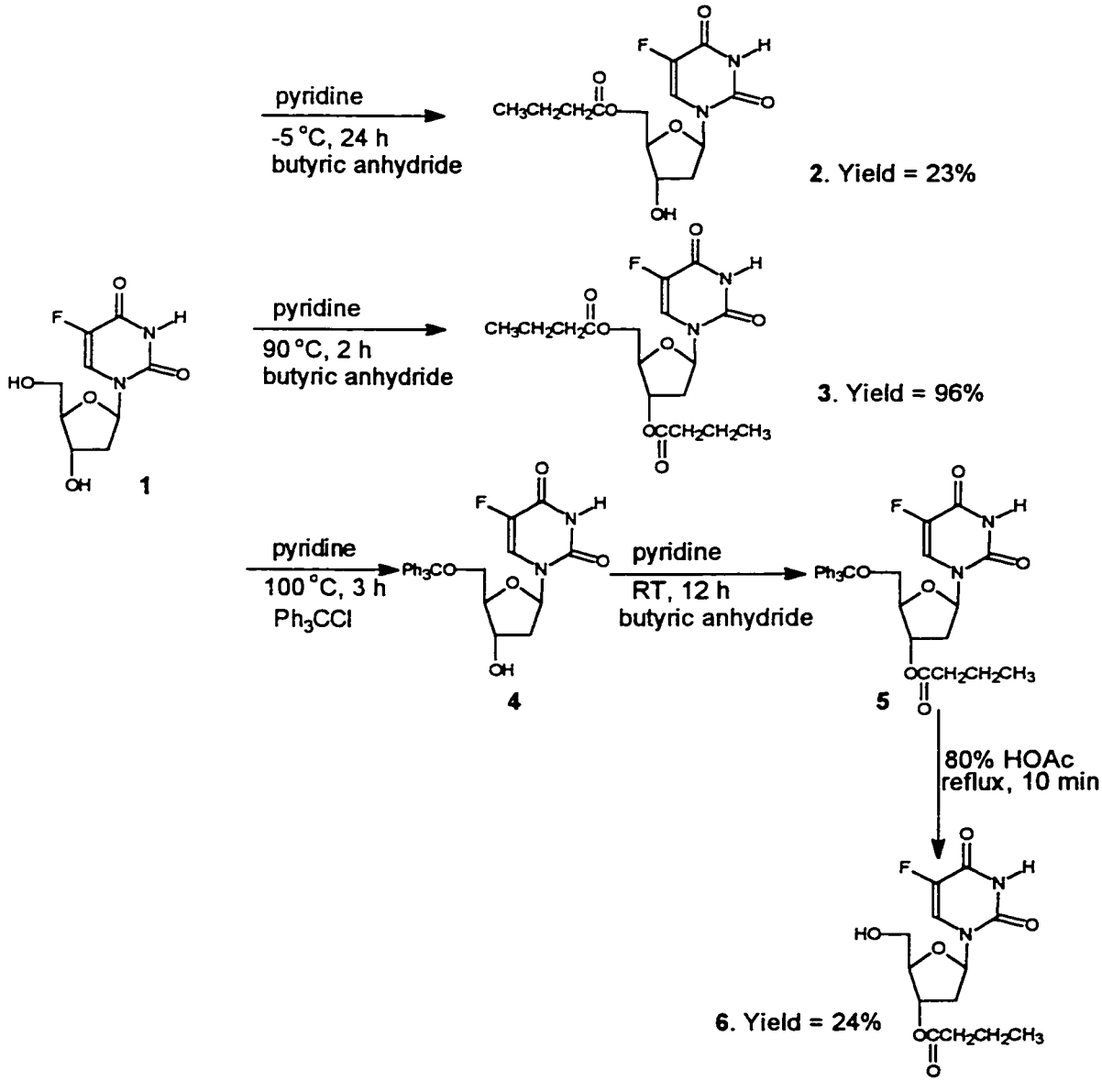


Table 3-1-1. Fluorinated pyrimidine nucleoside derivatives synthesized in this study

No	Compound	R_1	R_2	R_3
2	5'-*O*-BuFDU	$-CO(CH_2)_2CH_3$	H	H
3	3',5'-di-*O*-BuFDU	$-CO(CH_2)_2CH_3$	$-CO(CH_2)_2CH_3$	H
6	3'-*O*-BuFDU	H	$-CO(CH_2)_2CH_3$	H
9	3'-*O*-BuDFDU	H	$-CO(CH_2)_2CH_3$	F
10	5'-*O*-BuDFDU	$-CO(CH_2)_2CH_3$	H	F
11	3',5'-di-*O*-BuDFDU	$-CO(CH_2)_2CH_3$	$-CO(CH_2)_2CH_3$	F
17	3'-*O*-RFDU	H	$-C_{20}H_{27}O$	H
18	3',5'-di-*O*-RFDU	$-C_{20}H_{27}O$	$-C_{20}H_{27}O$	H
19	5'-PFDU	$(Cl_3C-CH_2O)_2-P(=O)-$	H	H
20	3'-*O*-Bu-5'-PFDU	$(Cl_3C-CH_2O)_2-P(=O)-$	$-CO(CH_2)_2CH_3$	H

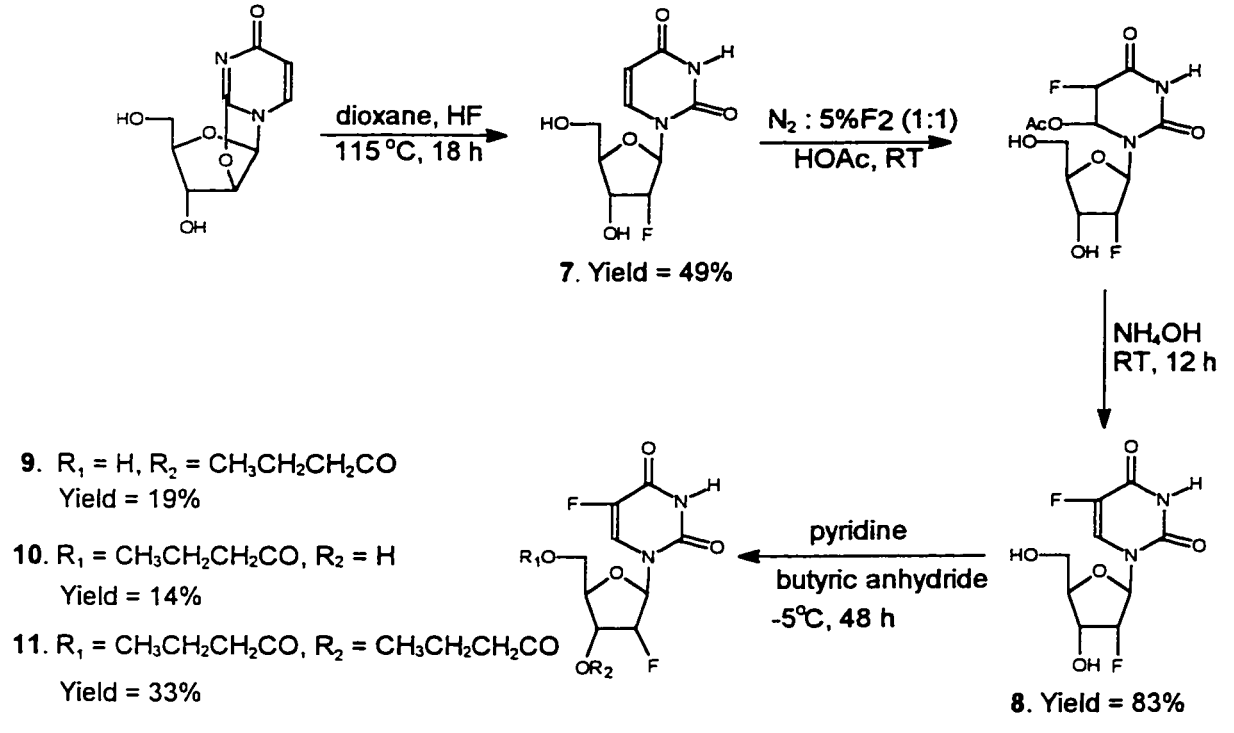
5'-*O*-Butanoyl-5-fluoro-2'-deoxyuridine (**2**) and 3',5'-di-*O*-butanoyl-5-fluoro-2'-deoxyuridine (**3**) were synthesized based on the literature method (Nishizawa et al. 1965). The 3'-*O*-butanoyl-5-fluoro-2'-deoxyurine (**6**) was prepared by a modified one pot reaction (Scheme 3-1-1).



Scheme 3-1-1. Syntheses of *O*-butanoyl esters (**2**, **3**, **6**) of FDU.

2'-Fluoro-2'-deoxyuridine (7) and 2',5-difluoro-2'-deoxyuridine (**8**, DFDU) were synthesized using the procedures described in the literature (Codington et al. 1964;

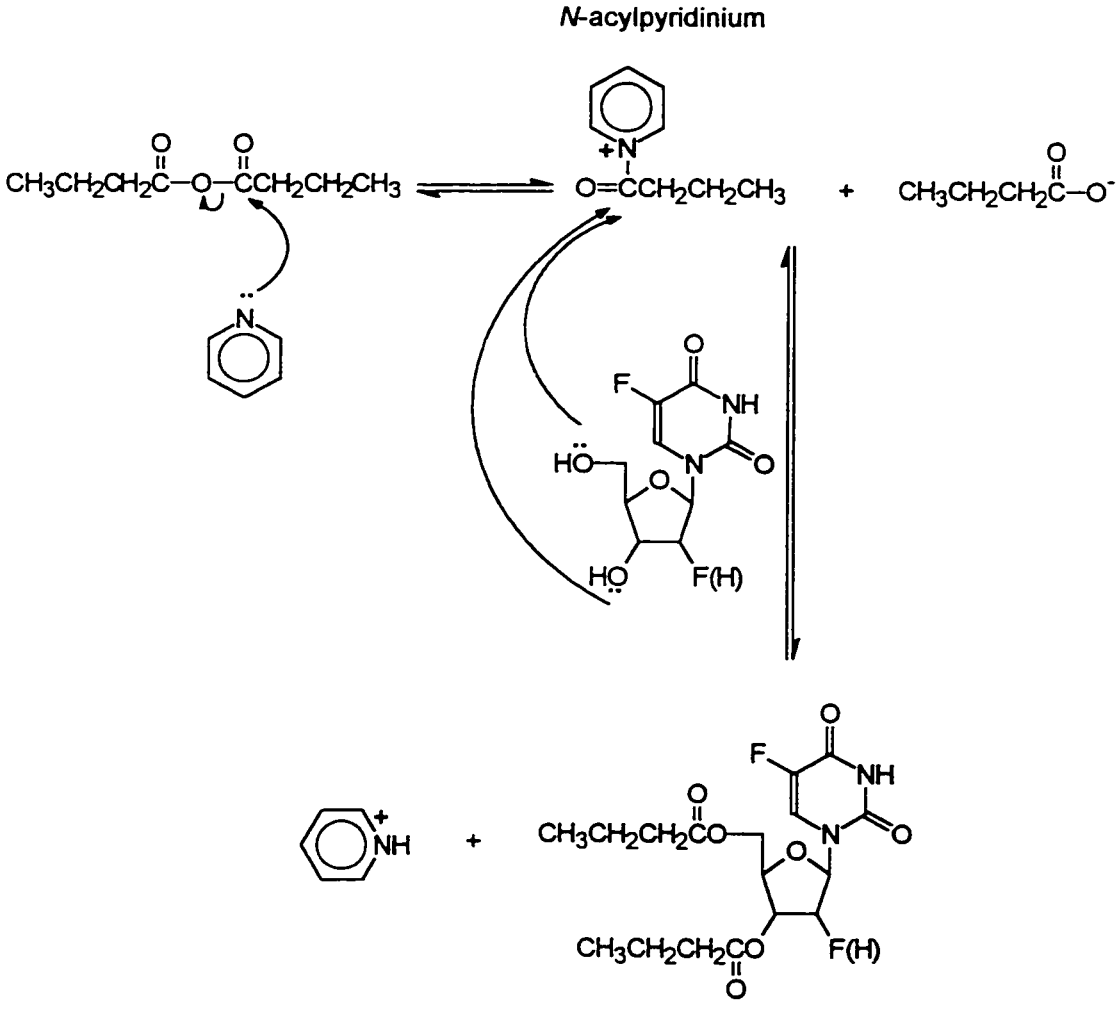
Abrams et al. 1985; Mercer et al. 1987). Reaction of DFDU (**8**) with butyric anhydride in pyridine at -5 °C, and separation of the products by silica gel column chromatography afforded 3'-*O*-butanoyl-2',5-difluoro-2'-deoxyuridine (**9**, 19%), 5'-*O*-butanoyi-2',5-difluoro-2'-deoxyuridine (**10**, 14%) and 3',5'-di-*O*-butanoyl-2',5-difluoro-2'-deoxyuridine (**11**, 33%), respectively (Scheme 3-1-2-1).



Scheme 3-1-2-1. Syntheses of *O*-butanoyl esters (**9**, **10**, **11**) of DFDU.

Compared to FDU (**1**), DFDU (**8**) was more readily esterified at the 3'-position. Therefore, increased steric hindrance at the 3'-position must be offset by the effects of 2'-F substituent. Since the mechanism of this reaction is nucleophilic acylation as shown in

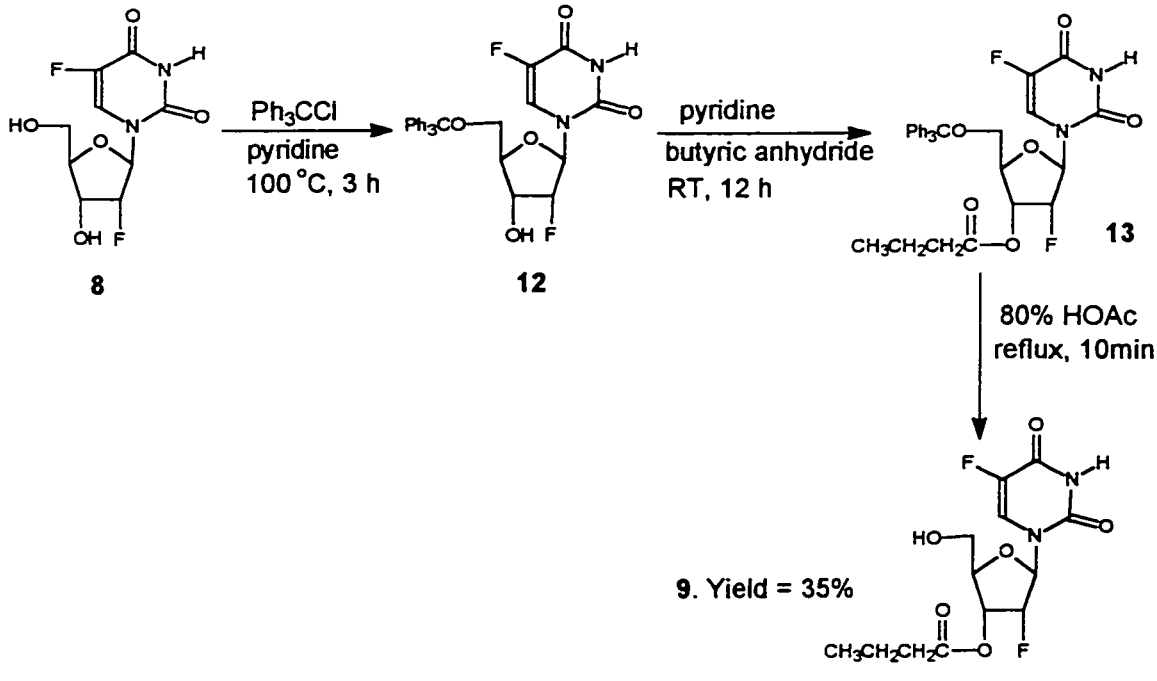
Scheme 3-1-2-2, the assumption is that the electron density at 3'-OH of DFDU might be increased following 2'-F substitution. However, this may not be the case because the relative electron density of DFDU at 3'-OH is even lower than that of FDU (-0.317 versus -0.327) based on a theoretical AM1 calculation (HyperChem, Version 4, Hypercube, Inc.), which is probably due to the inductive effect of 2'-F.



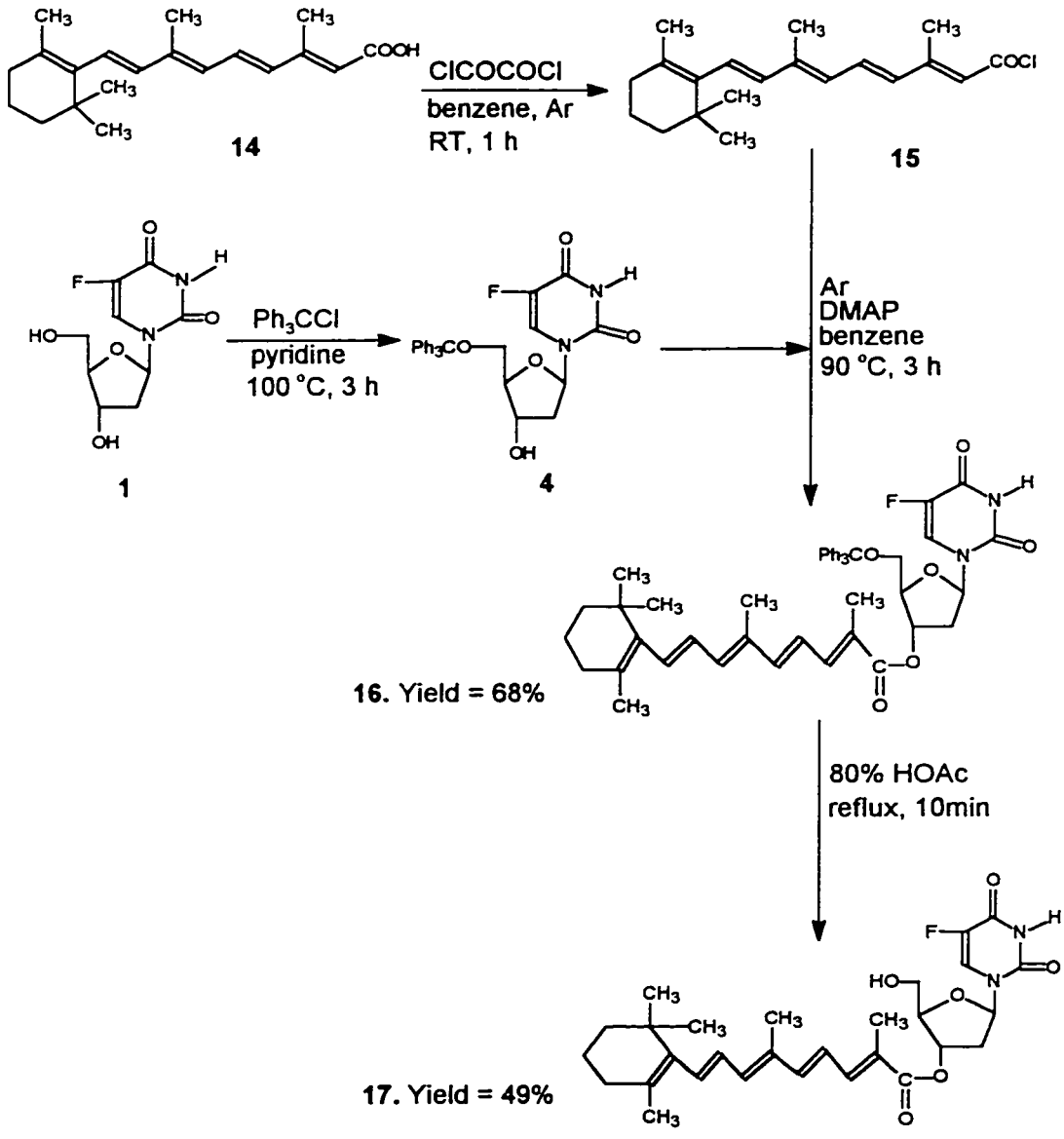
Scheme 3-1-2-2. Mechanism of nucleophilic acylation catalyzed by pyridine.

On the other hand, the 2'-F substituent could conceivably interact with the acylating agent, *N*-acylpyridinium, to position the acylating agent closer to the 3'-OH moiety, for fluorine has unshared pair of *p* electrons with high electron density. Consequently, the extent of esterification was quite similar at the 3'- and 5'-positions of DFDU (**8**) and three esters (**9**, 19%), (**10**, 14%) and (**11**, 33%) were obtained in a single reaction even at low temperature (-5 °C). In contrast, reaction of FDU with butyric anhydride/pyridine at -5 °C afforded mainly 5'-*O*-butanoyl ester (**2**, 23%).

Alternatively, 3'-*O*-butanoyl-2',5-difluoro-2'-deoxyuridine (**9**) was also synthesized in 35% yield by elaboration of DFDU (**8**) to the corresponding 5'-*O*-trityl derivative (**12**), followed by reaction with butyric anhydride, and then detritylation of (**13**) using 80% (v/v) acetic acid as illustrated in Scheme 3-1-3.

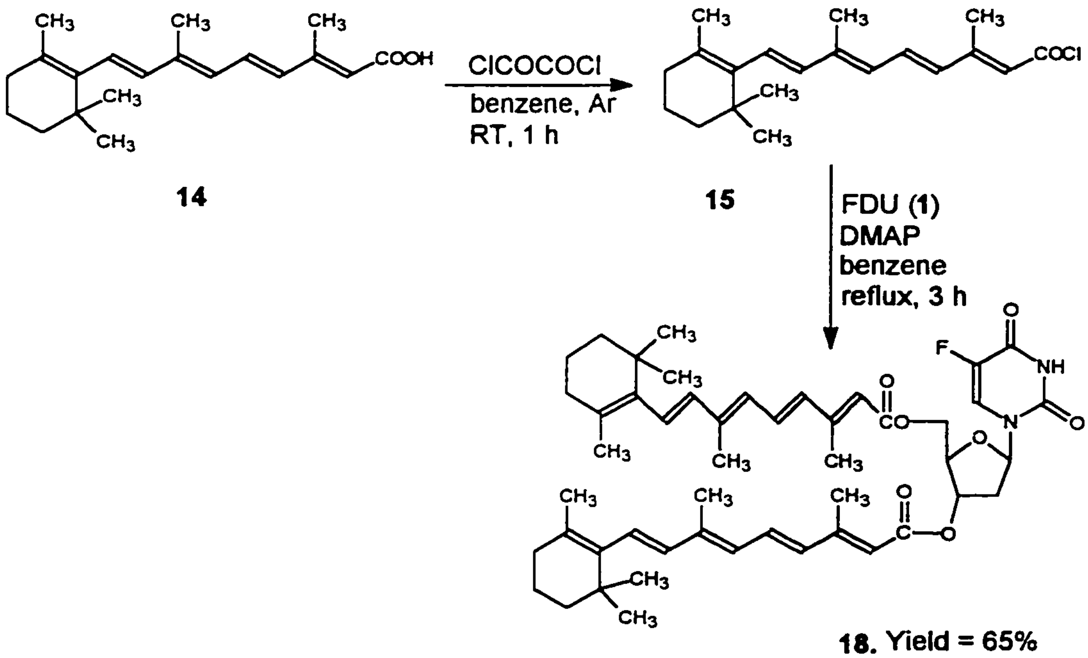


Scheme 3-1-3. Synthesis of 3'-*O*-butanoyl-2',5-difluoro-2'-deoxyuridine (**9**).



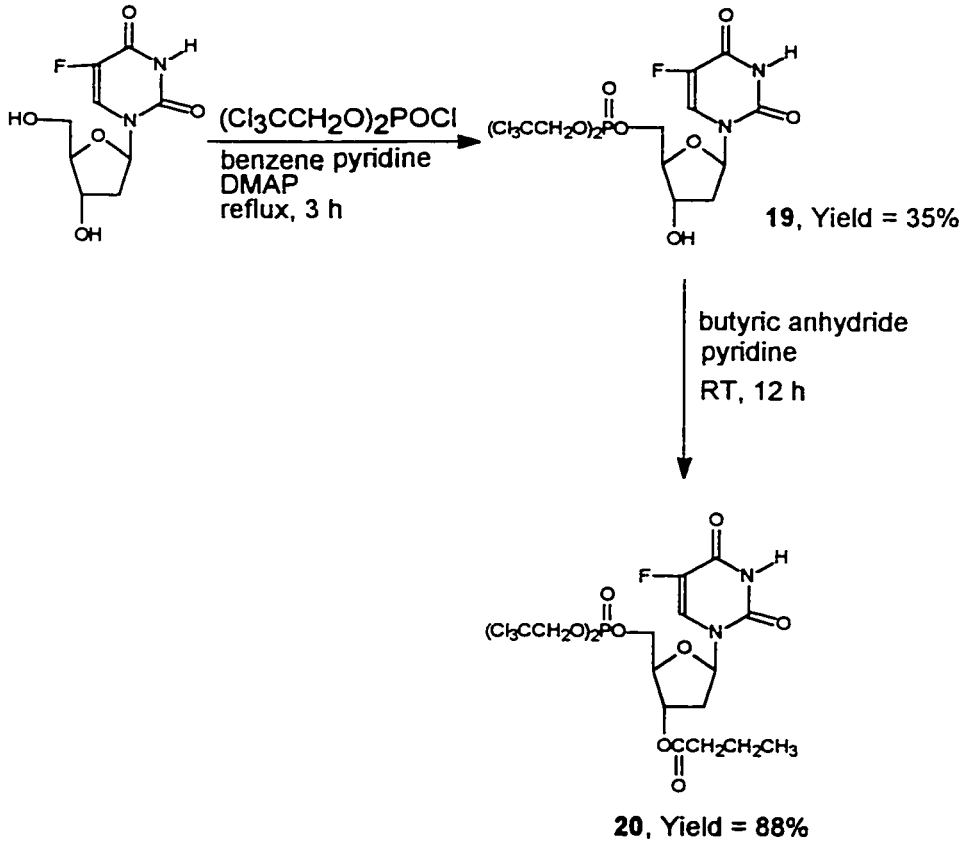
Scheme 3-1-4. Synthesis of 3'-*O*-retinoyl-5-fluoro-2'-deoxyuridine (**17**).

Reaction of all-*trans*-retinoic acid (**14**) with oxalyl chloride in benzene yielded retinoyl chloride (**15**), which was condensed with 5'-*O*-trityl-5-fluoro-2'-deoxyuridine (**4**) using 4-dimethylaminopyridine (DMAP) as a catalyst to yield 3'-*O*-retinoyl-5'-*O*-trityl-5-fluoro-2'-deoxyuridine (**16**, 68%). Detritylation of (**16**) using acetic acid (80% v/v) afforded 3'-*O*-retinoyl-5-fluoro-2'-deoxyuridine (**17**, 49%) as shown in Scheme 3-1-4. Similarly, condensation of retinoyl chloride (**15**) with FDU gave 3',5'-di-*O*-retinoyl-5-fluoro-2'-deoxyuridine (**18**, 65%) (Scheme 3-1-5).



Scheme 3-1-5. Synthesis of 3',5'-di-*O*-retinoyl-5'-fluoro-2'-deoxyuridine (**18**).

Reaction of FDU with *bis*(2,2,2-trichloroethyl)phosphorochloridate using DMAP as catalyst afforded 5'-*O*-*bis*(2,2,2-trichloroethyl)phosphoryl-5-fluoro-2'-deoxyuridine (**19**, 35%). Subsequent reaction of (**19**) with butyric anhydride/pyridine yielded 3'-*O*-butanoyl-5'-*O*-*bis*(2,2,2-trichloroethyl)phosphoryl-5-fluoro-2'-deoxyuridine (**20**, 88%) (see Scheme 3-1-6).



Scheme 3-1-6. Syntheses of masked neutral monophosphate nucleotide (**19**) and its 3'-*O*-butanoyl derivative (**20**).

3-2. *In Vitro* Anticancer Screen

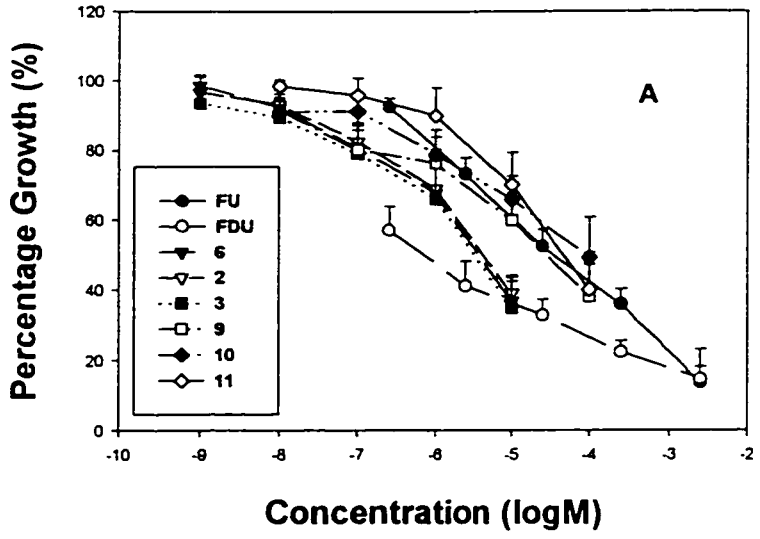
The butanoate, retinoate and *bis*(2,2,2-trichloroethyl)phosphate esters of FDU and DFDU together with FU and FDU were evaluated *in vitro* against a diverse panel of 60 human tumor cell lines in culture derived from nine cancer types (breast cancer, CNS cancer, colon cancer, leukemia, non-small cell lung cancer, melanoma, ovarian cancer, prostate cancer, and renal cancer) (Monks et al. 1991). The dose-response curves of each compound for each cell line were measured at five drug concentrations. This screen test was repeated at least twice at different times for each cell line and each compound except for FU, 3'-*O*-BuDFDU (**9**) and 5'-*O*-BuDFDU (**10**), which were screened only once. Every datum for the exposure of each cell line to each compound for each concentration was calculated as follows:

$$\text{If } (OD_{test} - OD_{tzero}) \geq 0, \text{ then } PG = 100 \times (OD_{test} - OD_{tzero}) / (OD_{ctrl} - OD_{tzero}).$$

$$\text{If } (OD_{test} - OD_{tzero}) < 0, \text{ then } PG = 100 \times (OD_{test} - OD_{tzero}) / OD_{tzero}.$$

Where OD_{tzero} = the average optical density measured just before exposure of the cells to the drugs; OD_{test} = the average optical density measured after 48 h exposure of the cells to the drugs; OD_{ctrl} = the average optical density measured after 48 h without exposure of the cells to the drug; PG = percentage growth.

Since cancer cell lines, even in one major cancer type, may not behave similarly, responses of certain cell lines to the drugs may not be the same. However all the PG data were pooled, averaged and re-graphed to prepare dose-response curves for each compound according to the nine major cancer types for convenience to examine and compare the individual compound anticancer activity against the major cancer types. The averaged dose-response curves for each compound listed in Table 3 together with the reference drugs FDU and FU against the nine major cancer types including 60 cell sublines are shown in Figures 3-2-1 to 3-2-9, in which error bars represent the standard errors of the mean.



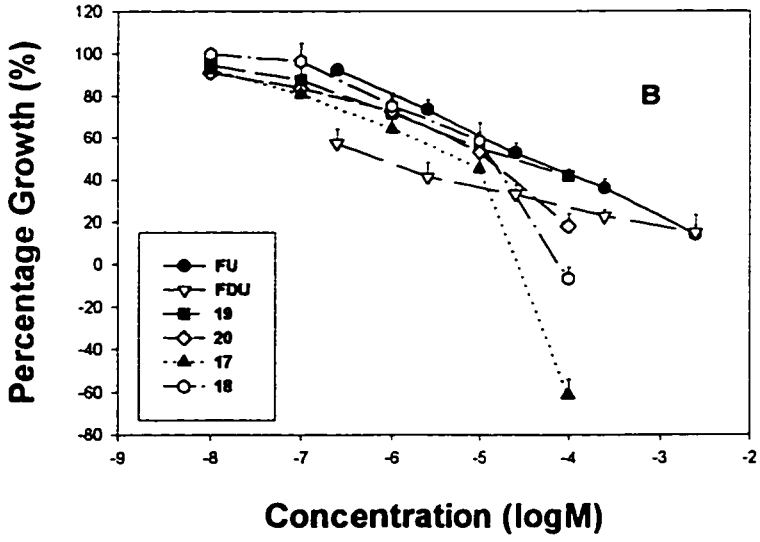
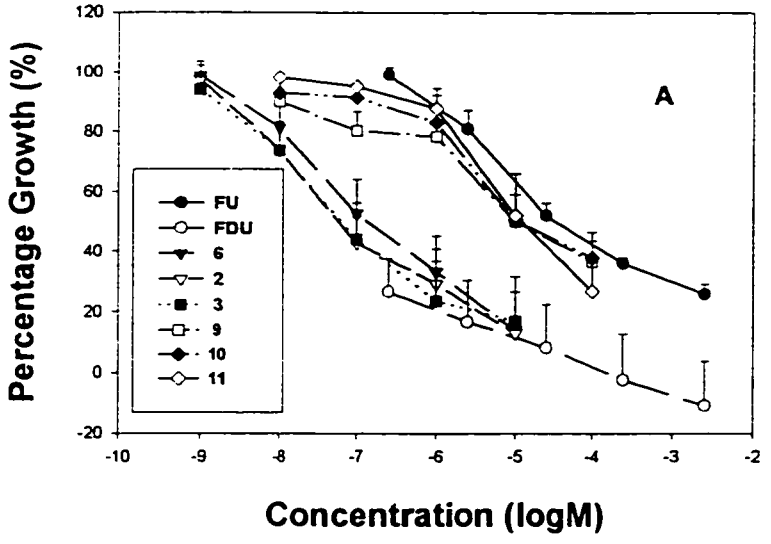


Figure 3-2-1 (**A**) and (**B**). Average growth inhibition of eight human breast cancer cell lines (MCF7; MCF7/ADR-RES; MDA-MB-231/ATCC; HS 578T; MDA-MB-435; MDA-N; BT-549; T-47D).



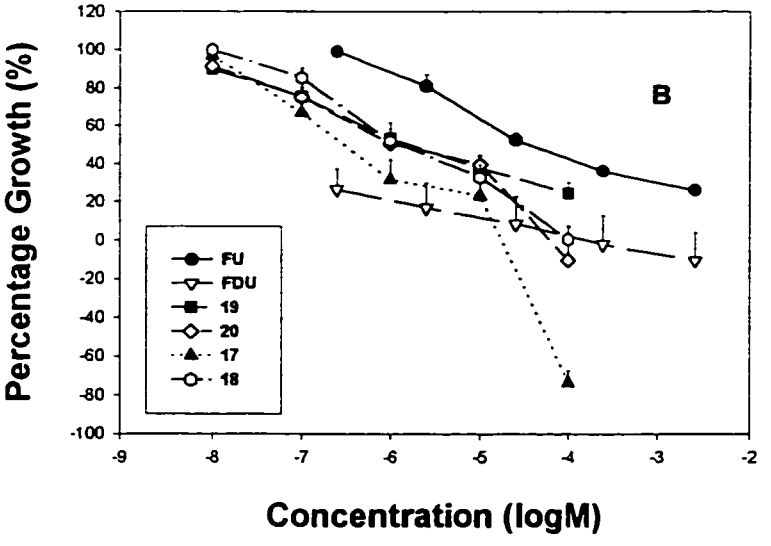
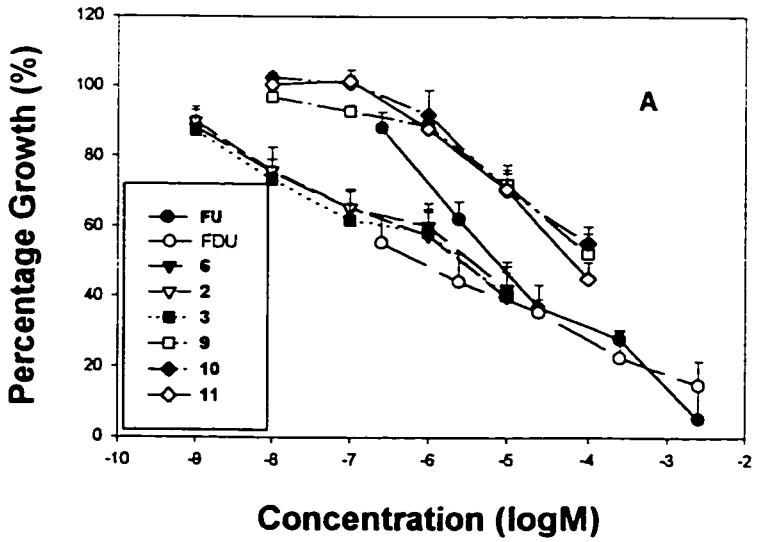


Figure 2-3-2 (**A**) and (**B**). Average growth inhibition of six human CNS cancer cell lines (SF-268; SF-295; SF-593; SNB-19; SNB-75; U251).



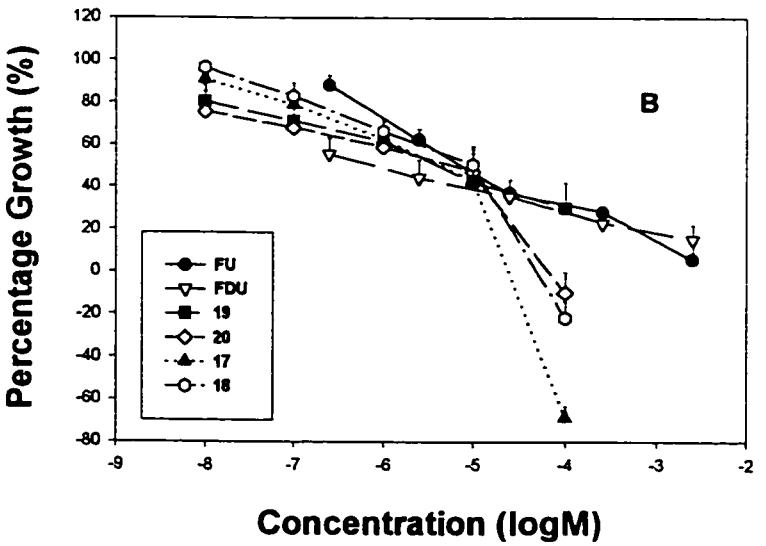
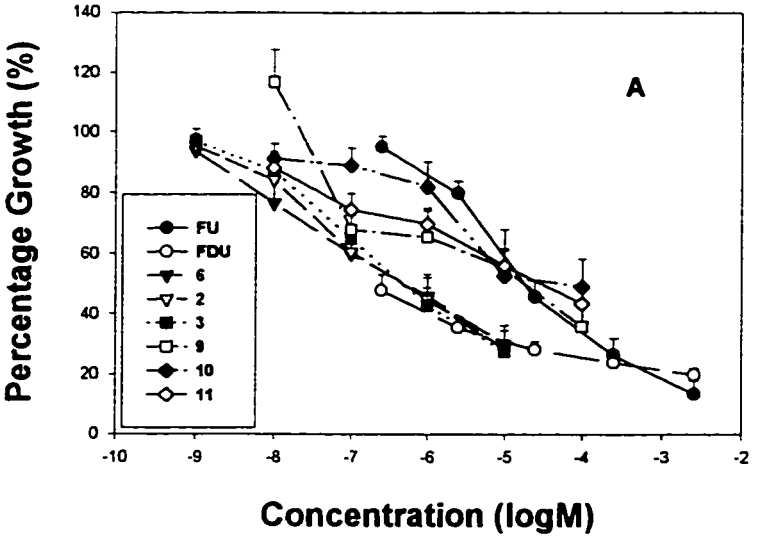


Figure 3-2-3 (**A**) and (**B**). Average growth inhibition of seven human colon cancer cell lines (COLO 205; HCC-2998; HCT-116; HCT-15; HT29; KM12; SW-620).



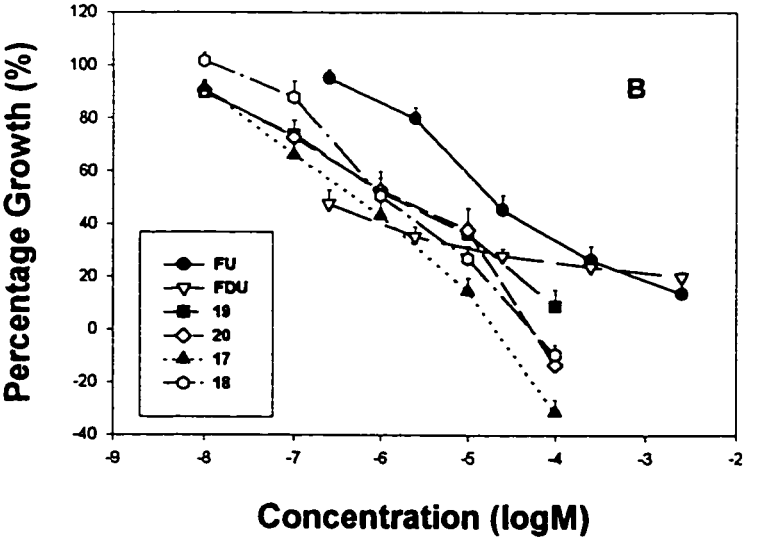
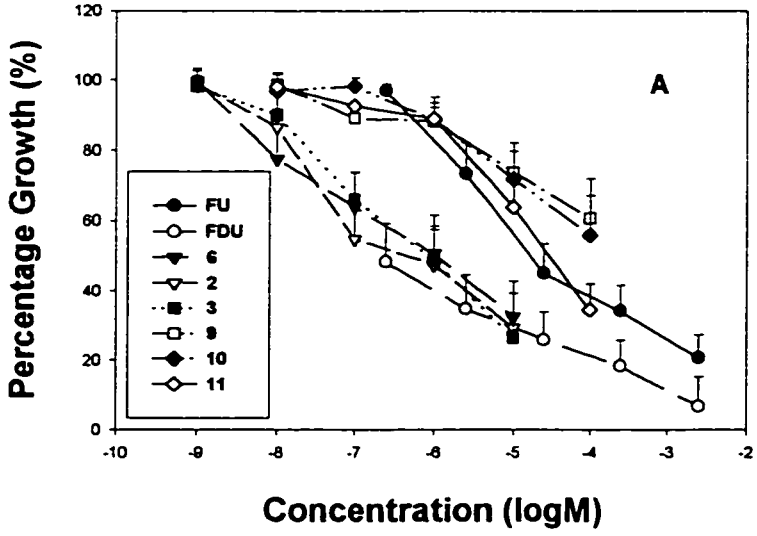


Figure 3-2-4 (**A**) and (**B**). Average growth inhibition of six human leukemia cell lines (CCRF-CEM; HL-60(TB); K-562; MOLT-4; SR; RPMI-8226).



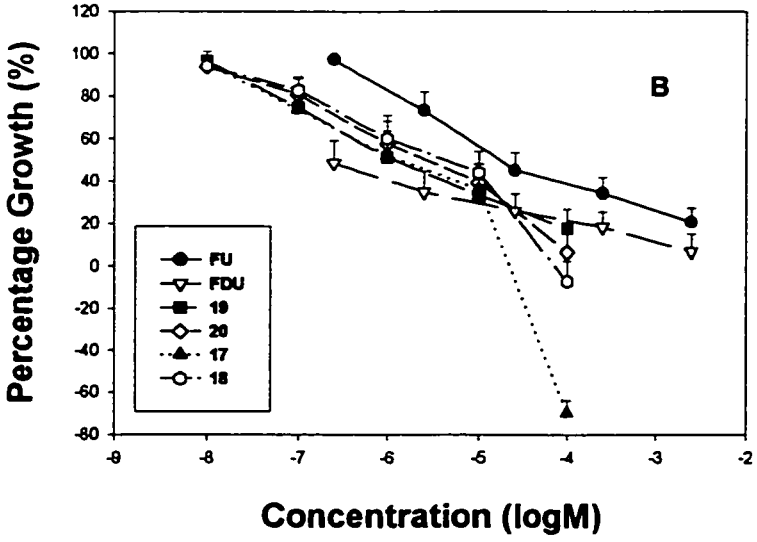
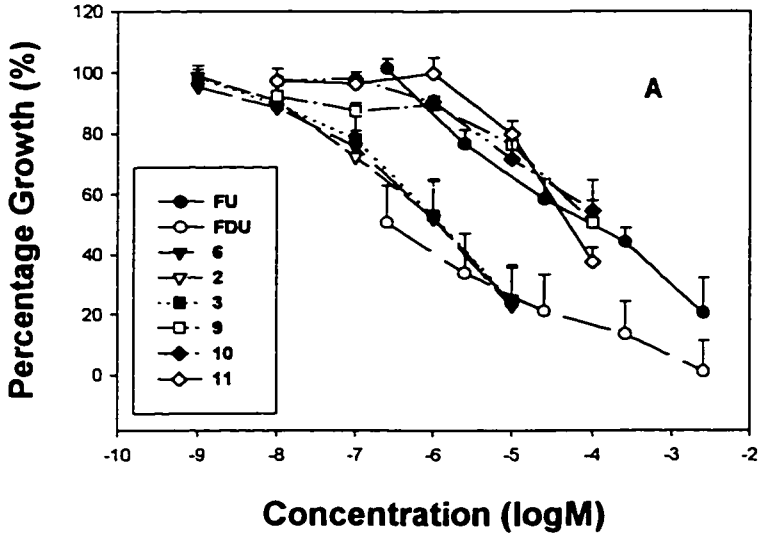


Figure 3-2-5 (**A**) and (**B**). Average growth inhibition of nine human non-small lung cancer cell lines (A549/ATCC; EKVX; HOP-62; HOP-92; NCI-H226; NCI-H23; NCI-H322M; NCI-H460; NCI-H522).



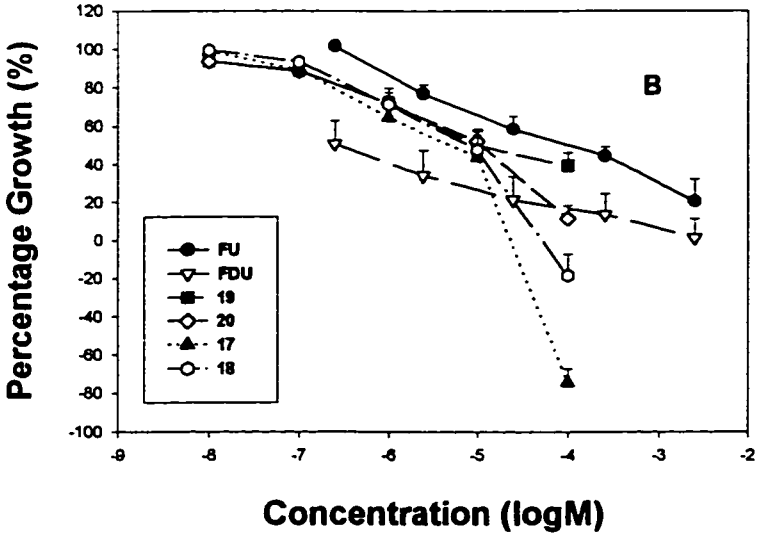
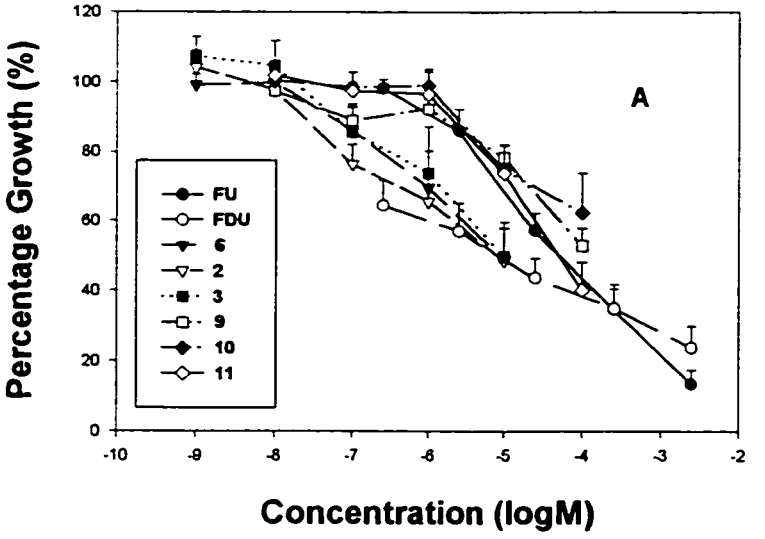


Figure 3-2-6 (**A**) and (**B**). Average growth inhibition of eight human melanoma cell lines (LOX IMVI; MALME-3M; M14; SK-MEL-2; SK-MEL-28; SK-MEL-5; UACC-257; UACC-62).



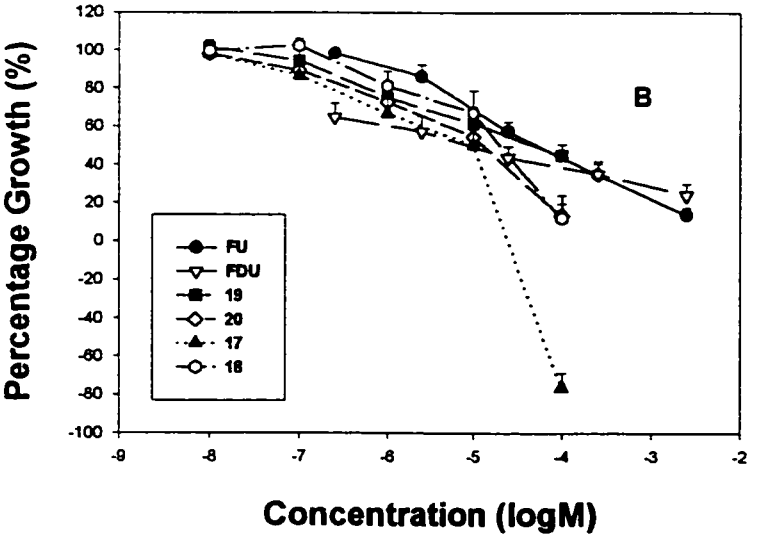
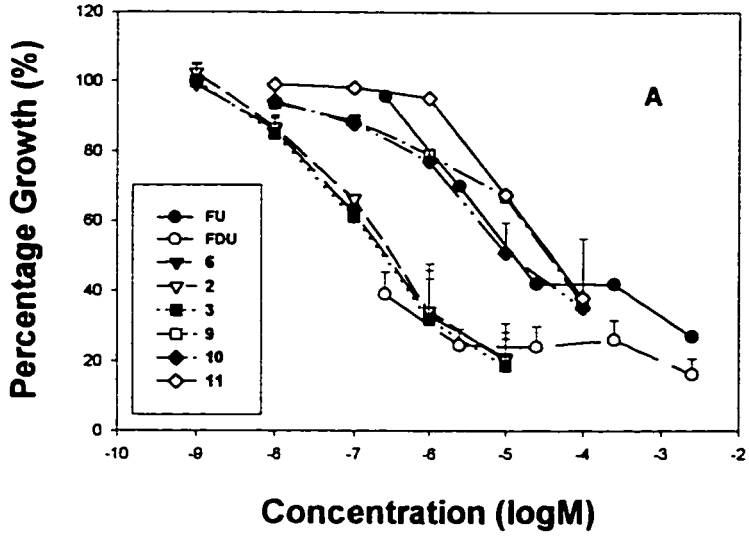


Figure 3-2-7 (**A**) and (**B**). Average growth inhibition of six human ovarian cancer cell lines (IGROV1; OVCAR-3; OVCAR-4; OVCAR-5; OVCAR-8; SK-OV-3).



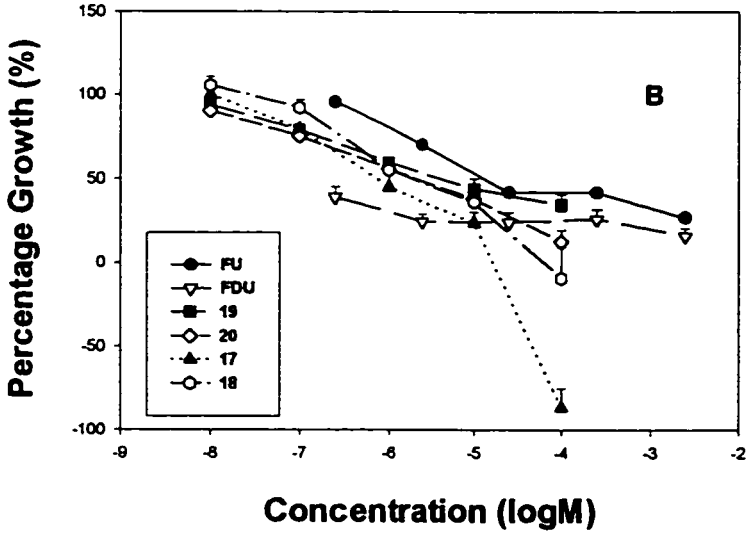
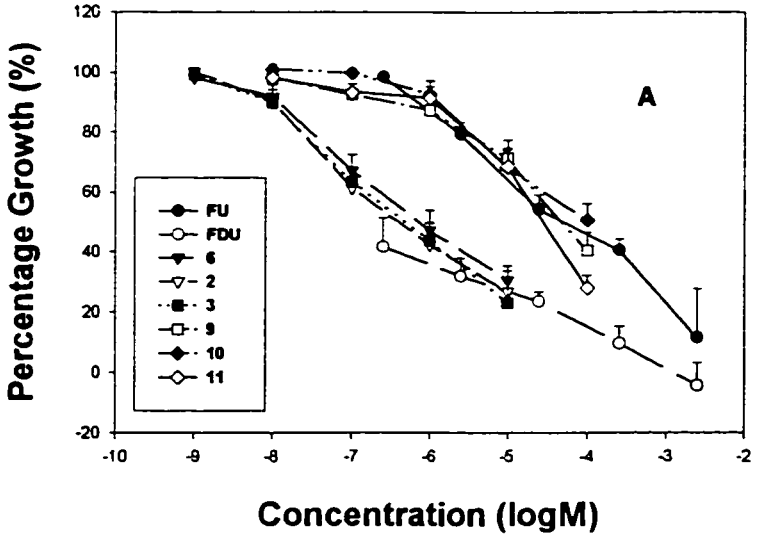


Figure 3-2-8 (**A**) and (**B**). Average growth inhibition of two human prostate cancer cell lines (PC-3; DU-145).



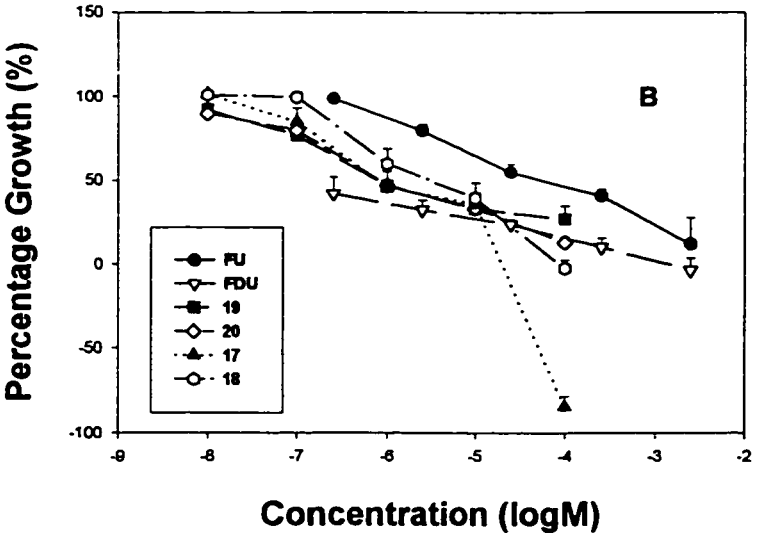


Figure 3-2-9 (**A**) and (**B**). Average growth inhibition of eight human renal cancer cell lines (786-0; A498; ACHN; CAKI-1; RXF-393; SN12C; TK-10; UO-31).

Based on the results obtained from this *in vitro* cytotoxic screen, a number of general profiles have been derived from the average dose-response graphs. The general potential for cell growth inhibition by butanoate esters of FDU (**2**, **3** and **6**) are not significantly different from each other and are similar to FDU, although they are better than FU. These similar potencies might be explained that the concentration of BA released from all these prodrugs (**2**, **3**, **6**) is lower than the effective concentration of BA required for pharmacological additive or synergistic efficacy with FDU such that the growth inhibition appears to be due to the activity of FDU alone. The butanoate esters of DFDU (**9-11**) exhibited similar cell growth inhibitions, with potencies close to that of FU. One possible explanation is that the 2'-F substituent might stabilize not only the glycosyl bond, but also to some extent the ester bonds so that cleavage of DFDU esters may be more difficult than that of FDU esters. Another hypothesis is that the cytotoxic mechanism of DFDU may be different from that of FDU.

A comparison of the retinoate esters of FDU shows that 3'-*O*-retinoyl-5-fluoro-2'-deoxyuridine (**17**) is more potent than 3',5'-di-*O*-retinoyl-5-fluoro-2'-deoxyuridine (**18**) in all tumor type panels, especially at the concentration $\geq 10^{-5}$ M. It should be noted that there are "concentration thresholds" for 3'-*O*-RFDU (**17**) and 3',5'-di-*O*-RFDU (**18**) (see Figures 3-2-1 (B) to 3-2-9 (B)). When the concentration is lower than that "threshold", the cell growth inhibition potency for the prodrugs 3'-*O*-RFDU (**17**) and 3',5'-di-*O*-RFDU (**18**) looks lower than or similar to that of FDU. When the concentration crosses the "threshold", the prodrugs significantly enhance the cytotoxicity with a considerable decrease of the cell growth curve slope. Possible explanations for this "threshold concentration" are as follows:

1. The prodrugs are enzymatically hydrolyzed slowly (Kawaguchi et al. 1985b). Therefore, in the drug screening time (48 hours), the hydrolysis may be incomplete. Moreover, according to the Michaelis-Menten equation, the hydrolysis velocity catalyzed by the enzyme is $V = V_{max}[S]/([S]+K_M)$, where V_{max} is the maximum reaction rate; [S] is the concentration of the prodrug; K_M is the Michealis constant. Assuming that

the condition satisfies $K_M >> [S]$, one would have $V \cong V_{max}[S]/K_M$, namely, $V \propto [S]$. Therefore, the hydrolytic rate would be slow at the low concentration of the prodrug and high at the high concentration.

2. The prodrug releases two active drugs with different anticancer mechanisms. Each drug has its own anti-neoplastic effective or synergistic concentration. If the concentration of one of the liberated active drugs is lower than the required minimum concentration for the cooperative effect, it would appear as if only one effective drug was present until crossing the "threshold".

However, this second explanation is not operative for *O*-retinoyl compounds, since 3'-*O*-retinoyl-5-fluoro-2'-deoxyuridine (**17**) is more potent than 3',5'-di-*O*-retinoyl-5-fluoro-2'-deoxyuridine (**18**) with respect to cell growth inhibition. This observation could be due, at least in part, to the different hydrolytic rates by esterases. In contrast to 3'-*O*-RFDU (**17**), 3',5'-di-*O*-RFDU (**18**) and 3'-*O*-Bu-5'-PFDU (**20**), FU or FDU retains the gentle slope without noticeable alteration within the wide range of drug concentrations used in this screen (see Figures 3-2-1 to 3-2-9), which implies that FU or FDU may not be able to kill all the cancer cells, possibly due to rapid drug catabolism and drug resistance caused by the heterogeneity of cancer cells.

In addition, it has been reported that both prostate cancer cell lines, PC-3 and DU-145 (see Figure 3-2-8), were resistant to RA alone (Jones et al. 1997). However, 3'-*O*-RFDU (**17**) is not only effective against PC-3 and DU-145 prostate cancer cell lines, but also much better than FDU (see Figure 3-2-8 (B)), which does indicate that the double-barreled prodrug 3'-*O*-RFDU (**17**) produced a synergistic effect when compared to FDU or RA alone.

A comparison of 5'-*O*-*bis*(2,2,2-trichloroethyl)phosphoryl-5-fluoro-2'-deoxyuridine (**19**) and 3'-*O*-butanoyl-5'-*O*-*bis*(2,2,2-trichloroethyl)phosphoryl-5-fluoro-2'-deoxyuridine (**20**) has shown that 3'-*O*-Bu-5'-PFDU (**20**) is superior to 5'-PFDU (**19**), which suggests that there may be a cooperative effect. Although "mM" concentration of BA is generally required for effect *in vitro* on cell differentiation and growth inhibition

(Miller et al. 1987; Breitman et al. 1994), a synergistic response may occur at lower concentrations of individual agents (Breitman et al. 1990; Breitman et al. 1994). Moreover, 5'-PFDU (**19**) is less potent than FDU with respect to cell growth inhibition. This implies that the masked neutral fluoronucleotide crosses the cell membrane and is slowly transformed to FdUMP, even slower than FDU. However, 3'-*O*-Bu-5'-PFDU (**20**) is more potent in cell growth inhibition than FDU at higher concentrations ($\geq 1 \times 10^{-5}$ M, see Figures 3-2-1 (B) to 3-2-9 (B)) but not at lower concentrations.

Caution should be taken in the interpretation of these screen results. Since this cancer cell growth inhibition screen was completed in only 48 hours, it is possible that the screening time used for exposure of the cells to the drug may be insufficient because:

1. some of the drug-induced biological processes may require a longer time such as delayed cell death (Alley et al. 1988);
2. some of the prodrugs may need a longer time to regenerate the active drugs by the enzymatic hydrolysis (Kawaguchi et al. 1985c)

Therefore, no compound should be ruled out completely on the basis of the *in vitro* screening data alone. To further elucidate these screen results and the mechanisms by which these prodrugs act, studies using different exposure time are needed.

However, the *in vitro* growth inhibition results have shown that two compounds, the nucleoside *O*-retinoyl ester (**17**) and the masked nucleotide *O*-butanoyl ester (**20**), were potentially more potent and had broader spectrum of anticancer activity than either FDU or FU alone. Therefore, further biological evaluations are warranted on the basis of these findings.

An alphanumeric summary of the NCI screening data [negative $\log_{10}$ GI50 (drug molar concentration causing 50% cell growth inhibition), TGI (drug molar concentration causing 100% growth inhibition), and LC50 (drug molar concentration causing 50% cell death or -50% cell growth) values] for each compound (**2**, **3**, **6**, **9-11** and **17-20**) is listed in Appendix 1 for each tumor-type panel with the individual cell line identifiers. The alphanumeric data can be used to reconstruct the original graphic data or any desired

modified format (Acton et al. 1994), and for related intermediate or future structure-activity calculations such as COMPARE analyses (Paull et al. 1989).

3-3. Effects of 3'-*O*-RFDU (17) and 3'-*O*-Bu-5'-PFDU (20) on *In Vitro* Differentiation and Apoptosis in Human HL-60 Acute Promyelocytic Leukemia (APL) Cells

Apart from the cytotoxic action of the fluorinated nucleoside or nucleotide, the second component of the double-barreled prodrug, namely RA or BA, was anticipated to be able to induce malignant cell differentiation and synergistic activation of cell apoptosis with the cytotoxic metabolite, FDU or FdUMP. Therefore, cell morphology and flow cytometric methods were used to study HL-60 APL cell differentiation and death after exposure of the cells to 3'-*O*-RFDU (**17**) or 3'-*O*-Bu-5'-PFDU (**20**) or the reference drugs (RA, NaBu and FDU). Since the HL-60 APL cell line was established from the peripheral blood of a patient with APL in 1977 (Collins et al. 1977), it has been used extensively as a model for assessing drugs that induce cell differentiation and apoptosis (Breitman et al. 1980; Martin et al. 1990; Breitman 1990a; Tarantilis et al. 1994; Terui et al. 1995; Zimra et al. 1997). This suggested that HL-60 APL cell line could serve as a model to evaluate the novel double-barreled prodrugs.

The morphology of HL-60 cells after 7 days of exposure to the prodrug or reference drug was evaluated by microscopic observation on the slides with Wright-stained cytospin preparation (Figure 3-3-1). In the absence of prodrug or drug, the HL-60 APL cells appear as predominantly typical promyelocytes with large round nuclei (Figure 3-3-1 (**A**)). The morphologic changes in HL-60 APL cells induced by the prodrugs showed irregular shapes for the nucleus and pyknotic changes in nuclear chromatin. However, the differentiated cell phenotypes, observed after treatment with the prodrugs, were not the same as that induced by RA or BA (see Figure 3-3-1) and significantly mixed with the morphological characteristics of cell death, such as highly condensed chromatin and the fragmented appearance of the nuclei. The differentiating cells treated with prodrug commit their pathway to death rather than terminal differentiation or maturation, which may be due to the toxic synergism of FDU or FdUMP. Consequently, the morphological changes

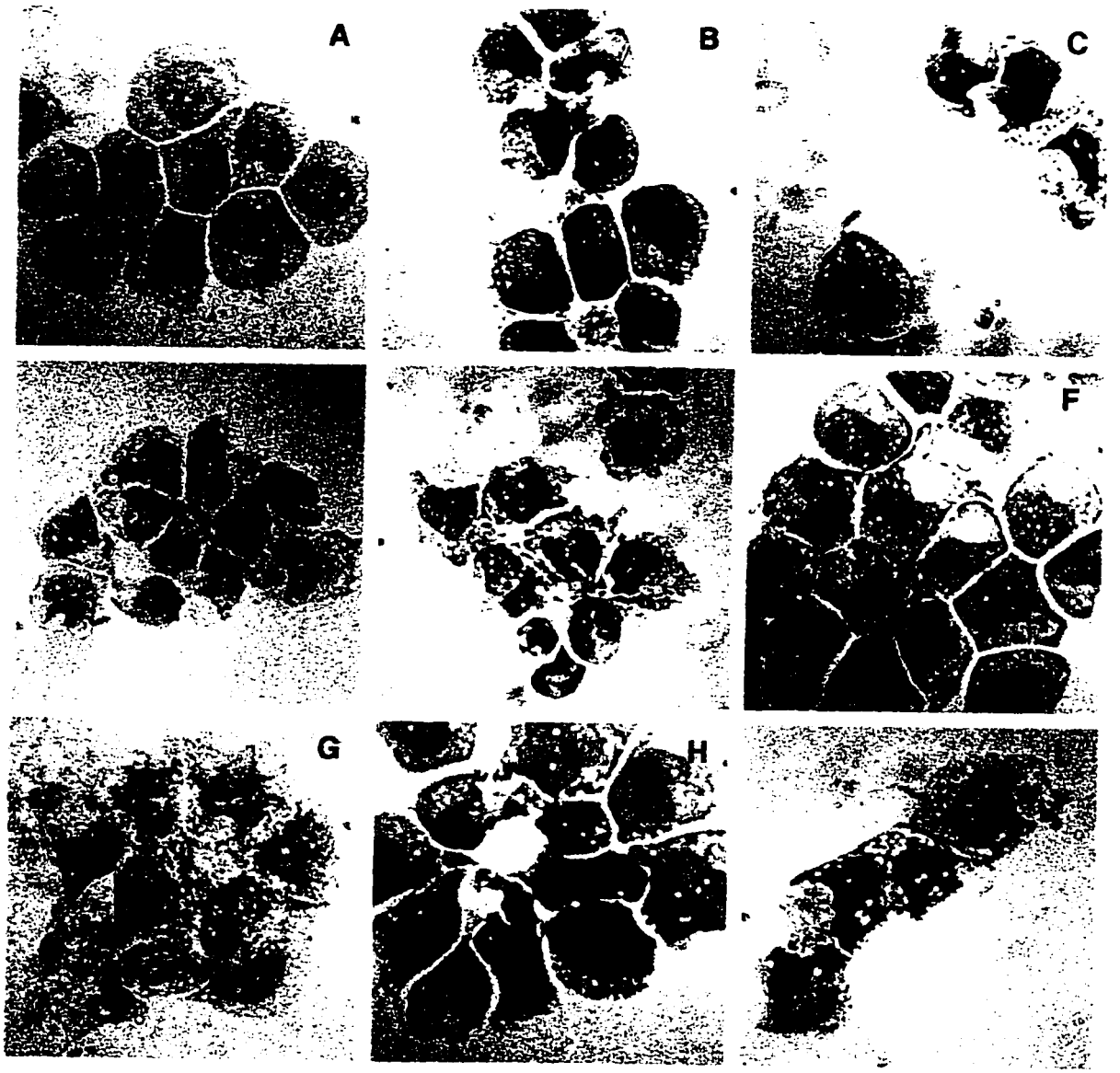


Figure 3-3-1. Representative morphology of HL-60 APL cells on Wright-stained cytospin slides after 7 days of incubation. (**A**) control; (**B**) treated with NaBu (1×10^{-4} M); (**C**) treated with NaBu (1×10^{-3} M); (**D**) treated with RA (1×10^{-6} M); (**E**) treated with RA (1×10^{-5} M); (**F**) treated with 3'-*O*-RFDU (**17**) (1×10^{-7} M); (**G**) treated with 3'-*O*-RFDU (**17**) (1×10^{-5} M); (**H**) treated with 3'-*O*-Bu-5'-PFDU (**20**) (1×10^{-6} M); (**I**) treated with 3'-*O*-Bu-5'-PFDU (**20**) (1×10^{-5} M).

of the cell differentiation were accompanied with a large amount of toxic granulation, neutrophil vacuolation and cell death, especially at the concentration $\geq 10^{-6}$ M. The morphologic changes were drug concentration-dependent and coincided with an increase in cell apoptosis, as determined by flow cytometry. Figure 3-3-2 shows the dose-response curves for differentiation in HL-60 APL cells determined by cell morphology.

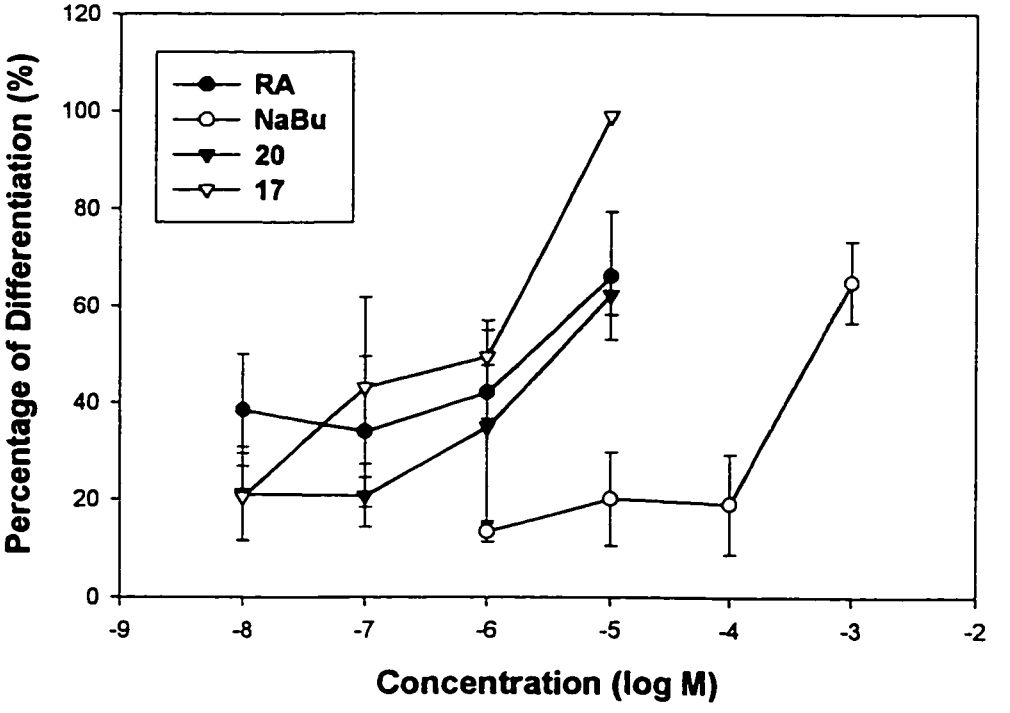
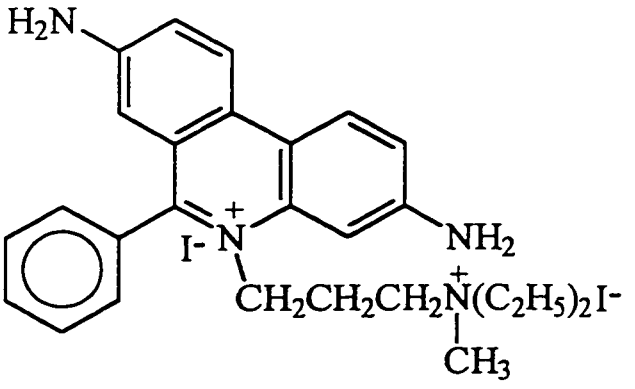


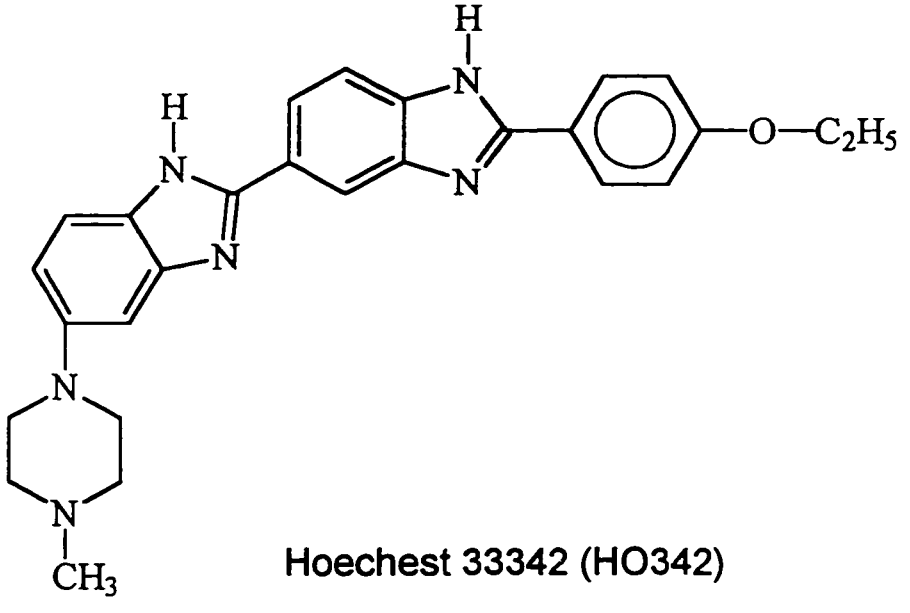
Figure 3-3-2. HL-60 APL cell differentiation after exposure of the cells to RA, NaBu, 3'-*O*-RFDU (17) and 3'-*O*-Bu-5'-PFDU (**20**) for 7 days. mean ± SD of control: 11% ± 4%. error bars represent SD.

Qualitative and quantitative cell deaths were analyzed by flow cytometry using the methodology described in the literature (Del Bino et al. 1991; Pollack et al. 1991; Dive et al. 1992). This method is based on the preserved integrity of the plasma membrane of the cells undergoing apoptosis, so that most of the membranes remain unchanged until the final stages of cell death or phagocytosis (Darzynkiewicz et al. 1992; Bosman et al. 1996). This is in contrast to cell necrosis, where one of the earliest changes is loss of membrane function and structural integrity. Therefore, the flow cytometry assay, based on the properties of DNA-binding fluorescent dyes, propidium iodide (PI) and Hoechst 33342 (HO342) (see Figure 3-3-3), and cell membrane permeability, can be useful for identification and quantification of viable, apoptotic and necrotic cells.

To determine the relative contribution of apoptosis and necrosis to cell death, HL-60 APL cells were exposed to 3'-*O*-RFDU (**17**) or 3'-*O*-Bu-5'-PFDU (**20**) or the reference compounds (FDU, RA and NaBu) at pre-determined concentrations and duration of the exposure. The cells were first exposed to charged PI which would be excluded by viable cells and the cells at early stages of apoptosis because their membrane integrity is preserved. PI stains DNA in the dead cells that have damaged membranes, producing a red fluorescence. Subsequent staining of cells with the neutral HO342 results in blue fluorescence for viable cells and the cells at early stages of apoptosis. Figure 3-3-4 illustrates the relative populations of viable, apoptotic and necrotic cells following cell staining first with PI and then with HO342 measured by flow cytometry. The early apoptotic cells (F area) had lower stainability with HO342 compared to viable cells due to DNA degradation. PI fluorescence of early apoptotic cells was also low because of dye exclusion by the cell membrane. Loss of cell membrane integrity and its function late in apoptosis resulted in increased stainability with PI (G area). Necrotic cells with a damaged plasma membrane had high PI and low HO342 fluorescence intensity (H area). The relative contribution of apoptosis and necrosis to cell death after exposure of HL-60 APL cells to 3'-*O*-RFDU (**17**) and 3'-*O*-Bu-5'-PFDU (**20**) along with reference drugs (FDU, RA and NaBu) determined by flow cytometry are summarized in Figure 3-3-5.



Propidium iodide (PI)



Hoechest 33342 (HO342)

Figure 3-3-3. Structures of the DNA-binding fluorescent dyes, PI and HO342.

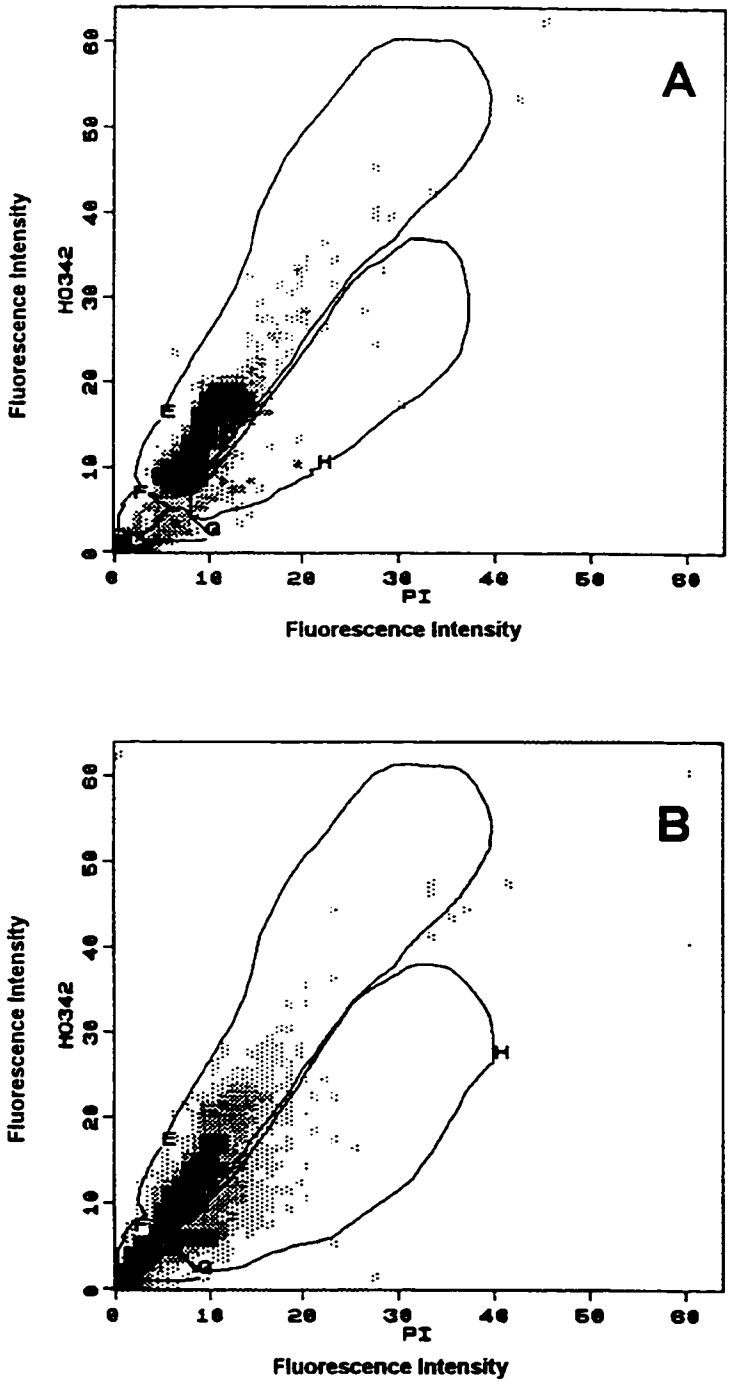


Figure 3-3-4. Viable, apoptotic and necrotic HL-60 APL cells stained with PI and HO342 under conditions where HO342 was applied after cell exposure to PI. (A) solvent-treated (control) for 48 hours; (B) 3'-*O*-RFDU-treated (1×10^{-5} M) for 48 hours. Apoptotic cells are located in the areas F (A: 0.4%, B: 22%) and G (A: 0.8%, B: 3.1%). Necrotic cells are located in the area H (A: 0.9%, B: 7.1%). All the viable cells including the cells in G_1, S and G_2M periods of the cell cycle are in the area E (A: 90%, B: 34%). The darkness represents the number of cells or DNA debris, i.e. the darker, the more cells or DNA debris.

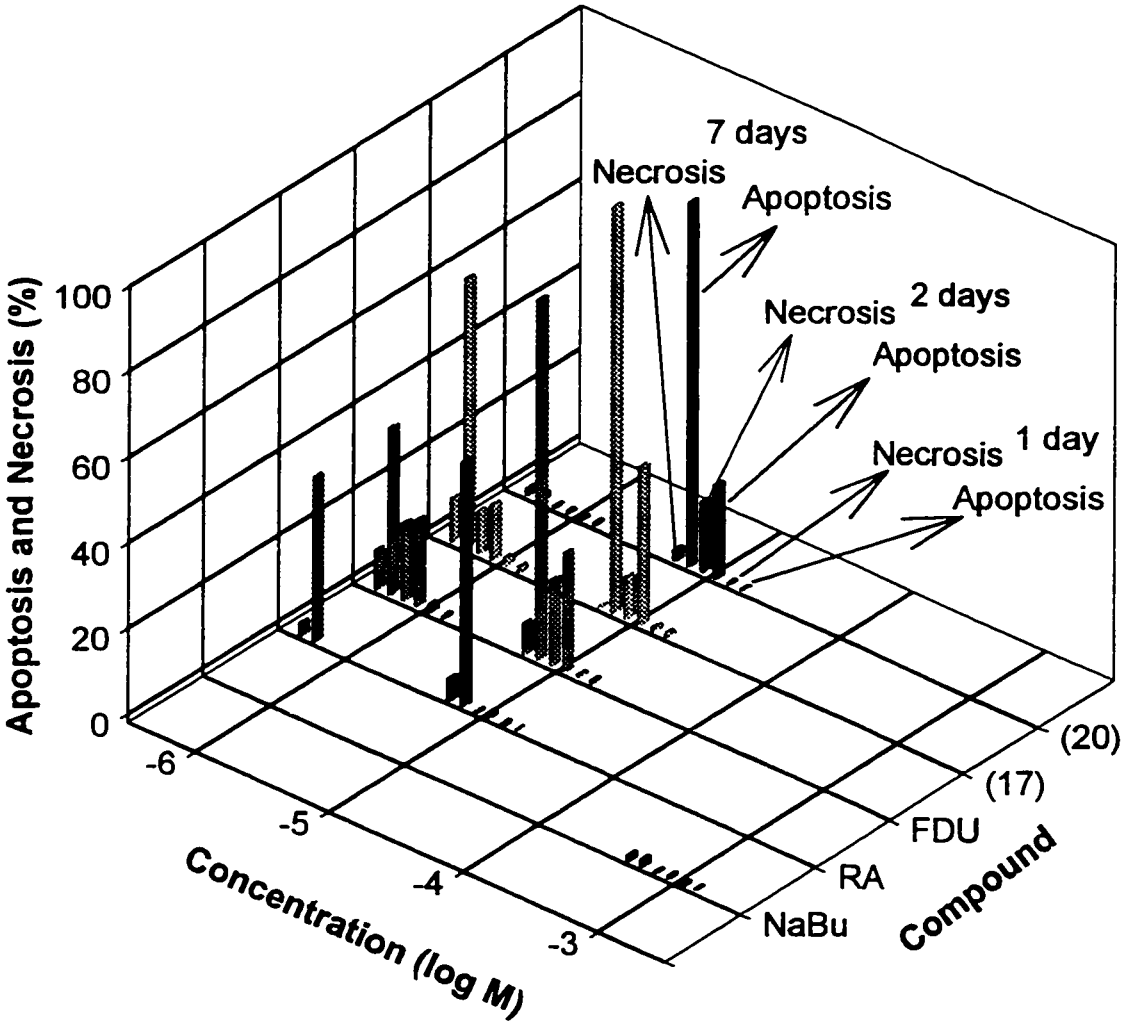


Figure 3-3-5. Relative contributions (%) of apoptosis and necrosis to the cell death after exposure of HL-60 APL cells to 3'-*O*-RFDU (**17**) and 3'-*O*-Bu-5'-PFDU (**20**) compared to FDU, RA and NaBu determined by flow cytometry.
Data have been normalized with reference to the respective control(drug-untreated) data.

Based on these morphological and flow cytometric studies, it is concluded that the novel prodrugs 3'-*O*-RFDU (**17**) and 3'-*O*-Bu-5'-PFDU (**20**), in addition to their cytotoxic actions, can induce malignant cell differentiation and lead the cell to apoptosis as expected. Among them, 3'-*O*-RFDU (**17**) was more cytotoxic than 3'-*O*-Bu-5'-PFDU (**20**). It is noteworthy that 3'-*O*-Bu-5'-PFDU (**20**) was much more effective in inducing cell differentiation and cell apoptosis than NaBu, which implies that the cytotoxic drug may potentiate the differentiation inducer and enhance the cell apoptosis (Ebert et al. 1994; Darzynkiewicz 1995).

Cell death after treatment with the prodrugs was found to be dose-dependent, as seen in the growth inhibition screens (see Figures 3-2-1 to 3-2-9), and time-dependent (see Figure 3-3-5). This indicates that cell death by either necrosis or apoptosis is not an immediate effect of exposure to these drugs. It is well documented that FDU is rapidly transported into cells, possibly via nucleoside transporters (Gati et al. 1984; Weckbecker 1991; Belt et al. 1993). However, after exposure of HL-60 APL cells to FDU for 24 hours, even at high concentration (1×10^{-5} M), the relative amount of dead cells including both necrotic cells and apoptotic cells were very low compared to that of viable cells (see Figure 3-3-5). After exposure of HL-60 APL cells to FDU (1×10^{-5} M) for 48 hours, the ratio of dead cells to viable cells is about 1:1. This time delay in cell death varied among the compounds tested, possibly due to their different cytotoxic mechanisms. RA-induced cell death occurred later than that caused by 3'-*O*-RFDU (**17**), 3'-*O*-Bu-5'-PFDU (**20**) and FDU at similar concentration (1×10^{-5} M). It was found that the major pathway for cell death of HL-60 APL cells after treatment with 3'-*O*-RFDU (**17**) and RA was apoptosis no matter what the exposure time was. On the other hand, although the apoptosis was also an important pathway for cell death after 48 hours of exposure to FDU or 3'-*O*-Bu-5'-PFDU (**20**), the relative contributions by necrosis and apoptosis were similar. However, at 7th day after treatment with FDU (1×10^{-5} M and 1×10^{-6} M) or 3'-*O*-Bu-5'-PFDU (**20**) (1×10^{-5} M), apoptosis in HL-60 APL cells was markedly higher than necrosis. It has been reported that HL-60 APL cells that are induced to differentiation die via apoptosis (Martin et al. 1990). Although it is not clear whether signal pathways that lead to cell death in differentiated cells are similar to those in normal human cells, it

has been observed that there is an increased expression of *bcl-2*, a well known anti-apoptotic gene, in early myeloid cells in bone marrow and that it decreases with the cell maturation, finally becoming almost undetectable in terminally differentiated neutrophils which subsequently undergo apoptosis (Naumovski et al. 1994). In the current study, it has been shown that NaBu was a very weak inducer of HL-60 APL cell death, even at a concentration of 1×10^{-3} M, although it could induce cell differentiation.

3-4. Responses of EMT-6 Murine Mammary Cancer Cells to RA and NaBu

Although *in vitro* biological evaluations are very important for studying and discovering potentially effective anticancer drugs, they suffer from severe limitations. *In vitro* methods are incapable of revealing many host mediated effects. For this reason, many drugs are effective *in vitro* but not *in vivo*, and consequently they are of clinical inefficacy. In addition to this, *in vitro* tests fail to give any estimate of therapeutic index. Thus, *in vivo* evaluation of prodrugs is necessary. In order to determine the *in vivo* response of transplantable EMT-6 mammary tumor in mice to the prodrugs 3'-*O*-RFDU (**17**) and 3'-*O*-Bu-5'-PFDU (**20**), the cytotoxic effects of RA and NaBu were first tested and confirmed *in vitro* in the EMT-6 cell line, using an assay that involves the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Mosmann 1983; Alley et al. 1988). The MTT assay is based on the property that a living cell is capable of reducing a tetrazolium salt, MTT, to a blue formazan product (Berridge et al. 1993). This reduced blue product, which is proportional to the number of viable cells, can be detected quantitatively using a colorimeter (Figure 3-4-1).

Figure 3-4-1 shows a linear relationship between the absorbance and the number of EMT-6 cells from 4,600 to 150,000 cells/mL. Both RA and NaBu exerted considerable growth-inhibitory effects on EMT-6 cells. These effects are dose-dependent and time-dependent (see Figure 3-4-2 and Table 3-4-1). Moreover, RA was effective at a much lower concentration than NaBu. The data (ED_{50}, dose required for 50% of effectiveness) for RA and BA in Table 3-4-1 are similar to the data reported in the literature (Kaleagasioglu et al. 1993; Breitman et al. 1994) although the different cell lines were used.

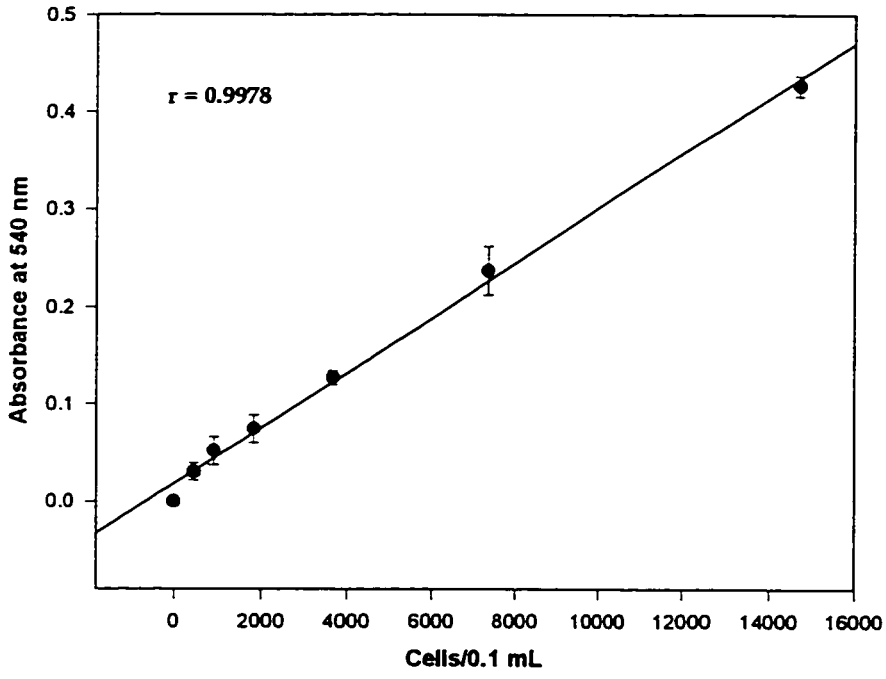


Figure 3-4-1. Linear correlation between EMT-6 cell number and optical density (absorbance) at 540 nm determined by the MTT assay. Error bars represent SD.

Table 3-4-1. Growth inhibition (ED_{50}) of EMT-6 murine mammary cancer cells

Compound	Exposure Time (h)	ED_{50} (mM)
RA	36	0.01
	80	0.04
NaBu	36	0.63
	80	1.6

ED_{50} for NaBu at 80 hours was larger than that at 36 hours. This may be explained that NaBu is a strong differentiation inducer but a weak cell growth inhibitor. Following the differentiation some of the viable cells may continue to enter the cell cycle and division. However, more experiments are needed to further confirm this phenomenon.

It has been reported that FU could significantly inhibit the growth of EMT-6 cells (Teicher et al. 1981). Because FDU shares similar cytotoxic pathways with FU (Dorr et al. 1994), it was expected that FDU would also inhibit the growth of EMT-6 cells.

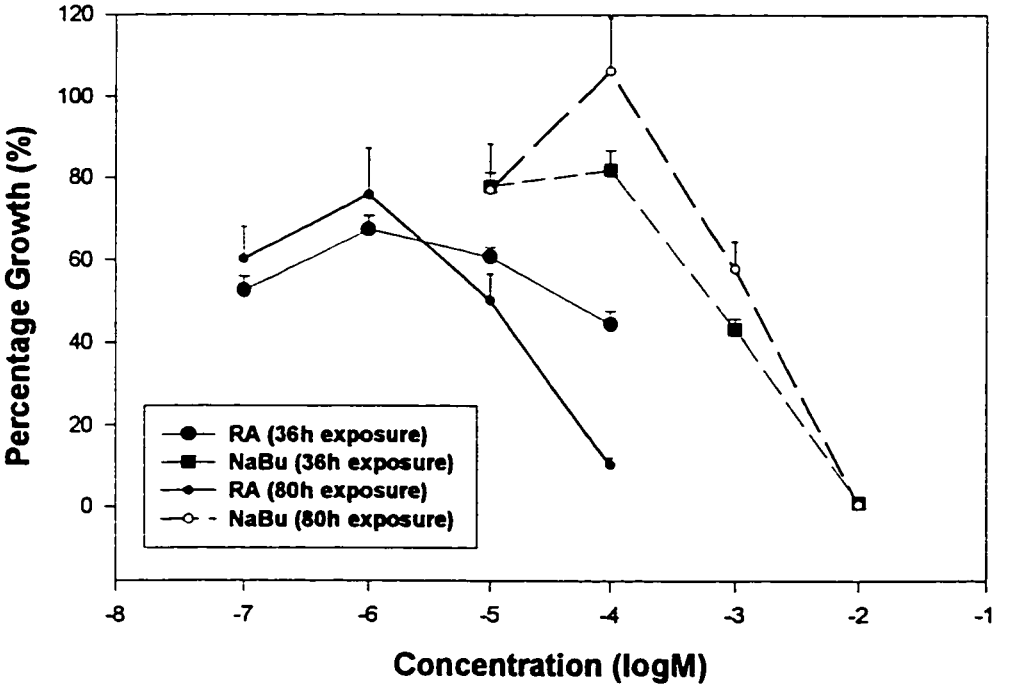


Figure 3-4-2. Growth inhibition of EMT-6 murine mammary cancer cells. Error bars represent SD.

3-5. Dose Escalation and Drug Toxicity Studies in Normal Mice

One of the major problems in current cancer chemotherapy is that most of the drugs used in clinics are not only toxic to the cancer but also toxic to the normal tissues. Adverse clinical side effects, such as those observed during the treatment with FDU or RA described in sections **1-3-2** and **1-4-2,** are well known in cancer chemotherapy.

Development of novel anticancer drugs with higher therapeutic indices still remains a challenge to scientists worldwide. From a pharmacokinetic viewpoint, the therapeutic window of a potential anticancer prodrug must be large enough to be clinically useful. Otherwise the prodrug may be worthless no matter how effective the prodrug is *in vitro*.

Thus, dose escalation studies for the novel double-barreled prodrugs 3'-*O*-RFDU (**17**) and 3'-*O*-Bu-5'-PFDU (**20**) were performed, together with the reference drugs (RA and NaBu alone or in combination with FDU) for comparison. These prodrugs and reference drugs were administered to healthy BDF1 female mice, which had been randomly assigned to test (3 mice for each drug-treated group and 4 mice for each control group). The highest dosage (7 days × 0.013 mmol/kg, i.p.) was determined based on the results from the *in vitro* anticancer screen data (see section **3-2**), which showed "threshold" concentrations (≥ 0.01 mM), and was also referred to the clinical dosage for FDU, which is 14 days × 0.0012 mmol/kg/day by hepatic artery infusion (Cao et al. 1993). Test compounds were administered by intraperitoneal (i.p.) injection starting from the dosage regimen of 7 days × 0.0001 mmol/kg (i.p.), in two-fold increment (7 days × (0.0001 mmol/kg × 2^n), n = 0, 1, 2, 3, 4, 5, 6, 7, i.p.), until the highest dosage regimen (7 days × 0.013 mmol/kg, i.p.) was reached. After dosing, the mice were monitored daily for any signs of acute or chronic toxicity, including restlessness, lethargy, inappropriate aggression, wobbling gait, weight loss and mortality, until the time of euthanasia at 60th day after the last drug administration. Because of mild morbidity (pain) in mice during RA dosing either alone or in combination with FDU, the dose escalation test for RA had to be suspended midway in the study. Although slow mouse weight increases were seen in all test groups at an early time after dosing compared to the control, no significant loss of mouse weight was observed compared to the control. The difference in mouse weight fluctuation between prodrug-treated and drug-untreated control was less that 10% (see Figures 3-5-3 to 3-5-4).

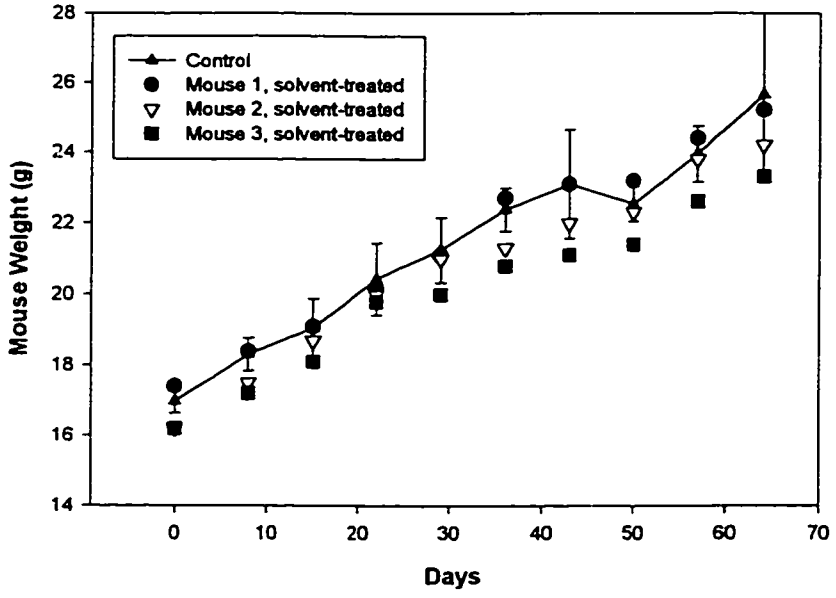


Figure 3-5-1. Mouse weight following solvent administration (7 days x 0.1 mL solvent / mouse, i.p.)

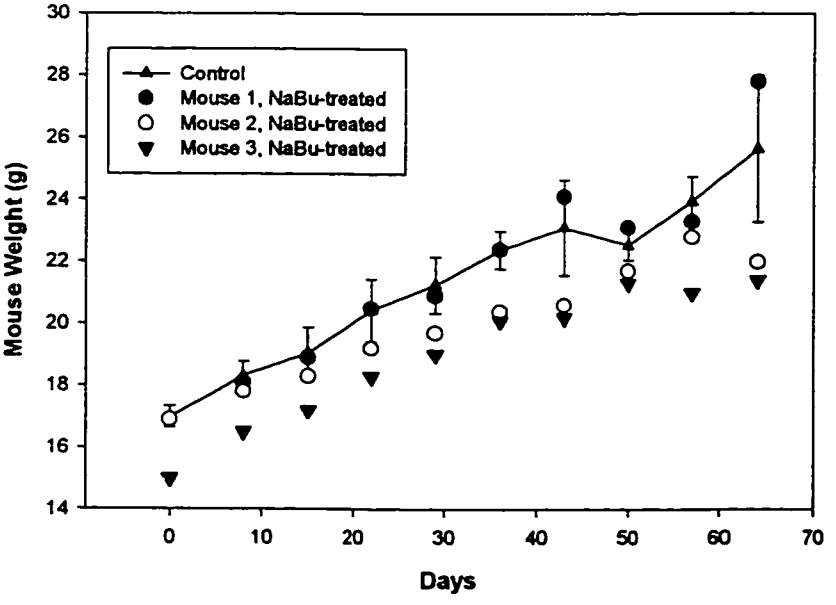


Figure 3-5-2. Mouse weight following NaBu administration (7 days x 0.013 mmol/kg, i.p.)

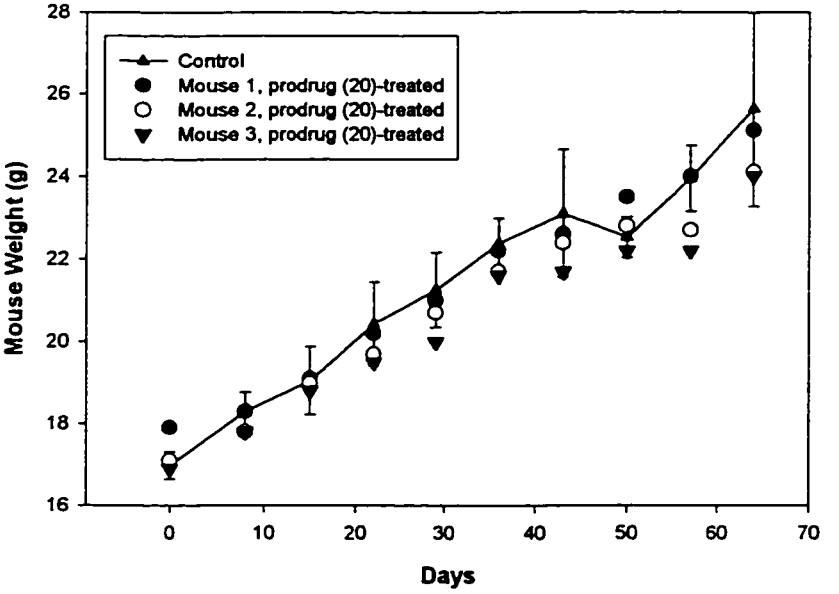


Figure 3-5-3. Mouse weight following prodrug (20) administration (7 days x 0.013 mmol/kg, i.p.)

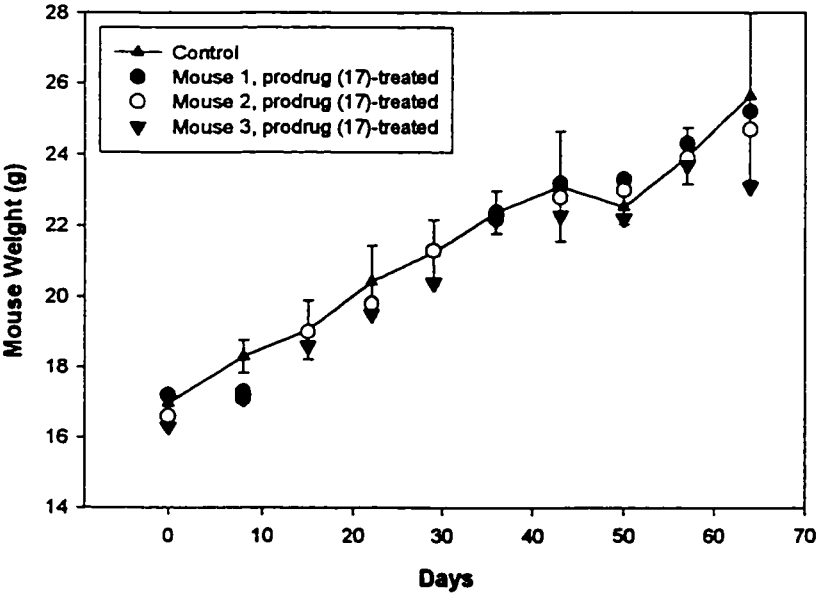


Figure 3-5-4. Mouse weight following prodrug (17) administration (7 days x 0.013 mmol/kg, i.p.)

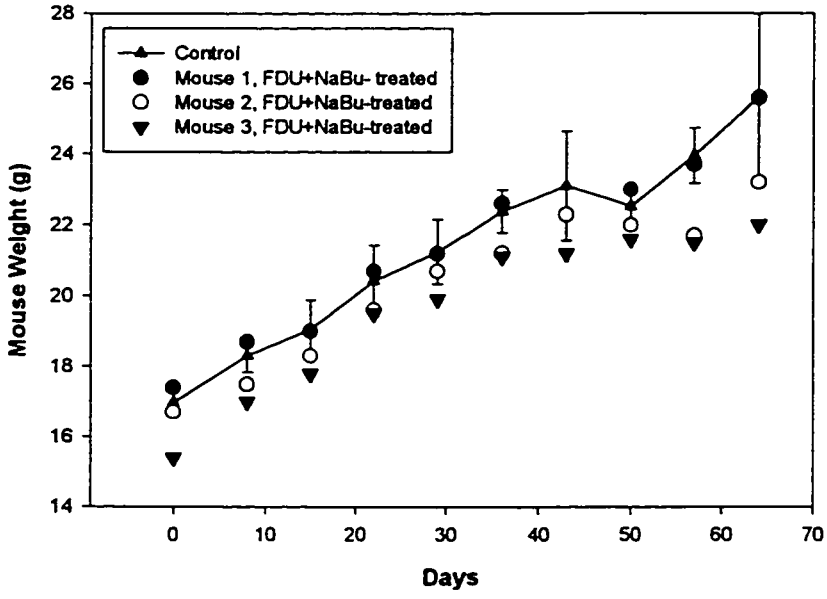


Figure 3-5-5. Mouse weight following FDU + NaBu coadministration (7 days x 0.013 mmol/kg, i.p.)

Clinical chemistry for serum and gross pathological and histopathological postmortem examinations of all mice (BDF1, female) given the highest dosage (7 days × 0.013 mmol/kg, i.p.) of 3'-*O*-RFDU (**17**) and 3'-*O*-Bu-5'-PFDU (**20**) were conducted by Dr. Nation at the Health Sciences Laboratory Animal Services, University of Alberta (HSLAS). The results of clinical chemistry analysis (Table 3-5-1) and gross pathological and histopathological postmortem examination showed that, under this dose schedule, there was no significant difference either in serum chemical parameters or in the gross and microscopic anatomy between drug-treated and control animals, concluded in Dr. Nation's report (Nation 1996). All data are within the normal ranges of healthy mice in HSLAS. However, caution should be taken due to the very few animals used in this study and needs further animal data to strengthen the conclusion.

Table 3-5-1. Clinical chemistry parameters obtained from BDF1 mice after 60 days from the last administration of 3'-*O*-RFDU (**17**) or 3'-*O*-Bu-5'-PFDU (**20**) (7 days × 0.013 mmol/kg, i.p.) compared to control

Group	**17**			**20**			Control			
Mouse No.	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*4*
ALT (iu/L)	32	N/A*	110	51	96	43	8	46	38	76
AP (iu/L)	133	N/A	170	173	145	182	265	176	170	175
TOT BILI (μM)	12	N/A	13	22	13	18	31	17	16	23
GLUC (mmol/L)	11.2	N/A	9.1	14.0	12.1	13.7	9.4	8.9	10.7	11.7
TP (g/L)	48	N/A	53	58	50	53	60	54	53	56
Alb (g/L)	26	N/A	29	31	28	30	33	30	29	30
Glob (g/L)	22	N/A	25	27	22	23	27	25	24	26
BUN (mmol/L)	7.1	N/A	7.3	6.3	8.6	7.0	8.3	9.8	8.8	6.0
Creat (μM)	27.6	N/A	25.4	25.8	24.4	23.1	20.4	22.8	24.8	23.1
Lipase (iu/L)	1349	N/A	1334	1514	1367	N/A	1377	1421	1677	N/A
CK (iu/L)	620	N/A	710	1541	104	275	904	646	185	159
OSM (mosmol/kg)	304.6	N/A	303.3	304.6	307.6	312.1	310.3	314.6	305.8	307.2
Na (mmol/L)	154	N/A	154	153	154	157	157	159	154	156
K (mmol/L)	8.0	N/A	7.9	10.0	7.8	8.7	9.5	8.3	7.9	8.3
Cl (mmol/L)	112	N/A	112	115	113	114	112	114	110	N/A
HCO_3^- (mmol/L)	32	N/A	32	25	30	29	37	33	34	32
Ca (mmol/L)	2.57	N/A	2.55	2.66	2.59	2.66	2.62	2.62	2.52	2.56
P (mmol/L)	3.16	N/A	2.76	N/A	N/A	3.10	3.41	2.93	2.66	2.92

ALT:	Alanine aminotransferase	AP:	Alkaline phosphatase
TOT BILI:	Total bilirubin	GLUC:	Glucose
TP:	Total protein	Alb:	Albumin
BUN:	Blood urea nitrogen	Glob:	Globulin
Creat:	Creatinine	OSM:	Osmolality
CK	Creatine kinase		

* N/A: not available because of insufficient sample for measurement.

3-6. Response of EMT-6 Murine Mammary Tumor to 3'-*O*-RFDU (17) and 3'-*O*-Bu-5'-PFDU (20) Compared to RA, NaBu and FDU Alone or in Combination

Evaluation of the *in vivo* anticancer efficacy for the prodrugs (**17**) and (**20**) was based on the change in drug-treated tumor volume compared to that in drug-untreated tumor volume (control). Tumors were grown subcutaneously (s.c.) in the flanks of female Balb/c mice. The test compounds were administered i.p., starting on the day 1 which was 24 hours after implantation of the EMT-6 tumor cells (10^6 cells/mouse). Two dosage regimens were evaluated (7 days × 0.0075 mmol/kg, i.p. and 7 days × 0.015 mmol/kg, i.p.). The end point used to determine the tumor response to the test compound was tumor growth delay *in situ*. Tumor diameter was measured along three perpendicular axes using a pair of calipers. The tumor volume was calculated using the volume formula for hemiellipsoids: $V=1/2(4\pi/3)(L/2)(W/2)(H)$, where L, W, and H are length, width and height of the tumor, respectively (Rockwell et al. 1972). Tumors were examined from the last time of dosing until the tumor volume reached 800 mm^3 or tumor gross hemorrhage and infiltration were observed.

Tumor growth delay for individual animals is shown in Figures 3-6-1 to 3-6-4, since mean values alone may fail to take into account the response of individual animals and their tumors to the drug therapy and sometimes even fail to identify the true efficacy of the drug therapy (Schabel et al. 1977). The mean growth delay has, however, been useful in determining the relative effectiveness of drug treatments, and this parameter was applied when the tumor volumes reached 100 mm^3, 200 mm^3, 300 mm^3, 400 mm^3 and 500 mm^3 (Table 3-6-1). Data are displayed as the difference between the time for the drug-treated group and the time for the control group to reach these specified tumor volumes. Complete regression (CR) or tumor free survivors were both excluded in the calculation of the mean tumor growth delay. There were some cures (CR) observed for both 3'-*O*-RFDU (7 days × 0.015 mmol/kg, i.p.) and 3'-*O*-Bu-5'-PFDU (7 days × 0.0075 mmol/kg, i.p.) (see Figures 3-6-2 (D) and 3-6-3 (E)). CR represents tumor growth regression below the limit of palpation after drug treatment.

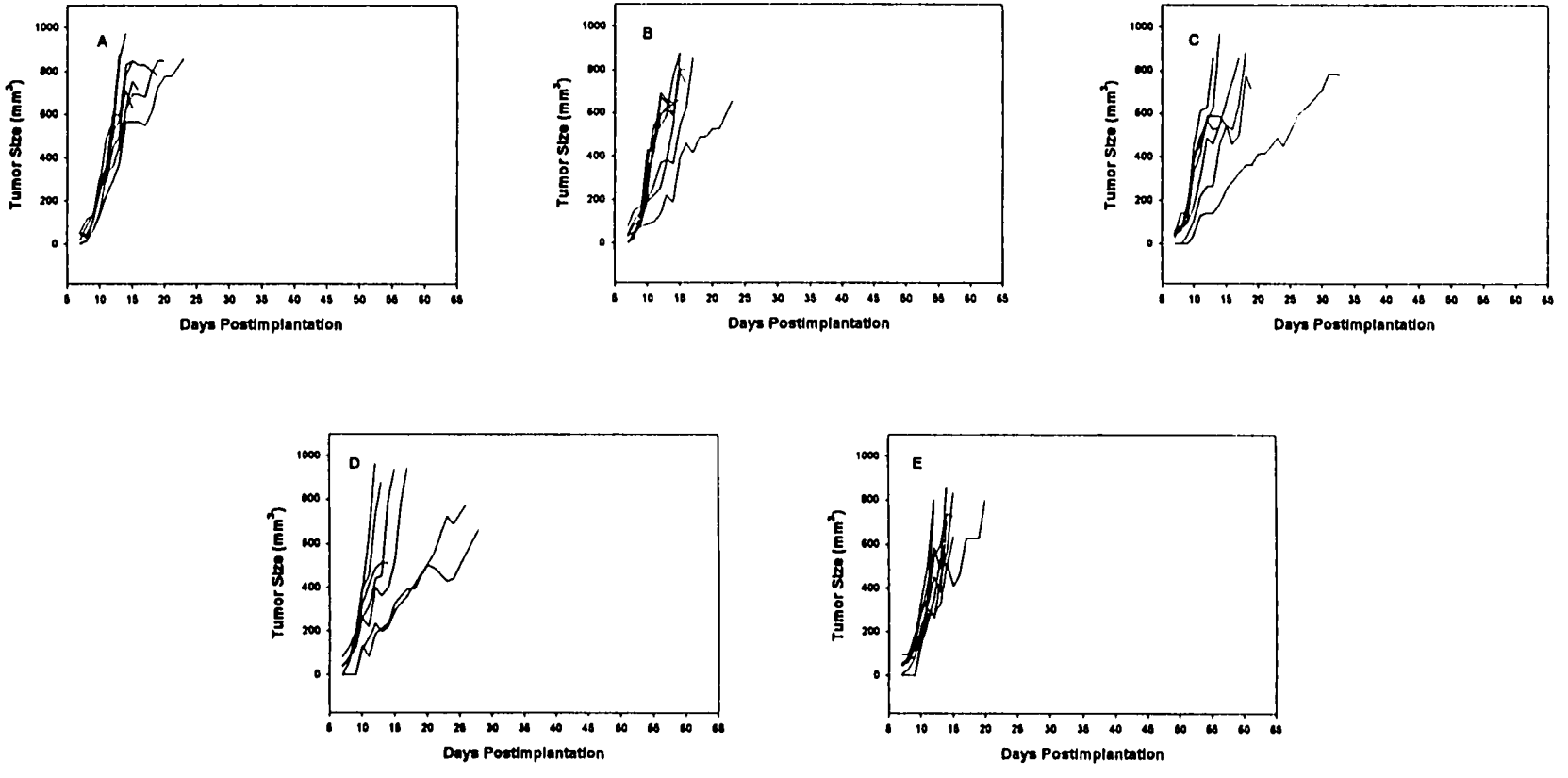


Figure 3-6-1. Individual tumor growth after s.c. implantation of 1×10^6 EMT-6 tumor cells at day 0 and i.p. injection of the test compound from day 1 to day 7, (**A**) solvent-treated control (n = 8, i.p.), (**B**) NaBu-treated (n = 8, 7 days × 0.0075 mmol/kg, i.p.), (**C**) NaBu-treated (n = 8, 7 days × 0.015 mmol/kg, i.p.), (**D**) RA-treated (n = 7, 7 days × 0.0075 mmol/kg, i.p.), (**E**) RA-treated (n = 8, 7 days × 0.015 mmol/kg, i.p.).

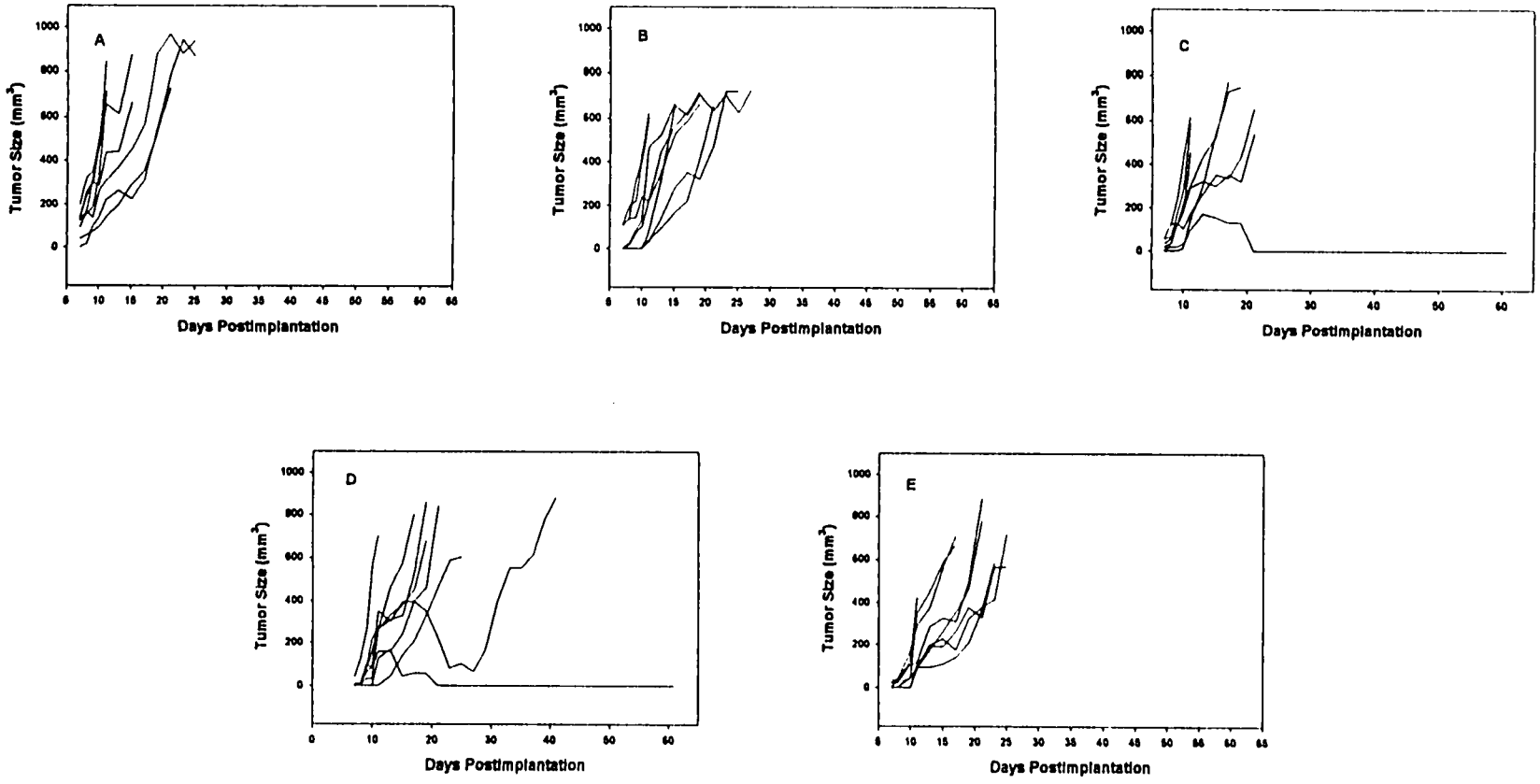


Figure 3-6-2. Individual tumor growth after s.c. implantation of 1 × 10^6 EMT-6 tumor cells at day 0 and i.p. injection of the test compound from day 1 to day 7, (**A**) solvent-treated control (n = 8, i.p.), (**B**) FDU+NaBu-treated (n = 8, 7 days × 0.0075 mmol/kg, i.p.), (**C**) FDU+NaBu-treated (n = 8, 7 days × 0.015 mmol/kg, i.p.), (**D**) (**20**)-treated (n = 8, 7 days × 0.0075 mmol/kg, i.p.), (**E**) (**20**)-treated (n = 8, 7 days × 0.015 mmol/kg, i.p.).

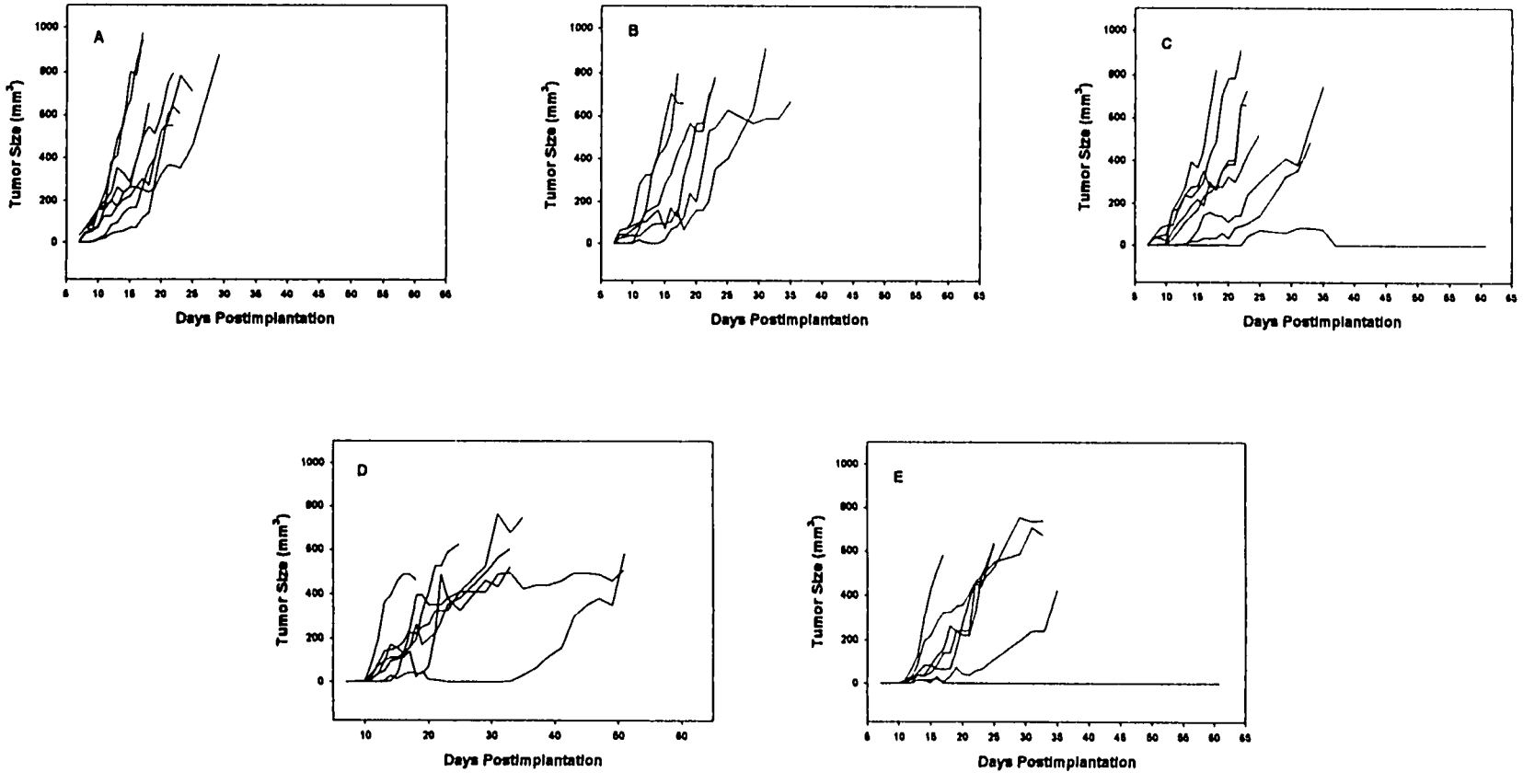


Figure 3-6-3. Individual tumor growth after s.c. implantation of 1×10^6 EMT-6 tumor cells at day 0 and i.p. injection of the test compound from day 1 to day 7, (**A**) solvent-treated control (n = 8, i.p.), (**B**) FDU+RA-treated (n = 6, 7 days × 0.0075 mmol/kg, i.p.), (**C**) FDU+RA-treated (n = 8, 7 days × 0.015 mmol/kg, i.p.), (**D**) (**17**)-treated (n = 7, 7 days × 0.0075 mmol/kg, i.p.), (**E**) (**17**)-treated (n = 7, 7 days × 0.015 mmol/kg, i.p.).

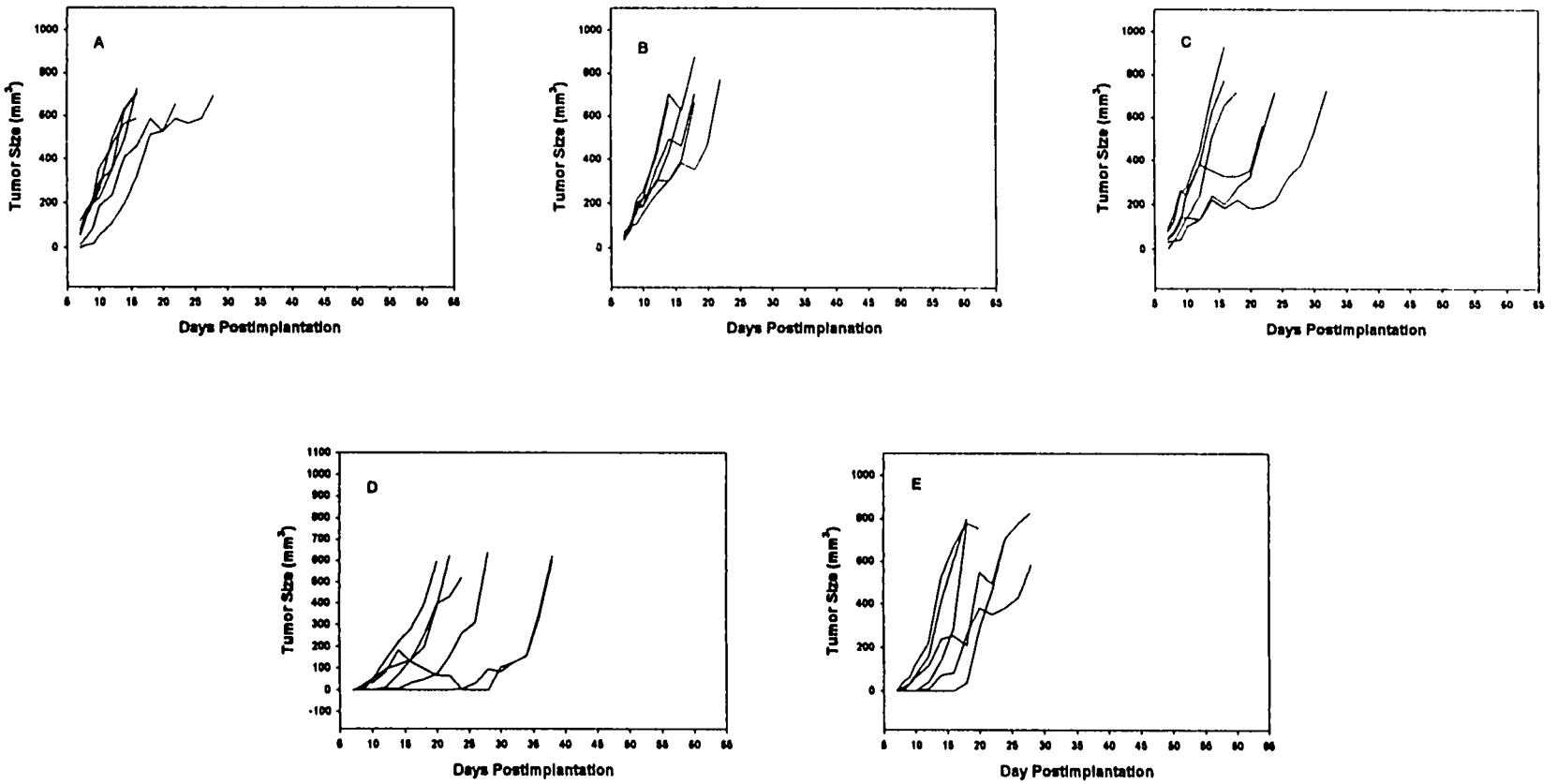


Figure 3-6-4. Individual tumor growth after s.c. implantation of 1×10^6 EMT-6 tumor cells at day 0 and i.p. injection of the test compound from day 1 to day 7, (**A**) solvent-treated control (n = 6, i.p.), (**B**) FDU-treated (n = 6, 7 days × 0.0075 mmol/kg, i.p.), (**C**) FDU-treated (n = 6, 7 days × 0.015 mmol/kg, i.p.), (**D**) (**17**)-treated (n = 6, 7 days × 0.0075 mmol/kg, i.p.), (**E**) (**20**)-treated (n = 6, 7 days × 0.015 mmol/kg, i.p.).

Table 3-6-1. *In vivo* tumor growth delay responses of EMT-6 murine mammary tumors to 3'-*O*-RFDU (**17**) and 3'-*O*-Bu-5'-PFDU (**20**), compared to FDU, RA, and NaBu alone or in combination

	Dose (mmol/kg)	No of Mice	Days±SD for tumor to grow to 100 mm^3 (Growth Delay)	Days±SD for tumor to grow to 200 mm^3 (Growth Delay)	Days±SD for tumor to grow to 300 mm^3 (Growth Delay)	Days±SD for tumor to grow to 400 mm^3 (Growth Delay)	Days±SD for tumor to grow to 500 mm^3 (Growth Delay)
Control		30	9±2	11±2	12±3	14±4	15±4
RA	0.0075	7	9±1 (0)	10±2 (-1)	12±2 (0)	13±3 (-1)	15±5 (0)
RA	0.015	8	9±1 (0)	10±1 (-1)	11±1 (-1)	12±1 (-2)	13±2 (-2)
NaBu	0.0075	8	9±1 (0)	10±2 (-1)	11±2 (-1)	12±2 (-2)	13±3 (-2)
NaBu	0.015	8	9±1 (0)	10±2 (-1)	11±2 (-1)	12±4 (-2)	14±5 (-1)
FDU	0.0075	6	8±0 (-1)	10±1 (-1)	12±1 (0)	14±3 (0)	16±3 (1)
FDU	0.015	6	8±1 (-1)	13±6 (2)	15±6 (3)	18±6 (4)	19±7 (4)
FDU+RA[#]	0.0075	6	14±4 (5)	16±4 (5)	17±5 (5)	18±4 (4)	20±5 (5)
FDU+RA *	0.015	7	13±5 (4)	17±6 (6)	20±5 (8)	23±6 (9)	24±7 (9)
FDU+NaBu	0.0075	8	10±2 (1)	11±3 (0)	12±3 (0)	14±4 (0)	15±4 (0)
FDU+NaBu *	0.015	7	9±1 (0)	10±1 (-1)	11±2 (-1)	14±4 (0)	14±4 (-1)
3'-O-RFDU [#]	0.0075	13	19±9 (10)	22±9 (11)	24±9 (12)	26±9 (12)	30±11 (15)
3'-O-RFDU *, [#]	0.015	6	17±4 (8)	19±6 (8)	21±7 (9)	23±7 (9)	24±6 (9)
3'-O-Bu-O-5'-PFDU *	0.0075	7	13±7 (4)	14±7 (3)	16±7 (4)	17±7 (3)	19±7(4)
3'-O-Bu-5'-PFDU	0.015	14	12±3 (3)	13±3 (2)	16±3 (4)	18±4 (4)	19±5 (4)

* One tumor was cured (CR) and was not counted in this group for tumor growth delay.

[#] One mouse did not develop a palpable tumor after EMT-6 cell implantation and was not counted in this group for tumor growth

Table 3-6-2. Statistical evaluation when tumors grew to the size of 500 mm^3, treated with prodrugs (**17**) and (**20**) and drugs (FDU, RA and NaBu) used alone or in combination

Test Compound	Dose (mmol/kg)	Mean±SD (Day)	P value (unpaired T-test, test compound -treated *vs.* control)	P value (unpaired T-test, test compound -treated *vs.* FDU alone)	P value (Unpaired T-test, test compound -treated *vs.* RA alone)	P value (Unpaired T-test, test compound -treated *vs.* NaBu alone)	P value (Unpaired T-test, prodrug-treated *vs.* Combination -treated)
Control		14.73±3.73					
RA	0.0075	15.14±5.46	0.42781				
	0.015	13.00±1.69	0.03338				
NaBu	0.0075	12.88±2.95	0.07921				
	0.015	13.88±4.97	0.32966				
FDU	0.0075	15.67±2.66	0.24201				
	0.015	18.67±6.98	0.11560				
FDU+RA	0.0075	19.50±4.64	0.02661	0.05859	0.07400		
	0.015	23.57±6.60	0.00570	0.11154	0.00246		
FDU+NaBu	0.0075	14.50±4.14	0.44395	0.26712		0.19140	
	0.015	14.43±4.24	0.43259	0.11539		0.40975	
17	0.0075	30.08±10.87	0.00012	0.00022	0.00035		0.00428
	0.015	24.33±6.47	0.00685	0.08773	0.00348		0.41893
20	0.0075	18.71±6.97	0.09396	0.15810		0.03692	0.09688
	0.015	18.79±4.54	0.00411	0.48521		0.01894	0.02472

CR was also seen in some animals treated with the combination of FDU+NaBu or FDU+RA (see Figures 3-6-2 (C) and 3-6-3 (C)). Yet no CR was observed in the animals treated with any of the drugs (FDU, RA, and NaBu) alone in this study. Although RA and BA were established *in vitro* as effective inhibitor of EMT-6 tumor cells (see Figure 3-4-1 and Table 3-4-2), neither RA or BA showed even moderate anticancer activity *in vivo* based on the mean values of tumor growth delay of EMT-6 tumor (see Table 3-6-1) and unpaired T-test (see Table 3-6-2). RA (7 days × 0.015 mmol/kg, i.p.) was even worse than

control based on the T-test in Table 3-6-2. One possible explanation is that RA may produce severe toxicity which consequently may weaken the mouse immune system. However, moderate tumor growth delay was observed in some individual animals followed by the treatment of either RA or BA (see Figure 3-6-1). FDU (7 days × 0.015 mmol/kg, i.p.) showed moderate anticancer activity against the EMT-6 tumor *in vivo* (see Table 3-6-1). It is evident that pharmacokinetic and metabolic factors played a critical role in determining the *in vivo* responses of the EMT-6 tumor to these drugs. The combined use of FDU with NaBu was not cooperative in this experiment, probably because the plasma concentration of NaBu was too low.

3'-*O*-RFDU (**17**) was the most potent compound among the agents tested in the tumor growth delay study (see Tables 3-6-1 and 3-6-2). Prodrug (**17**) showed marked prolongation of tumor growth delay compared to not only FDU or RA used alone but also the combination of FDU and RA ($P < 0.01$, using unpaired t-test, dose schedule was 7 days × 0.0075 mmol/kg, i.p.). 3'-*O*-Bu-5'-PFDU (**20**) also significantly increased tumor growth delay compared to the combination of FDU and NaBu ($P < 0.05$, using unpaired t-test, dose schedule was 7 days × 0.015 mmol/kg, i.p.). Thus it is demonstrated that the prodrug is potentially better than the combined use of the drugs, as postulated. For further insight into the anticancer activity of the prodrugs and their superiority to the physical combined use of the drugs, studies on responses using other tumor models to evaluate the prodrugs, together with prodrug pharmacokinetic and metabolic studies are necessary.

On the other hand, although the combined use of drugs may have lower anticancer activity than that of the corresponding double-barreled prodrug (see Tables 3-6-1 and 3-6-2), the physical combination of FDU and RA (7 days × 0.015 mmol/kg, i.p.) was shown much better than using the drugs alone ($P < 0.01$, unpaired t-test, see Table 3-6-2). This finding confirms the concept that cytotoxic-differentiation therapy may be synergistic.

It has been reported that, when a solid neoplasm in animals is treated by chemotherapy, the extent of tumor regression might be a poor indicator of the actual decrease of tumor cell viability (Lloyd 1975). In fact, the decrease in cell viability, as determined by bioassay, may be several orders greater than the regression of the tumor

size indicated. Therefore, the log cell kill was estimated using the equation described in the literature (Corbett et al. 1982).

log kill/dose = average tumor growth delay (day) ÷ (3.32 × Td × No. of doses)

where Td is the average doubling time of control tumors, which was estimated as 1.5 days for the growth of EMT-6 tumors from 100 mm^3 to 200 mm^3 in the growth delay experiment. The term "log kill/dose" is defined as follows: log kill/dose = -log F/dose, where F is the fraction of the tumor cells surviving after drug treatment.

Table 3-6-3. Estimation of average log kill/dose and fraction of tumor cell survival (F) in mice after drug treatment

Compound(s)	Dose (mmol/kg)	average growth delay	log kill/dose	F
RA	0.0075	0	N/A *	N/A
	0.015	-1	N/A	N/A
NaBu	0.0075	-1	N/A	N/A
	0.015	-1	N/A	N/A
FDU	0.0075	0	N/A	N/A
	0.015	2	0.057	0.88
FDU+RA	0.0075	5	0.14	0.72
	0.015	7	0.20	0.63
FDU+NaBu	0.0075	0	N/A	N/A
	0.015	-1	N/A	N/A
3'-*O*-RFDU	0.0075	12	0.34	0.45
	0.015	9	0.26	0.55
3'-*O*-Bu-5'-PFDU	0.0075	4	0.11	0.78
	0.015	3	0.086	0.82

* N/A: not available, when tumor growth delay ≤ 0, the log kill/dose cannot be calculated.

3-7. Pharmacokinetic Studies of 3'-*O*-RFDU (17) and its Primary Metabolites FDU and RA in Balb/c Mice Bearing EMT-6 Murine Mammary Tumors

The prodrugs designed in this study were postulated to be biotransformable, with a potential to improve the pharmacokinetics of both active constituent drugs. 3'-*O*-RFDU (**17**) was chosen for the pharmacokinetic study because it showed marked anticancer activity compared to other agents examined in the preliminary experiments, including significant growth inhibition of malignant cells (section **3-2**), delay in the growth of EMT-6 solid tumors (section **3-6**) and low toxicity in normal mice (section **3-5**).

3-7-1. HPLC Separation of 3'-*O*-RFDU (17) and its Metabolites RA and FDU in Biological Samples

A gradient elution reverse phase HPLC method was developed to separate and quantitate 3'-*O*-RFDU (**17**) and its primary metabolites RA and FDU. Samples were analyzed using UV detection at 270 nm for FDU, and at 350 nm for 3'-*O*-RFDU and RA. With the HPLC system used in our lab, this necessitated manually switching the UV detector wavelength from 270 nm to 350 nm once during each run. Figure 3-7-1 shows the representative chromatograms for separation of FDU (at 270 nm), 3'-*O*-RFDU (**17**) (at 350 nm) and RA (at 350 nm) in mouse plasma.

Quantitation was performed using the internal standard method. FDU was quantitated only in plasma, but not in other biological samples, because the high background absorbance of the tissue extracts at 270 nm and the low concentration of FDU released from 3'-*O*-RFDU (**17**) severely affected both qualitative and quantitative detection of FDU. Unfortunately, there is no absorbance for FDU at the higher wavelength such as 350 nm, although the background absorbance was significantly decreased at 350 nm wavelength.

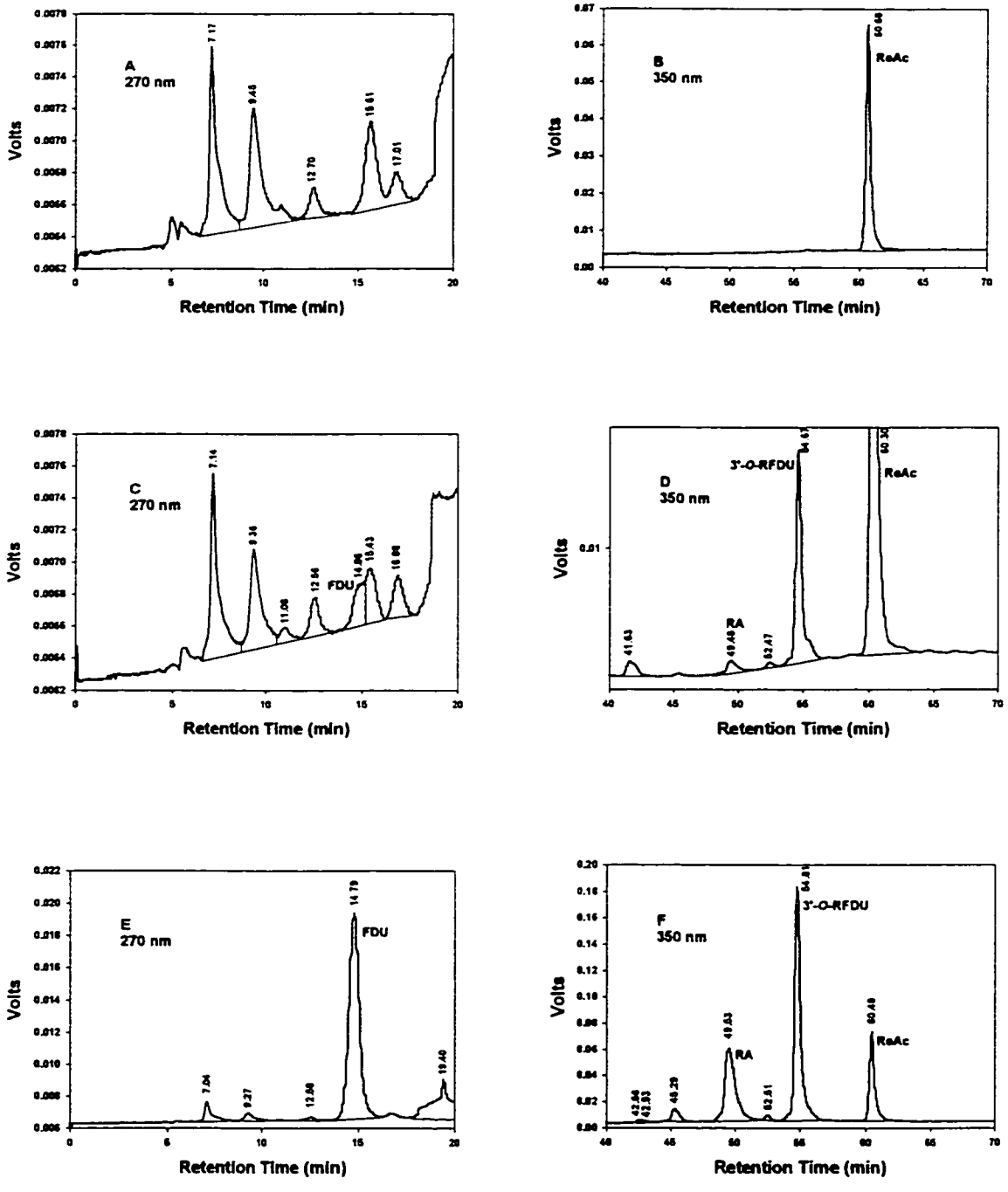


Figure 3-7-1. Representative HPLC chromatograms. (**A**) and (**B**): control plasma at 5 min after i.v. injection of the vehicle; (**C**) and (**D**): 3'-*O*-RFDU (**17**)-treated plasma at 5 min after i.v. injection of 3'-*O*-RFDU (12.5 μmol/kg); (**E**) and (**F**): mouse blank plasma spiked with FDU, 3'-*O*-RFDU, RA and ReAc (internal standard).

3-7-2. Percentage Recovery and Calibration Curves for 3'-*O*-RFDU (17) and its Metabolites RA and FDU in Biological Samples

Percentage recovery for the extraction procedure was determined for each mouse tissue (except urine) by comparing detector response to standards with the response to extracted standards from the spiked tissues.

Table 3-7-2. Recoveries (% mean ± SE[#], n = 6) of ReAc* (internal standard), 3'-*O*-RFDU (17) and its metabolites RA and FDU from spiked mouse tissues

	Plasma	Liver	Lung	Kidney	Tumor
ReAc	99.5±3.18	78.1±3.05	81.2±3.05	77.2±2.59	79.5±2.50
3'-*O*-RFDU	95.2±3.22	84.8±3.45	93.2±9.99	80.1±7.42	79.3±11.4
RA	65.3±7.10	46.9±13.9	65.5±12.0	26.3±8.05	12.2±3.27
FDU	25.1±1.01	21.6±4.91	60.3±8.42	22.1±9.10	11.1±1.18

[#] Standard error
* ReAc = retinyl acetate

Standard solutions used for making the calibration curves were prepared using the same procedures as those used for unknown sample treatment except that known amounts of 3'-*O*-RFDU (17), FDU, RA and ReAc (internal standard) were added to the blank samples. Due to different extraction recoveries for different biological tissues, individual calibration curves were prepared for each tissue type used in this study. The calibration curves for 3'-*O*-RFDU (17), RA and FDU were linear over the range of 0-5 μg per injection. When the calibration data (peak area ratios (area of drug / area of internal standard) versus concentrations) for FDU, RA and 3'-*O*-RFDU (17) were fitted a linear squares regression analysis, the average correlation coefficient ± SD was 0.9950 ± 0.0071.

3-7-3. Assay Precision, Accuracy and Limits of Quantitation for 3'-*O*-RFDU (17) and its Metabolites RA and FDU in Biological Samples

The precision and accuracy of the assay were evaluated by analyzing three standard samples (blank biological samples containing known amounts of 3'-*O*-RFDU (17), FDU, RA and ReAc) for each tissue type on different days. The measured concentrations of each compound were compared to the amount of the compound added (theoretical concentration). The accuracy is presented as the percentage difference from the theoretical concentration. The precision is described as standard deviation for the three measurements. Summaries of assay precision and accuracy are shown in Table 3-7-3.

The limit of detection for 3'-*O*-RFDU (17) was 0.2 μg in 1 mL (3.8×10^{-1} μM) of plasma. The minimum detectable amount for RA was 0.1 μg in 1 mL (3.3×10^{-1} μM) of plasma and for FDU was 0.3 μg in 1 mL (1.2 μM) of plasma.

In conclusion, this HPLC method is suitable for the separation and quantitation of RA and 3'-*O*-RFDU (17) in biological samples, such as liver, lung, kidney, solid tumor and urine. However, only in plasma, FDU, a water-soluble compound, along with the RA and prodrug (17), water-insoluble compounds, could be simultaneously identified and quantified. The results obtained from the recovery, accuracy and precision experiments show that this HPLC method is not ideal for the detection of FDU in biological samples. Since a complicated gradient elution was required for the separation, additional balance time for the column was necessary after each separation was completed, such that two hours was required for each injection of an assay sample.

3-7-4. Time-course of Concentrations for 3'-*O*-RFDU (17), FDU and RA in Different Tissues

Plasma and four tissue concentration-time curves were obtained after an i.v. bolus dose and a plasma concentration-time curve was obtained following a p.o. administration of 3'-*O*-RFDU (17). These time-course curves are shown in Figures 3-7-2 to 3-7-7. Only for plasma were concentrations of FDU measured and the concentration-time profiles of FDU in plasma were shown in Figures 3-7-2 and 3-7-3. 3'-*O*-RFDU (17) or RA could not be detected in urine.

Table 3-7-3. Analytical precision and accuracy for 3'-*O*-RFDU (**17**) and its metabolites, RA and FDU, in different mouse tissues

Tissue	3'-O-RFDU			RA			FDU		
	Theoretical concentration	Concentration (mean±SD, n=3)	Accuracy (%)	Theoretical concentration	Concentration (mean±SD, n=3)	Accuracy (%)	Theoretical concentration	Concentration (mean±SD, n=3)	Accuracy (%)
Plasma	0.19 (μmol/mL)	0.19±0.0083 (μmol/mL)	0.13	0.083 (μmol/mL)	0.10±0.015 (μmol/mL)	26	0.10 (μmol/mL)	0.10±0.0036 (μmol/mL)	0.47
Liver	0.19 (μmol/g)	0.19±0.050 (μmol/g)	2.6	0.083 (μmol/g)	0.073±0.022 (μmol/g)	12	0.10 (μmol/g)	0.11±0.077 (μmol/g)	5.8
Lung	0.19 (μmol/g)	0.19±0.014 (μmol/g)	0.13	0.083 (μmol/g)	0.064±0.033 (μmol/g)	23	0.10 (μmol/g)	0.080±0.037 (μmol/g)	20
Kidney	0.19 (μmol/g)	0.20±0.015 (μmol/g)	2.7	0.083 (μmol/g)	0.10±0.015 (μmol/g)	23	0.10 (μmol/g)	0.36±0.45 (μmol/g)	255
Tumor	0.19 (μmol/g)	0.19±0.029 (μmol/g)	0.74	0.083 (μmol/g)	0.054±0.026 (μmol/g)	34	0.10 (μmol/g)	0.11±0.066 (μmol/g)	5.9
Urine	0.019 (μmol)	0.017±0.0038 (μmol)	13.1	0.0083 (μmol)	0.0082±0.00040 (μmol)	1.5	0.010 (μmol)	ND*	N/A#

* Not detectable
# Not available

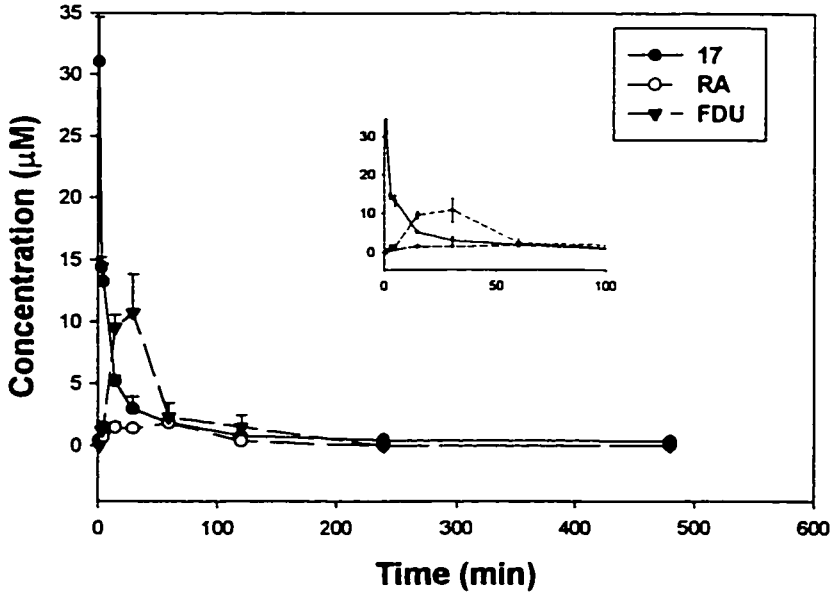


Figure 3-7-2. 3'-*O*-RFDU (17), RA and FDU concentration-time profiles in mouse plasma after a single i.v. bolus injection of (17) (12.5 μmol/kg). Error bars represent SD (n = 3).

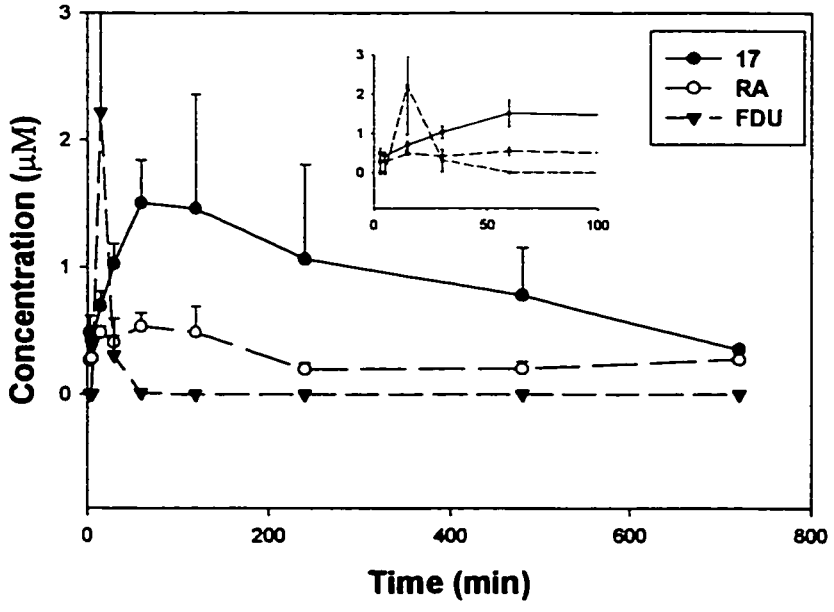


Figure 3-7-3. 3'-*O*-RFDU (17), RA and FDU concentration-time profiles in mouse plasma after a single p.o. dose of (17) (13.7 μmol/kg). Error bars represent SD (n = 3).

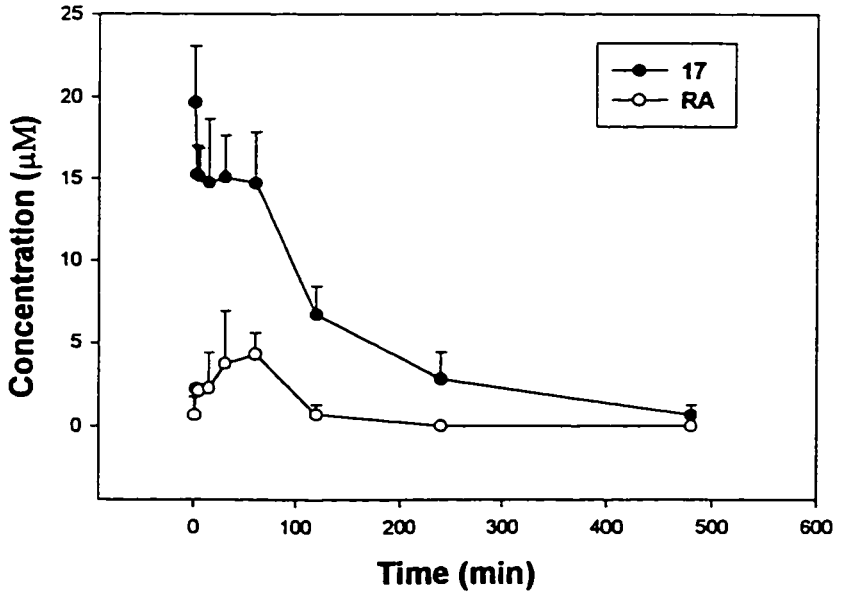


Figure 3-7-4. 3'-*O*-RFDU (**17**) and RA concentration-time profiles in mouse kidney after a single i.v. bolus injection of (**17**) (12.5 μmol/kg). Error bars represent SD (n = 3).

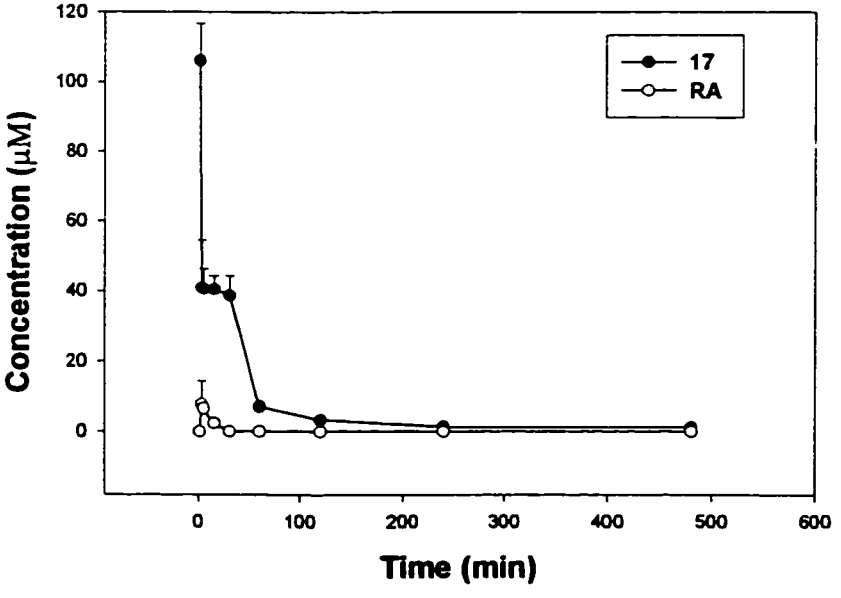


Figure 3-7-5. 3'-*O*-RFDU (**17**) and RA concentration-time profiles in mouse liver after a single i.v. bolus injection of (**17**) (12.5 μmol/kg). Error bars represent SD (n = 3).

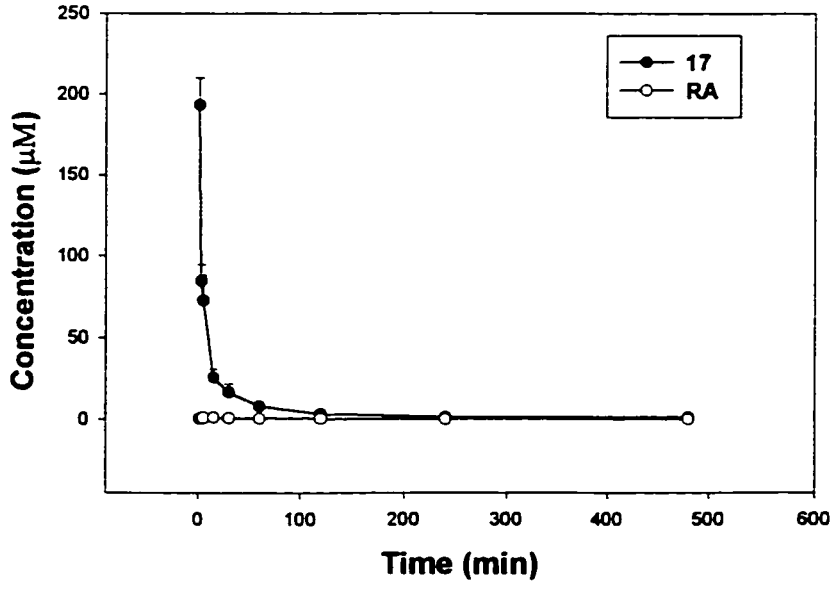


Figure 3-7-6. 3'-*O*-RFDU (17) and RA concentration-time profiles in mouse lung after a single i.v. bolus injection of (17) (12.5 μmol/kg). Error bars represent SD (n = 3).

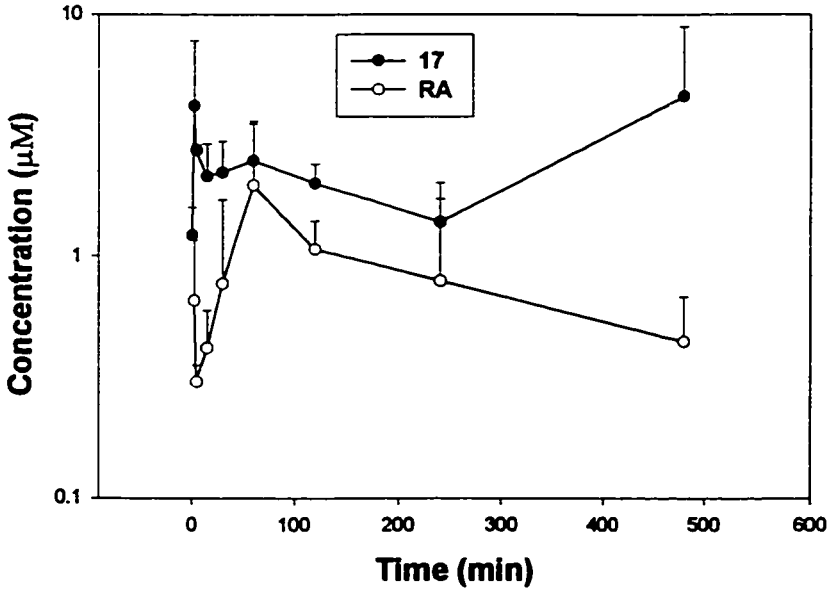


Figure 3-7-7. 3'-*O*-RFDU (17) and RA concentration-time profiles in EMT-6 tumors of mice after a single i.v. bolus injection of (17) (12.5 μmol/kg). Error bars represent SD (n = 3).

3-7-5. Pharmacokinetic Analysis

All pharmacokinetic parameters were calculated based on the mean concentration value (n=3) for each measured time point. The data were analyzed by computer using WinNonlin (Version 1.1, Scientific Consulting Inc.). The concentration-time profiles for 3'-*O*-RFDU (**17**), RA and FDU, following an i.v. bolus injection, are shown in Figure 3-7-2. Concentration-time profiles for 3'-*O*-RFDU (**17**) following an i.v. bolus dose were best fit by an open three-compartment model, whereas concentration-time profiles for both RA and FDU after a single i.v. bolus dose of 3'-*O*-RFDU (**17**) were best described by a one compartment model for metabolite. Therefore, plasma concentration-time profiles are characterized by the following equations:

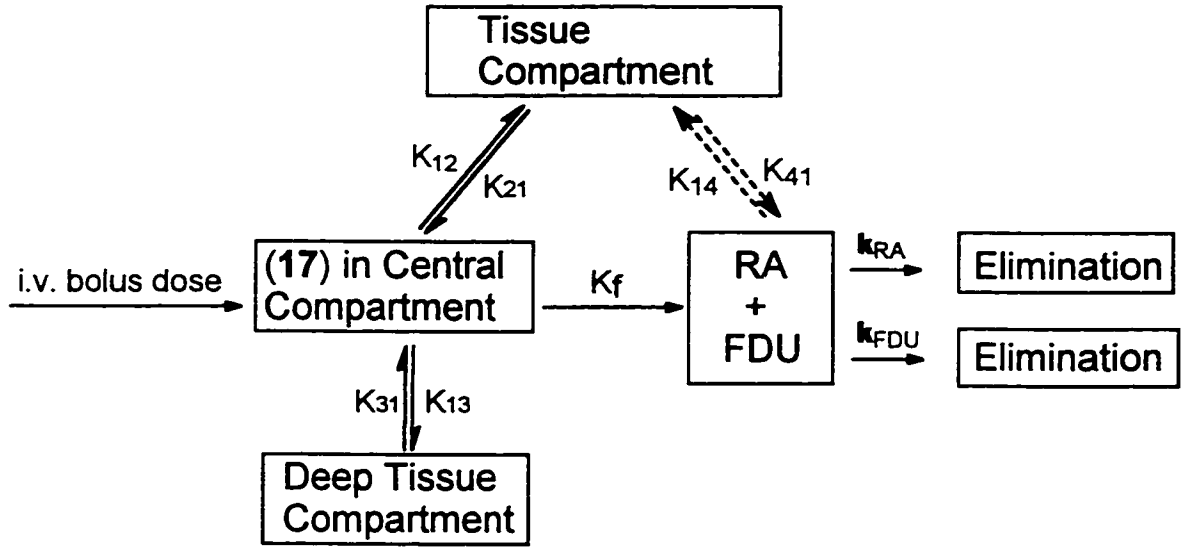
$$C_{17} = A \times EXP(-\alpha t) + B \times EXP(-\beta t) + C \times EXP(-\gamma t)$$

$$C_{RA} = [D \times K_f / V_{RA}(K_f - k_{RA})] \times [EXP(-k_{RA}t) - EXP(-K_f t)]$$

$$C_{FDU} = [D \times K_f / V_{FDU}(K_f - k_{FDU})] \times [EXP(-k_{FDU}t) - EXP(-K_f t)]$$

Where C_{17}, C_{RA}, and C_{FDU} are the concentrations of 3'-*O*-RFDU (**17**), RA and FDU at any time "t", and D is the dose of 3'-*O*-RFDU (**17**). k_{RA} and k_{FDU} are the elimination rate constants for RA and FDU, respectively. K_f is the formation rate constant for RA and FDU, or the hydrolytic rate constant for 3'-*O*-RFDU (**17**). The rate constants K_{12} and K_{21} represent the first order rate transfer constants for the movement of 3'-*O*-RFDU (**17**) from central compartment to tissue compartment (K_{12}) and from tissue compartment to central compartment (K_{21}). The rate constants K_{13} and K_{31} represent the first order rate transfer constants for the movement of 3'-*O*-RFDU (**17**) from central compartment to deep tissue compartment (K_{13}) and from deep tissue compartment to central compartment (K_{31}). The rate constants K_{14} and K_{41} represent the first order rate transfer constants for the movement of FDU+RA released from 3'-*O*-RFDU (**17**) from central compartment to tissue compartment (K_{14}) and from tissue compartment to central compartment (K_{41}). V_{RA} and V_{FDU} are the distribution volumes for RA and FDU, respectively. A, B and C correspond to the y axis intercepts of extrapolated lines for the central, tissue and deep tissue compartments, respectively. α, β and γ are the first-order rate constants for central,

tissue and deep tissue compartments. Parameters calculated based on these models are shown in Table 3-7-5-1 together with the pharmacokinetic parameters of 3'-*O*-RFDU (**17**) and RA in tumor as estimated by a one-compartment model.



Scheme 3-7-5. Compartment model for 3'-*O*-RFDU (**17**) and its primary metabolites RA and FDU.

Table 3-7-5-1. Pharmacokinetic parameters* for 3'-*O*-RFDU (**17**) and its metabolites FDU and RA in mice following a single i.v. bolus dose of 3'-*O*-RFDU (**17**) (12.5 μmol/kg)

	$AUC_{0\rightarrow480}$ (μM/L·min)	CL (mL/min·kg)	Vd (mL)	$T_{1/2\alpha}$ (min)	$T_{1/2\beta}$ (min)	$T_{1/2\gamma}$ (min)	Tmax (min)	Cmax (μM)
3'-O-RFDU	809±241	0.308±0.0920	6.83±1.79	2.05±0.747	25.1±7.13	532±417	0	36.5±9.57
RA	144±27.9	N/A[$]	N/A	N/A	N/A	N/A	21.6±5.91	1.63±0.352
FDU	426±254	N/A	N/A	N/A	N/A	N/A	18.7±25.8	10.8±8.45
3'-O-RFDU(tu[#])	3400±5330	N/A	N/A	N/A	N/A	N/A	8.63±3.90	2.37±0.425
RA(tu[#])	702±418	N/A	N/A	N/A	N/A	N/A	29.2±53.3	1.25±0.383

* Values = means ± SE, n = 3, as determined by WinNonlin

[#] tu = tumor

[$] N/A: not available since there were no sufficient data for calculation.

The concentration-time profiles for 3'-*O*-RFDU (**17**) and its metabolites RA and FDU following a single p.o. dose (13.7 μmol/kg) are shown in Figure 3-7-3. The concentration-time profiles for 3'-RFDU (**17**) following a single oral dose were best described by an open one-compartment model. The pharmacokinetic parameters for 3'-*O*-RFDU (**17**) and RA following a p.o. dose are shown in Table 3-7-5-2. The peak concentration was achieved about 1 hour after p.o. administration. The bioavailability of 3'-*O*-RFDU (**17**) was 90%, as calculated by $AUC_{0\rightarrow720}$(p.o.)/$AUC_{0\rightarrow480}$(i.v.). Although the time periods used for calculation of AUC are different for p.o. and i.v. (720 min versus 480 min), $AUC_{0\rightarrow\infty}$(i.v.)/$AUC_{0\rightarrow480}$(i.v.) ≅ 1 and $AUC_{0\rightarrow\infty}$(p.o.)/$AUC_{0\rightarrow720}$(p.o.) ≅ 1 were estimated by WinNonlin, so that the calculation for the bioavailability of 3'-*O*-RFDU (**17**) is meaningful.

Table 3-7-5-2. Pharmacokinetic parameters* for 3'-*O*-RFDU (**17**) and its metabolite RA in mice following a p.o. dose of 3'-*O*-RFDU (**17**) (13.7 μmol/kg)

	$AUC_{0\rightarrow720}$ (μM/L·min)	Tmax (min)	Cmax (μM)
3'-*O*-RFDU	802±122	62.1±14.0	1.40±0.215
RA	312±125	17.4±8.30	0.394±0.0759

* Values = means ± SE, n=3, as determined by WinNonlin.

3-7-6. Fraction of Dose Remained at the Injection Site (Tail Vein of Mice)

It was important to determine the fraction of 3'-*O*-RFDU (**17**) that might be deposited at the injection site since the tail vein of mice are very small and 3'-*O*-RFDU is very lipophilic. Measurements indicated that less than 0.3% of the dose remained at the injection site (mouse tail vein) as determined by HPLC (mean 0.27%, SD = 0.19, n = 3, Samples were collected at 5 min after an i.v. injection. mean 0.16%, SD = 0.14, n = 3, Samples were collected at 4 hours after an i.v. injection).

3-7-7. Discussion of Pharmacokinetics for 3'-*O*-RFDU (17) and its Metabolites RA and FDU

It was postulated that 3'-*O*-RFDU (17) would be cleaved by esterases *in vivo* to release two active drugs, FDU and RA, which in turn would be converted to a variety of metabolites.

In tumor (Figure 3-7-7), the concentration-time profile for the metabolite RA was essentially parallel to that of 3'-*O*-RFDU (17), which implies that the prodrug may be hydrolyzed in tumor. In addition, based on pharmacokinetic modeling using WinNonlin (shown in Scheme 3-7-5), $K_{12} / K_{14} \approx 19$, $k_{RA} / K_{14} \approx 21$ and $k_{FDU} / K_{14} \approx 7100$ were obtained. Where K_{12} is the transfer rate constant for 3'-*O*-RFDU (17) for the movement of the prodrug from the central compartment to the tissue compartment; K_{14} represents the sum of transfer rate constants of RA and FDU for the movement of RA and FDU from the central compartment to the tissue compartment; k_{RA} is the elimination rate constant of RA; k_{FDU} is the elimination rate constant of FDU. These data indicate that 3'-*O*-RFDU (17) is taken up more readily by tissues than RA and FDU ($K_{12} >> K_{14}$) and RA and FDU are more rapidly eliminated from the circulation system compared to their movement into the tissue ($k_{RA} >> K_{14}$ and $k_{FDU} >> K_{14}$). Therefore, the pharmacokinetic model indicates that the tumor takes up 3'-*O*-RFDU (17) directly and then generates the active drugs (RA and FDU) by hydrolysis locally, rather than absorbing the drugs (RA and FDU) from the blood, since k_{RA}, k_{FDU} and K_{12} are much larger than K_{14}. To further confirm this postulate, the pharmacokinetics for 3'-*O*-RFDU (17) should be determined using a dual labeled compound, such as C-14-labelled FDU and H-3-labelled RA. The parallel decrease of 3'-*O*-RFDU (17) and RA concentrations in tumor and plasma also indicates that the elimination of RA is rate-limited by its formation. This is consistent with the elimination half-life for 3'-*O*-RFDU (17) (532 min) which is much longer than that for RA (19 min); the concentration of 3'-*O*-RFDU (17) was always higher than that of RA (see Figures 3-7-2 and 3-7-7).

Rapid hydrolysis of 3'-*O*-RFDU (17) produced a peak concentration for FDU in approximately 30 min after an i.v. bolus dose. The peak reflects the balance between rates

of formation and elimination. It is known that FDU undergoes rapid elimination ($T_{1/2}$ = 7 min) from the plasma (LaCreta 1987) compared to the $T_{1/2}$ (19 min) for RA (Adamson et al. 1992). However, the concentration of FDU in plasma reached much higher levels than that of RA (Figure 3-7-2). The explanation for this observation could be that the volume of distribution for FDU must be smaller than that for RA, or that FDU tends to bind less to tissues than RA.

The concentration-time profiles for 3'-*O*-RFDU (**17**) in both liver (Figure 3-7-5) and kidney (Figure 3-7-4) following an i.v. bolus dose of compound (**17**) display three distinct phases: an initial rapid distribution phase, a plateau elimination phase and a linear elimination phase. It is possible that hydrolysis of prodrug (**17**) by esterases occurs in a variety of tissues including blood, kidney, liver and tumor. However, the rates of ester hydrolysis are quite different in different organs (Kawaguchi et al. 1985a). The plateau phase was not observed in blood, probably due to the high activity of esterases in the blood (Kawaguchi et al. 1985a). The liver and kidney had the plateau phases for prodrug (**17**) elimination prior to the linear elimination phases, although they lasted only for a very short duration, which indicates that the concentration of the prodrug or the rate of elimination (slope of the curve in Figures 3-7-5 and 3-7-4) at that phase did not change or changed very slowly with time. This is a characteristic of saturated elimination. However, to gain further insight into 3'-*O*-RFDU (**17**) elimination, pharmacokinetic studies at different doses should be performed.

On the other hand, tumor retained prodrug (**17**) for a relatively long time, and consequently appeared to accumulate 3'-*O*-RFDU (**17**). The AUC for prodrug (**17**) in tumor was estimated to be much greater than that in plasma (3400 μM/L·min and 809 μM/L·min). The elimination half-life (532 min) for 3'-*O*-RFDU (**17**) was much longer than that for either FDU ($T_{1/2}$ = 7 min in man) or FU ($T_{1/2}$ = 13 min in man) (Heggie et al. 1987) or RA ($T_{1/2}$ = 19 min in monkey). This implies that the double-barreled prodrug proceeded to a depot, from which the active drugs were released.

Following a p.o. dose, FDU reached its maximum concentration more rapidly than 3'-*O*-RFDU (**17**) (Figure 3-7-3). This observation might be due to lower uptake of FDU by tissues compared with prodrug (**17**) uptake, since the prodrug is more lipophilic than

FDU. In contrast, RA is fat soluble and therefore it is more susceptible to be taken up and metabolized in the tissues, consequently a smaller amount of RA may be released into the blood (Curley et al. 1996) compared to that of FDU. The bioavailability of 3'-*O*-RFDU (**17**) was 90% following a p.o. dose, which implies that first pass metabolism of 3'-*O*-RFDU (**17**) was minor.

3-8. Summary and Conclusions

A group of potential anticancer double-barreled prodrugs have been synthesized. The results of primary *in vitro* anticancer screening showed that retinoyl esters of FDU (**17**, **18**) and 3'-*O*-butanoyl-5'-*O*-*bis*(trichloroethyl)phosphoryl-5-fluoro-2'-deoxyuridine (**20**) are more potent than FDU. There clearly is an additive or synergistic action produced by the drugs generated by these novel prodrugs. Interestingly, it has been reported that all-*trans*-retinoic acid *N*-conjugates of cytosine arabinoside and adenine arabinoside are more potent in HL-60 cell growth inhibition *in vitro*, compared to the parent nucleosides (Manfredini et al. 1997). Effects of 3'-*O*-RFDU (**17**) and 3'-*O*-Bu-5'-PFDU (**20**) on the induction of cell differentiation and cell death in HL-60 APL cells showed that these prodrugs could induce malignant cell differentiation, but instead of terminal differentiation, the cells undergoing differentiation were accompanied with enhanced cell apoptosis which demonstrated that induction of cell differentiation may potentiate cell apoptosis. Apoptosis was the major pathway for HL-60 APL cell death caused by 3'-*O*-RFDU (**17**), which is similar to RA. However, necrosis played an important part in the HL-60 APL cell death upon treatment with 3'-*O*-Bu-5'-PFDU (**20**), which is similar to FDU. The process of HL-60 APL cell death required a certain period of time, which was dependent on the drug. Tumor growth delay experiments showed that cytotoxic-differentiation therapy could be synergistic. The tumor growth delay studies also demonstrated that 3'-*O*-RFDU (**17**) and 3'-*O*-Bu-5'-PFDU (**20**) were better than the combined use of FDU+RA and FDU+NaBu, respectively, which was probably due to improvement of drug pharmacokinetics. Another advantage of the prodrugs (**17**) and (**20**) is that they showed low toxicity to normal mice (see section **3-5**). Both *in vitro* and *in vivo* experiments indicated that, among the novel compounds synthesized in this study, 3'-*O*-RFDU (**17**) was the most potent compound,

and that 3'-*O*-RFDU (17) exhibited a broad spectrum of anticancer activity although the potential anticancer activity of other compounds listed in Table 3-1-1 cannot be ruled out.

The plasma pharmacokinetics for 3'-*O*-RFDU (17) in mice bearing EMT-6 tumors was best described by a three compartment model. Its primary metabolites, FDU and RA, in the plasma fit a one compartment model. 3'-*O*-RFDU (17) had a longer plasma half-life compared to either FDU or RA and had high bioavailability (90%), which implies that the prodrug may be potentially used for p.o. route. Tissue analyses showed that 3'-*O*-RFDU (17) could accumulate in tumor, which may be associated with the significant increase in tumor growth delay, and the rapid elimination of 3'-*O*-RFDU (17) by liver and kidney may also be relevant to the low toxicity of this prodrug observed in the dose escalation experiment and in the examinations of clinical chemistry for serum and histopathology. The prodrug, rather than the released drug in the circulation, being taken up by the tumor may be another reason for the low toxicity to normal tissues.

In conclusion, 3'-*O*-RFDU (17) has been shown to be a bioactivable double-barreled prodrug that releases two active drugs (FDU and RA) which provide cytotoxic-differentiation synergism. 3'-*O*-RFDU is potentially useful for cancer chemotherapy based on its relatively low toxicity, broad spectrum of anticancer activity, potent induction of cell apoptosis and improved pharmacokinetics compared to either FDU or RA.

4. EXPERIMENTAL SECTION

4-1. General Materials and Methods

All reagents used in organic synthesis were of reagent grade quality. All reagents used for *in vitro* tests were cell culture grade quality. All reagents used for the pretreatment of biological tissue samples were reagent grade and solvents used for HPLC were HPLC grade quality. 5-Fluoro-2'-deoxyuridine (FDU), *N,N*-dimethylaminopyridine (DMAP), *bis*(2,2,2-trichloroethyl)phosphorochloridate, all-*trans*-retinoic acid (RA), propidium iodide (PI), Hoechest 33342 (HO342), ammonium acetate (SigmaUltra) and retinyl acetate (ReAc, the internal standard for HPLC analysis) were purchased from the Sigma Chemical Co. Fetal bovine serum (FBS) and trypan blue were purchased from the GIBCO BRL Co. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was kindly provided by the Experimental Oncology Division, Cross Cancer Institute, Edmonton, Alberta. Waymouth cell culture medium was kindly provided by Dr. G. Miller. RPMI 1640 cell culture medium was kindly provided by Dr. Suresh. Butyric anhydride was purchased from the Eastman Kodak Company. All the retinoyl compounds were synthesized under an argon atmosphere in the dark or with the lights dimmed. Sodium butanoate (NaBu) was prepared by the reaction of butyric acid with sodium hydroxide and its purity was confirmed by ^{1}H NMR spectrometry.

Melting points were determined using a Thomas Hoover capillary apparatus and were uncorrected. Nuclear magnetic resonance spectra (^{1}H NMR, ^{13}C NMR, ^{19}F NMR) were acquired using a Bruker AM-300 spectrometer. CF_3CO_2D was used as an internal standard for ^{19}F NMR. The assignments of all exchangeable protons (O*H*, N*H*) was confirmed by addition of D_2O. ^{13}C NMR spectra were acquired using the *J*-modulated spin echo technique where methyl and methine carbon resonances appear as positive peaks and methylene and quaternary carbons appear as negative peaks. Elemental analyses were performed by the Chemistry Department, University of Alberta. Preparative silica gel column chromatography was performed using EM SCIENCE silica gel 60 (70-230 mesh). Thin layer chromatography (TLC) for analysis was performed using Polygram® Sil G/UV_{254} (0.25mm thickness).

HL-60 (ATCC CCL 240) cells and EMT-6 cells were obtained from the Cross Cancer Institute, Edmonton, Alberta. HL-60 cells are a promyelocytic cell line derived from a 36-year-old Caucasian female with acute promyelocytic leukemia (Collins et al. 1977). BDF1 female mice were purchased from Charles River Canada. Balb/c female mice were supplied by HSLAS. The animals used for tumor growth delay experiments were housed in a germ-free room. The animals used for dose escalation and pharmacokinetic studies were housed in conventional animal rooms.

The HPLC analytical system used in this study was composed of two Waters 501 solvent delivery pumps, a Waters 486 tunable absorbance detector, a Waters interface module and a Baseline 810 chromatography data processing system.

4-2. Synthesis of 3'-*O*-Butanoyl-5-fluoro-2'-deoxyuridine (6)

A solution of FDU (102 mg, 0.41 mmol) and triphenylchloromethane (126 mg, 0.44 mmol) in anhydrous pyridine (5 mL) was stirred at room temperature for 6 h, followed by heating at 100 °C for 3 h. After cooling, butyric anhydride (339 μL, 2.2 mmol) was added and the reaction was allowed to proceed for 12 h at room temperature with stirring. After removal of the solvent *in vacuo*, toluene (2 × 5 mL) was added and immediately removed by evaporation. Acetic acid (80% v/v, 4 mL) was added to the residue and the solution was heated at reflux for 10 minutes. After removal of the solvent *in vacuo*, the residue was chromatographed on a silica gel column using gradient elution (0-50% v/v EtOAc in $CHCl_3$), followed by crystallization from water to afford the product (**6**) (31 mg, 24%). mp 135-136 °C (Lit. mp 135-136 °C (Nishizawa et al. 1965)); ^{1}H NMR ($CDCl_3$): δ 0.98 (t, J = 7.4 Hz, 3H, CH_2CH_3), 1.68 (sextet, J = 7.4 Hz, 2H, $CH_2CH_2CH_3$), 2.04 (t, $J_{5',5'OH}$ = 3.6 Hz, 1H, O*H*), 2.26-2.36 (m, 1H, H-2'), 2.35 (t, J = 7.4Hz, 2H, $COCH_2CH_2$), 2.43-2.50 (m, J_{gem} = 14.0 Hz, 1H, H-2'), 3.99 (dd, $J_{5',5'OH}$ = 3.6 Hz, $J_{4',5'}$ = 2.2 Hz, 2H, H-5'), 4.13 (dd, $J_{4',5'}$ = 2.2 Hz, $J_{3',4'}$ = 1.9 Hz, 1H, H-4'), 5.36 (sextet, $J_{2',3'}$ = 6.0 Hz, $J_{3',4'}$ = 1.9 Hz, 1H, H-3'), 6.34 (m, 1H, H-1'), 8.05 (d, $J_{6,5F}$ = 6.3 Hz, 1H, H-6), 8.37 (br s, 1H, N*H*).

4-3. Synthesis of 5'-*O*-Butanoyl-5- fluoro-2'-deoxyuridine (2)

Butyric anhydride (101 μL, 0.62 mmol) was added to a solution of FDU (152 mg, 0.62 mmol) in anhydrous pyridine (8 mL) at -5 °C (NaCl + ice water bath). The reaction was continued at ≤ 0 °C for 24 h. After removal of the solvent *in vacuo*, toluene (2 × 5 mL) was added, and immediately removed by evaporation. The residue was then chromatographed on a silica gel column using gradient elution (0-75% v/v EtOAc in $CHCl_3$), followed by crystallization from water to afford the product (**2**) (45 mg, 23%). mp 110-111 °C (Lit. mp 109 °C (Nishizawa et al. 1965)); ^{1}H NMR ($CDCl_3$): δ 1.00 (t, J = 7.4 Hz, 3H, CH_2CH_3), 1.71 (sextet, J = 7.4 Hz, 2H, $CH_2CH_2CH_3$), 2.14-2.23 (m, 1H, H-2'), 2.38 (s, 1H, O*H*), 2.40 (t, J = 7.4 Hz, 2H, $COCH_2CH_2$), 2.48-2.56 (m, 1H, H-2'), 4.18 (m, 1H, H-4'), 4.31 and 4.48 (two dd, J_{gem} = 12 Hz, $J_{4',5'}$ = 6 Hz, 2H, H-5'), 4.41 (m, 1H, H-3'), 6.26 (m, 1H, H-1'), 7.74 (d, $J_{6,5F}$ = 6 Hz, 1H, H-6), 8.78 (br s, 1H, N*H*).

4-4. Synthesis of 3',5'-Di-*O*-butanoyl-5-fluoro-2'-deoxyuridine (3)

Butyric anhydride (150 μL, 0.92 mmol) was added to a solution of FDU (99 mg, 0.40 mmol) in anhydrous pyridine (8 mL). The reaction was allowed to proceed at 90 °C for 2 h. After removal of the solvent *in vacuo*, toluene (2 × 5 mL) was added and immediately removed by evaporation. The residue was then chromatographed on a silica gel column using gradient elution (0-30% v/v EtOAc in $CHCl_3$), followed by crystallization from ethanol to obtain product (**3**) (105 mg, 68%). mp 116-117 °C (Lit. mp 115-116 °C (Nishizawa et al. 1965)); ^{1}H NMR ($CDCl_3$): δ 0.97 and 0.98 (two overlapping t, J = 7.1 Hz, 6H, $2CH_2CH_3$), 1.68 (two overlapping sextet, J = 7.1 Hz, 4H, $2CH_2CH_2CH_3$), 2.15 (m, 1H, H-2'), 2.32 and 2.34 (two overlapping t, J = 7.1 Hz, 4H, $2COCH_2CH_2$), 2.53 (m, J_{gem} = 14.3 Hz, 1H, H-2'), 4.27-4.34 (m, 2H, H-4', H-5"), 4.42-4.47 (dd, J_{gem} = 12.1 Hz, $J_{4',5'}$ = 2.8 Hz, 1H, H-5'), 5.23 (m, 1H, H-3'), 6.31 (m, 1H, H-1'), 7.68 (d, $J_{6,5F}$ = 6.1 Hz, 1H, H-6), 8.92 (br s, 1H, N*H*).

4-5. Synthesis of 2'-Fluoro-2'-deoxyuridine (7)

2,2'-Anhydro-1-β-D-arabinofuranosyluracil (2.44 g, 10.8 mmol) was dissolved in freshly dried dioxane (225 mL) in a stainless steel pressure reaction container, which was cooled in an acetone-dry ice bath. The air in the container was exhausted *in vacuo* to facilitate transfer of liquid hydrogen fluoride (20 mL), which was condensed from hydrogen fluoride gas in a cylinder, into the reaction container. The container was then tightly closed and heated in an oil bath at 115 ± 5 °C for 18 h. After cooling to room temperature, the reaction mixture was poured into H_2O (80 mL) and neutralized with $CaCO_3$ to pH 6-7. After filtration and removal of the solvent *in vacuo*, the residue was chromatographed on a silica gel column using gradient elution (2-6% v/v methanol in chloroform), followed by crystallization from ethanol to afford the product (7) (1.15 g, 45%). mp 147 °C (Lit. mp 150-151 °C (Codington et al. 1964)); ^{1}H NMR (DMSO-d_6): δ 3.56-3.60 and 3.73-3.78 (two m, J_{gem} = 12.6 Hz, 2H, H-5'), 3.86 (m, 1H, H-4'), 4.10-4.18 (m, $J_{2'F,3'}$ = 21.1 Hz, 1H, H-3'), 5.03 (ddd, $J_{2',2'F}$ = 53.0 Hz, $J_{2',3'}$ = 4.4 Hz, $J_{1',2'}$ = 2.2 Hz, 1H, H-2'), 5.26 (br s, 1H, O*H*-5'), 5.62 (d, $J_{5,6}$ = 8.3 Hz, H-5), 5.69 (d, $J_{3',3'OH}$ = 6.1 Hz, 1H, O*H*-3'), 5.89 (dd, $J_{1',2'F}$ = 17.3 Hz and $J_{1',2'}$ = 2.2 Hz, 1H, H-1'), 7.90 (d, $J_{5,6}$ = 8.3 Hz, 1H, H-6), 11.39 (br s, 1H, N*H*); ^{13}C NMR (DMSO-d_6): δ59.3 (C-5'), 67.4 (d, $J_{3',2'F}$ = 17.4 Hz, C-3'), 83.2 (C-4'), 87.1 (d, $J_{1',2'F}$ = 34.9 Hz, C-1'), 93.5 (d, $J_{2',2'F}$ = 185.2 Hz, C-2'), 101.5 (C-6), 140.3 (C-5), 150.2 (C-2 *C*=O), 163.0 (C-4 *C*=O); ^{19}F NMR (DMSO-d_6): δ -128 (ddd, $J_{2'H,2'F}$ = 53.0 Hz, $J_{2'F,3'}$ = 21.1 Hz, $J_{1',2'F}$ = 17.3 Hz, 1F, F-2').

4-6. Synthesis of 2',5-Difluoro-2'-deoxyuridine (8)

2'-Fluoro-2'-deoxyuridine (7) (0.547 g, 2.2 mmol) in anhydrous acetic acid (70 mL) was placed in a dry, nitrogen flushed, three-necked flask. Fluorine was added to the solution by bubbling 2.5% fluorine in nitrogen into the vessel at room temperature. The reaction was monitored by TLC, using 15% methanol in chloroform as development solvent, until (7) was exhausted. After removal of the solvent *in vacuo*, the residue was coevaporated with ethanol (2 × 5 mL), dried *in vacuo*, followed by adding methanol (100 mL) and 28% ammonia (5 mL). The mixture was stirred at room temperature for 12 h.

After removal of the solvent *in vacuo*, the residue was chromatographed on a silica gel column and eluted with 5% (v/v) methanol in chloroform to afford (**8**) (0.482 g, 82%) as a white foam. ^{1}H NMR (DMSO-d_6): δ3.59-3.63 and 3.79-3.83 (two m, J_{gem} = 11.6 Hz, 2H, H-5'), 3.88 (m, 1H, H-4'), 4.17 (m, $J_{3',2'F}$ = 22.0 Hz, 1H, H-3'), 5.01 (ddd, $J_{2',2'F}$ = 53.0 Hz, $J_{2',3'}$ = 4.4 Hz, $J_{1',2'}$ = 1.7 Hz, 1H, H-2'), 5.41 (br s, 1H, O*H*-5'), 5.73 (d, $J_{3',3'OH}$ = 6.1 Hz, 1H, O*H*-3'), 5.85 (dd, $J_{1',2'F}$ = 16.5 Hz, 1H, H-1'), 8.34 (d, $J_{6,5F}$ = 7.4 Hz, H-6), 11.91 (br s, 1H N*H*); ^{13}C NMR (DMSO-d_6): δ58.7 (C-5'), 66.8 (d, $J_{3',2'F}$ = 16.3 Hz, C-3'), 83.1 (C-4'), 87.0 (d, $J_{1',2'F}$ = 33.8 Hz, C-1'), 93.5 (d, $J_{2',2'F}$ = 185.2 Hz, C-2'), 124.4 (d, $J_{6,5F}$ = 36.0 Hz, C-6), 139.8 (d, $J_{5,5F}$ = 228.8 Hz, C-5), 148.7 (C-2 *C*=O), 157.0 (d, $J_{4,5F}$ = 26.1 Hz, C-4 *C*=O); ^{19}F NMR (DMSO-d_6): δ -93.8 (d, $J_{6,5F}$ = 7.4 Hz, 1F, F-5), -128.7 (ddd, $J_{2',2'F}$ = 53.0 Hz, $J_{3',2'F}$ = 22.0 Hz, $J_{1',2'F}$ = 16.5 Hz, 1F, F-2').

4-7. Synthesis of 3'-*O*-Butanoyl-2',5-difluoro-2'-deoxyuridine (9), 5'-*O*-Butanoyl-2',5-difluoro-2'-deoxyuridine (10) and 3',5'-Di-*O*-butanoyl-2',5-difluoro-2'-deoxyuridine (11)

A solution of 2',5-difluoro-2'-deoxyuridine (**8**) (184 mg, 0.70 mmol) in freshly dried pyridine (20 mL) was stirred 15 min at -10 °C and then butyric anhydride (130 μL, 0.79 mmol) was added. The reaction mixture was maintained at -5 °C for 48 h with stirring. After the solvent was removed *in vacuo*, the residue was coevaporated with toluene (2 × 5 mL) prior to purification by silica gel column chromatography using gradient elution (0-2.5% v/v methanol in $CHCl_3$). Three fractions were separated and collected, which exhibited R_f values (TLC, MeOH:$CHCl_3$, 1:19, v/v, as development solvent) of 0.66 (**11**), 0.38 (**9**) and 0.27 (**10**), respectively (66% total yield).

4-7-1. 3'-*O*-Butanoyl-2',5-difluoro-2'-deoxyuridine (9)

Product (**9**) (44 mg, 19%) was obtained as a white solid upon precipitation from EtOAc-*n*-hexane: mp 155-156 °C; ^{1}H NMR ($CDCl_3$): δ 0.98 (t, J = 7.4 Hz, 3H, CH_2CH_3), 1.68 (sextet, J = 7.4 Hz, 2H, $CH_2CH_2CH_3$), 2.40 (t, J = 7.4 Hz, 2H, $COCH_2CH_2$), 3.13 (br s, 1H, O*H*), 3.82 and 4.05 (two dd, J_{gem} = 12.4 Hz, $J_{4',5'}$ = 1.8 Hz, 2H, H-5'), 4.27 (m,

$J_{3',4'}$ = 5.9 Hz, 1H, H-4'), 5.16-5.40 (m, 2H, H-2', H-3'), 6.01 (dd, $J_{1',2'F}$ = 15.9 Hz, $J_{1',2'}$ = 2.2 Hz, 1H, H-1'), 8.05 (d, $J_{5F,6}$ = 6.2 Hz, 1H, H-6), 9.80 (s, 1H, N*H*); ^{13}C NMR ($CDCl_6$): δ 13.5 (CH_2CH_3), 18.3 (CH_2CH_3), 35.6 ($COCH_2$), 60.5 (C-5'), 69.2 (d, $J_{2'F,3'}$ = 15.4 Hz, C-3'), 82.1 (C-4'), 88.8(d, $J_{1',2'F}$ = 34.0 Hz, C-1'), 91.1 (d, $J_{2',2'F}$ = 194.5 Hz, C-2'), 125.1 (d, $J_{5F,6}$ = 34.6 Hz, C-6), 140.6 (d, $J_{5,5F}$ = 237.5 Hz, C-5), 148.9 (C—2 *C*=O), 157.2 (d, J = 27.0 Hz, C-4 *C*=O), 173.1 (COO); ^{19}F NMR ($CDCl_3$): δ -88.4 (d, $J_{5F,6}$ = 6.2 Hz, 1F, F-5), -127.6 (ddd, $J_{2',2'F}$ = 52.6 Hz, $J_{1',2'F}$ = 15.9 Hz, $J_{3',2'F}$ = 14.7 Hz, 1F, F-2'). Anal. Calcd. for $C_{13}H_{16}F_2N_2O_6$: C, 46.71; H, 4.79; N, 8.38. Found: C, 46.87; H, 4.80; N, 8.00.

4-7-2. 5'-*O*-Butanoyl-2',5-difluoro-2'-deoxyuridine (10)

Product (**10**) (34 mg, 14%) was obtained as a white solid upon precipitation from EtOAc-*n*-hexane: mp 40-60 °C; ^{1}H NMR ($CDCl_3$): δ0.96 (t, J = 7.4 Hz, 3H, CH_2CH_3), 1.67 (sextet, J = 7.4 Hz, 2H, $CH_2CH_2CH_3$), 2.37 (t, J = 7.4 Hz, 2H, $COCH_2CH_2$), 3.37 (br s, 1H, O*H*), 4.25-4.34 (m, 2H, H-3', H-4'), 4.46 (t, $J_{4',5'}$ = 13.1 Hz, 2H, H-5'), 5.08 (m, $J_{2',2'F}$ = 52.6 Hz, 1H, H-2'), 5.91 (dd, $J_{1',2'F}$ = 16.7 Hz, 1H, H-1'), 7.78 (d, $J_{5F,6}$ = 6.0 Hz, 1H, H-6), 9.90 (s, 1H, N*H*); ^{13}C NMR ($CDCl_3$): δ13.5 (CH_2CH_3), 18.3 (CH_2CH_3), 35.8 ($COCH_2$), 61.9 (C-5'), 68.8 (d, $J_{2'F,3'}$ = 16.7 Hz, C-3'), 80.8 (C-4'), 88.9 (d, $J_{1',2'F}$ = 35.4 Hz, C-1'), 93.3 (d, $J_{2',2'F}$ = 187.5 Hz, C-2'), 124.2 (d, $J_{5F,6}$ = 34.6 Hz, C-6), 140.6 (d, $J_{5,5F}$ = 238.0 Hz, C-5), 148.9 (C-2 *C*=O), 157.2 (d, $J_{4,5F}$ = 26.8 Hz, C-4 *C*=O), 173.1 (COO); ^{19}F NMR ($CDCl_3$): δ -88.7 (d, $J_{5F,6}$ = 6.2 Hz, 1F, F-5), -126.6 (ddd, $J_{2',2'F}$ = 52.6 Hz, $J_{2'F,3'}$ = 21.1 Hz, $J_{1',2'F}$ = 16.7 Hz, 1F, F-2'). Anal. Calcd. for $C_{13}H_{16}F_2N_2O_6$: C, 46.71; H, 4.79; N, 8.38. Found: C, 46.62; H, 4.72; N, 8.01.

4-7-3. 3',5'-Di-*O*-butanoyl-2',5-difluoro-2'-deoxyuridine (11)

Product (**11**) (99 mg, 33%) was obtained as a white solid upon precipitation from $CHCl_3$-*n*-hexane: mp 87-88 °C; ^{1}H NMR ($CDCl_3$): δ 0.97 (t, J = 7.4 Hz, 6H, $2CH_2CH_3$), 1.69 (sextet, 4H, $2CH_2CH_2H_3$), 2.37 and 2.40 (two overlapping t, J = 7.4 Hz, 4H, $2COCH_2CH_2$), 4.35-4.50 (m, 3H, H-4', H-5'), 5.07 (ddd, $J_{2'F,3'}$ = 18.6 Hz, $J_{3',4'}$ = 8.2 Hz, $J_{2',3'}$ = 4.5 Hz, 1H, H-3'), 5.26 (dd, $J_{2',2'F}$ = 51.6 Hz, $J_{2'3'}$ = 4.5 Hz, 1H, H-2'), 5.96 (d,

$J_{1',2'F}$ = 16.8 Hz, 1H, H-1'), 7.73 (d, $J_{5F,6}$ = 6.1 Hz, 1H, H-6), 9.16 (s, 1H, N*H*); ^{13}C NMR ($CDCl_3$): δ 13.4 and 13.5 ($2CH_2\mathit{C}H_3$), 18.3 ($2\mathit{C}H_2CH_3$), 35.5 and 35.8 ($2CO\mathit{C}H_2$), 61.4 (C-5'), 68.8 (d, $J_{2'F,3'}$ = 15.8 Hz, C-3'), 78.8 (C-4'), 89.4 (d, $J_{1',2'F}$ = 37.5 Hz, C-1'), 90.9 (d, $J_{2',2'F}$ = 194.2 Hz, C-2'), 123.8 (d, $J_{5F,6}$ = 34.4 Hz, C-6), 140.7 (d, $J_{5,5F}$ = 239.1 Hz, C-5), 148.3 (C-2 *C*=O), 156.4 (d, $J_{4,5F}$ = 27.1 Hz, C-4 *C*=O), 172.5 and 172.8 (2*C*OO); ^{19}F NMR ($CDCl_3$): δ -88.2 (d, $J_{5F,6}$ = 6.1 Hz, F-5), 125.2 (ddd, $J_{2',2'F}$ = 51.6 Hz, $J_{2'F,3'}$ = 18.6 Hz, $J_{1',2'F}$ = 16.8 Hz, 1F, F-2'). Anal. Calcd. for $C_{17}H_{22}F_2N_2O_7$: C, 50.49; H, 5.44; N, 6.93. Found: C, 50.55; H, 5.53; N, 6.81.

4-7-4. Synthesis of 3'-*O*-Butanoyl-2',5-difluoro-2'-deoxyuridine (9)

2',5-Difluoro-2'-deoxyuridine (**8**) (92 mg, 0.35 mmol) and triphenylchloromethane (117 mg, 0.41 mmol) were dissolved in anhydrous pyridine (5 mL). The solution was stirred at room temperature for 6 h, followed by heating at 100 °C for 3 h. After cooling, butyric anhydride (285 μL, 1.9 mmol) was added and the reaction was allowed to proceed for 12 h at room temperature with stirring. After removal of the solvent *in vacuo*, toluene (2 × 5 mL) was added and immediately removed by evaporation. Acetic acid (80% v/v, 4 mL) was added to the residue and the solution was heated at reflux for 10 min. After removal of the solvent *in vacuo*, the residue was chromatographed on a silica gel column using gradient elution (0-50% EtOAc in $CHCl_3$), followed by precipitation using EtOAc-*n*-hexane to afford product (**9**) (41 mg, 35.3%), which was identical (TLC, mp and ^{1}H NMR) to that described in the previous procedure.

4-8. Synthesis of 5'-*O*-Trityl-5-fluoro-2'-deoxyuridine (4)

A solution of FDU (245 mg, 1.0 mmol) and triphenylchloromethane (387 mg, 1.4 mmol) in anhydrous pyridine (8 mL) was stirred at room temperature for 12 h, followed by heating at 100 °C for 3 h. After cooling to room temperature, the reaction mixture was poured onto ice-water (40 mL) with stirring. After removal of the solvent by filtration, the solid was dried *in vacuo*, followed by crystallization from benzene to afford (**4**) (330 mg, 60%). mp 149-151 °C (Lit. mp 150-151 °C (Nishizawa et al. 1965)) ^{1}H NMR (CD_3COCD_3): δ 2.04-2.16 and 2.26-2.36 (two m, 2H, H-2'), 3.16-3.22 and 3.24-3.30

(two dd, J_{gem} = 12 Hz, $J_{4',5'}$ = 3 Hz, 2H, H-5'), 3.95 (m, 1H, H-4'), 4.43 (m, 1H, H-3'), 6.13 (m, 1H, H-1'), 7.06-7.28 (m, 15H, phenyl group), 7.66 (d, $J_{5F,6}$ = 6 Hz), 9.92 (br s, 1H, N*H*).

4-9. Synthesis of 3'-*O*-Retinoyl-5'-*O*-trityl-5-fluoro-2'-deoxyuridine (16)

Oxalyl chloride (82 μL, 0.92 mmol) was added to a solution of RA (185 mg, 0.62 mmol) in dry benzene (10 mL). The reactants were stirred at room temperature for 1 h, followed by removing the solvent *in vacuo*. The residue was dissolved in anhydrous benzene (5 mL). A solution of (**4**) (201 mg, 0.41 mmol) and DMAP (79 mg, 0.64 mmol) in dry benzene (10 mL) was added at 0 °C. The reaction was maintained at 0 °C for 1 h followed by heating at 90 °C for 3 h. The reaction mixture was cooled to room temperature and washed with water (3 × 15 mL). After removal of the organic solvent *in vacuo*, the residue was applied on a silica gel column and eluted with 10% EtOAc in $CHCl_3$. The collected fractions having R_f 0.62 (TLC, EtOAc-*n*-hexane, 1:1 v/v, as development solvent) yielded (**16**) (250 mg, 79%). ^{1}H NMR ($CDCl_3$): δ 1.04 (s, 6H, cyclohexene C-6 *Me*), 1.42-1.68 (m, 4H, cyclohexene H-4 and H-5), 1.72 (s, 3H, cyclohexene C-2 *Me*), 2.00-2.08 (m, 5H, retinoyl C-7 *Me* and cyclohexene H-3), 2.34-2.66 (m, 5H, H-2' and retinoyl C-3 *Me*), 3.34-3.43 and 3.57-3.66 (dd, J_{gem} = 12.0 Hz, $J_{4',5'}$ = 3.0 Hz, 2H, H-5'), 4.21 (m, 1H, H-4'), 5.54 (m, 1H, H-3'), 5.79 (s, 1H, retinoyl H-2), 6.12-6.38 (m, 5H, H-1' and retinoyl H-4, H-6, H-8 and H-9), 7.06 (dd, $J_{4,5}$ = 15.0 Hz, $J_{5,6}$ = 12.0 Hz, 1H, retinoyl H-5), 7.20-7.50 (m, 15H, phenyl group), 7.86 (d, $J_{5F,6}$ = 6.0 Hz, 1H, H-6) 8.14 (s, 1H, N*H*). Product (**16**) was used immediately in the preparation of 3'-*O*-RFDU (**17**).

4-10. Synthesis of 3'-*O*-Retinoyl-5-fluoro-2'-deoxyuridine (17)

A solution of (**16**) (250 mg, 0.32 mmol) in acetic acid (80%, 10 mL) was heated at reflux for 10 min. After removal of the solvent *in vacuo*, the residue was applied on a silica gel column, with gradient elution (0-3% v/v methanol in $CHCl_3$). The collected fractions (R_f 0.39; TLC, EtOAc-$CHCl_3$, 1:1 v/v, as development solvent) were evaporated to dryness *in vacuo*. Precipitation from acetone-H_2O afforded (**17**) (100 mg, 46%) as a

yellow solid: mp 79-80 °C; ^{1}H NMR ($CDCl_3$): δ 1.04 (s, 6H, two cyclohexene C-6 *Me*), 1.46-1.67 (m, 4H, cyclohexene H-4 and H-5), 1.72 (s, 3H, cyclohexene C-2 *Me*), 2.01-2.11 (m, 5H, retinoyl C-7 *Me* and cyclohexene H-3), 2.28-2.54 (m, 5H, H-2' and retinoyl C-3 *Me*), 4.00-4.07 (m, 2H, H-5'), 4.16-4.22 (m, 1H, H-4'), 5.36-5.42 (m, 1H, H-3'), 5.79 (s, 1H, retinoyl H-2), 6.13-6.37 (m, 5H, H-1' and retinoyl H-4, H-6, H-8 and H-9), 7.06 (dd, $J_{4,5}$ = 15.0 Hz, $J_{5,6}$ = 11.4 Hz, 1H, retinoyl H-5), 8.08 (d, $J_{5F,6}$ = 6.3 Hz, 1H, H-6) 9.54 (br s, 1H, N*H*); ^{13}C NMR ($CDCl_3$): δ 13.0 (retinoyl C-7 *Me*), 14.1 (retinoyl C-3 *Me*), 19.3 (cyclohexene C-4), 21.8 (cyclohexene C-2 *Me*), 29.0 (two cyclohexene C-6 *Me*), 33.2 (cyclohexene C-3), 34.3 (cyclohexene C-6), 38.0 (C-2'), 39.7 (cyclohexene C-5), 62.7 (C-5'), 73.7 (C-3'), 85.4 (C-1'), 85.8 (C-4'), 116.8 (retinoyl C-2), 124.5 (d, $J_{5F,6}$ = 34.0 Hz, C-6), 129.2 and 129.3 (retinoyl C-6 and C-9), 130.2 (cyclohexene C-2), 132.0 (retinoyl C-5), 134.5 (retinoyl C-4), 137.1 (retinoyl C-8), 137.6 (cyclohexene C-1), 140.4 (retinoyl C-7), 140.6 (d, $J_{5,5F}$ = 236.4 Hz, C-5), 148.5 (C-2, *C*=O), 155.1 (retinoyl C-3), 156.7 (d, $J_{4,5F}$ = 26.2 Hz, C-4 *C*=O), 166.6 (*C*OO). The assignments of ^{1}H and ^{13}C NMR for the retinoic ester moiety are in accordance with those previously assigned to retinoic acid (Waterhous et al. 1990) and methyl retinoate (Curley et al. 1989). Anal. Calcd. for $C_{29}H_{37}FN_2O_6$: C, 65.83; H, 7.00; N, 5.30. Found: C, 64.42; H, 6.91; N, 5.08.

4-11. Synthesis of 3',5'-Di-*O*-retinoyl-5- fluoro-2'-deoxyuridine (18)

Oxalyl chloride (60 μL, 0.67 mmol) was added to a solution of RA (134 mg, 0.45 mmol) in dry benzene (8 mL). The reactants were stirred at room temperature for 1 h. After removal of the solvent *in vacuo*, the residue was dissolved in anhydrous benzene (5 mL), and then a solution of (**1**) (53 mg, 0.22 mmol) and DMAP (84 mg, 0.68 mmol) in dry benzene (5 ml) was added at 0 °C. The reaction was allowed to proceed at 0 °C for 1 h prior to heating at reflux for 3 h. After removal of the solvent *in vacuo*, the residue was purified by chromatography on a silica gel column with gradient elution (0-15%, v/v, EtOAc in $CHCl_3$). The collected fractions (R_f 0.64; TLC, EtOAc-$CHCl_3$, 1:3 v/v, as development solvent) was evaporated to dryness *in vacuo*, followed by precipitation from acetone-H_2O to afford (**18**) (114 mg, 65%) as a yellow solid: mp 98-100 °C; ^{1}H NMR ($CDCl_3$): δ 1.04 (s, 12H, four cyclohexene C-6 *Me*), 1.46-1.70 (m, 8H, cyclohexene H-4

and H-5), 1.73 (s, 6H, two cyclohexene C-2 *Me*), 2.02-2.23 (m, 11H, H-2', two retinoyl C-7 *Me*, two cyclohexene H-3), 2.37 and 2.40 (two s, 6H, two retinoyl C-3 *Me*), 2.50-2.60 (m, 1H, H-2"), 4.30-4.36 (m, 1H, H-4'), 4.37-4.55 (m, 2H, H-5'), 5.26-5.32 (m, 1H, H-3'), 5.75 and 5.78 (two s, 2H, two retinoyl H-2), 6.13-6.24 (m, 9H, H-1', retinoyl H-4, H-6, H-8 and H-9), 7.00-7.12 (m, 2H, retinoyl H-5), 7.80 (d, $J_{5F,6}$ = 6.0 Hz, 1H, H-6) ; ^{13}C NMR ($CDCl_3$): δ 13.0 (2C, two retinoyl C-7 *Me*), 14.1 (2C, two retinoyl C-3 *Me*), 19.3 (2C, two cyclohexene C-4), 21.8 (2C, two cyclohexene C-2 *Me*), 29.0 (4C, four cyclohexene C-6 *Me*), 33.2 (2C, two cyclohexene C-3), 34.3 (2C, two cyclohexene C-6), 38.3 (C-2'), 39.7 (2C, two cyclohexene C-5), 62.8 (C-5'), 73.3 (C-3'), 83.2 (C-1'), 85.5 (C-4'), 116.0 and 116.6 (two retinoyl C-2), 123.5 (d, $J_{5F,6}$ = 34.9 Hz, C-6), 129.2 and 129.3 (two retinoyl C-6 and two retinoyl C-9), 130.2 (2C, two cyclohexene C-2), 132.1 and 132.3 (two retinoyl C-5), 134.4 and 134.5 (two retinoyl C-4), 137.1 (2C, two retinoyl C-8), 137.7 (2C, two cyclohexene C-1), 140.5 (2C, two retinoyl C-7), 140.7 (d, $J_{5,5F}$ = 238.1 Hz, C-5), 148.5 (C-2 *C*=O), 155.2 and 155.8 (two retinoyl C-3), 156.3 (d, $J_{4,5F}$ = 27.2 Hz, C-4 *C*=O), 166.0 and 166.2 (two *C*OO). Anal. Calcd. for $C_{49}H_{63}FN_2O_7$: C, 72.55; H, 7.83; N, 3.46. Found: C, 72.08; H, 7.85; N, 3.40.

4-12. Synthesis of 5'-*O*-*Bis*(2,2,2-trichloroethyl)phosphoryl-5-fluoro-2'-deoxyuridine (19)

A solution of *bis*(2,2,2-trichloroethyl)phosphorochloridate (312 mg, 0.82 mmol) in dry benzene (5 mL) was added with stirring to a solution of (1) (101 mg, 0.41 mmol) and DMAP (88 mg, 0.71 mmol) in a mixture of anhydrous pyridine (5 mL) and benzene (5 mL). The resulting suspension was stirred at room temperature for 12 h prior to heating at reflux for 3 h. After removal of the solvent *in vacuo*, toluene (2 × 10 mL) was added and immediately removed by evaporation, and then the residue was loaded on a silica gel column with gradient elution (1-5% v/v methanol in $CHCl_3$). Collected fractions (R_f 0.43; TLC, EtOAc as development solvent) were evaporated to dryness *in vacuo*, followed by crystallization from $CHCl_3$ to afford (**19**) (84 mg, 35%) as a white solid: mp 148-149 °C; ^{1}H NMR (CD_3OD): δ 2.20-2.40 (m, 2H, H-2'), 4.06-4.15 (m, 1H, H-4'), 4.38-4.52 (m, 3H, H-3', H-5'), 4.76-4.84 (m, 4H, two Cl_3CCH_2O), 6.23 (dd, $J_{1',2'} = J_{1',2''}$ = 6.7 Hz, 1H,

H-1'), 7.83 (d, $J_{5F,6}$ = 6.6 Hz, 1H, H-6); ^{13}C NMR (CD_3OD): δ 40.5 (C-2'), 69.9 (d, $J_{5',P}$ = 5.9 Hz, C-5'), 71.6 (C-3'), 78.5 (d, $J_{CH2O,P}$ = 4.3 Hz, 2C, two CH_2OP), 86.1 (d, $J_{4',P}$ = 7.6 Hz, C-4'), 87.2 (C-1'), 95.9 (d, $J_{Cl3C,P}$ = 10.8 Hz, 2C, two Cl_3C), 125.9 (d, $J_{5F,6}$ = 34.9 Hz, C-6), 141.9 (d, $J_{5,5F}$ = 234.2 Hz, C-5), 150.6 (C-2 *C*=O), 159.3 (d, $J_{4,5F}$ = 25.1 Hz, C-4). Anal. Calcd. for $C_{13}H_{14}FN_2O_8P$: C, 26.49; H, 2.38; N, 4.75. Found: C, 26.60; H, 2.44; N, 4.61.

4-13. Synthesis of 5'-*O*-*Bis*(2,2,2-trichloroethyl)phosphoryl-3'-*O*-butanoyl-5-fluoro-2'-deoxyuridine (20)

Butyric anhydride (96 μL, 0.587 mmol) was added to a solution of (**19**) (80 mg, 0.136 mmol) in anhydrous pyridine (10 mL) and the reaction was allowed to proceed at room temperature for 12 h. After removal of the solvent *in vacuo*, toluene (2 × 10 mL) was added and immediately removed by evaporation. The residue was applied to a silica gel column with gradient elution (0-3% v/v methanol in $CHCl_3$). Collected fractions (R_f 0.41; TLC, MeOH-$CHCL_3$, 1:19 v/v, as development solvent) were evaporated to dryness *in vacuo*, followed by crystallization from EtOH-H_2O to afford (**20**) (79 mg, 88%) as white needles: mp 118-119 °C; ^{1}H NMR ($CDCl_3$): δ 0.98 (t, *J* = 7.4 Hz, 3H, CH_2CH_3), 1.68 (sextet, *J* = 7.4 Hz, 2H, $CH_2CH_2CH_3$), 2.13-2.23 (ddd, J_{gem} = 14.3 Hz, $J_{2',3'}$ = 9.1 Hz, $J_{1',2'}$ = 6.9 Hz, 1H, H-2'), 2.35 (t, *J* = 7.4 Hz, 2H, $COCH_2CH_2$), 2.48-2.55 (ddd, J_{gem} = 14.3 Hz, $J_{2'',3'}$ = 5.5 Hz, $J_{1',2''}$ = 1.4 Hz, 1H, H-2'), 4.22 (m, 1H, H-4'), 4.46-4.63 (two m, J_{gem} = 11.3 Hz, 2H, H-5'), 4.63-4.76 (m, 4H, two Cl_3CCH_2O), 5.33 (m, 1H, H-3'), 6.32-6.37 (m, 1H, H-1'), 7.73 (d, $J_{5F,6}$ = 6.1 Hz, 1H, H-6), 8.90 (br s, 1H, N*H*); ^{13}C NMR ($CDCl_3$): δ 13.6 (CH_2CH_3), 18.3 (CH_2CH_3), 35.9 ($COCH_2$), 37.4 (C-2'), 68.3 (d, $J_{5',P}$ = 6.5 Hz, C-5'), 73.8 (C-3'), 77.4 (CH_2OP, overlapping with solvent $CDCl_3$), 83.1 (d, $J_{4',P}$ = 7.6 Hz, C-4'), 85.2 (C-1'), 94.4 (d, $J_{Cl3C,P}$ = 10.9 Hz, Cl_3C), 123.3 (d, $J_{5F,6}$ = 34.9 Hz, C-6), 140.7 (d, $J_{5,5F}$ = 239.7 Hz, C-5), 148.6 (C-2 *C*=O), 156.2 (d, $J_{4,5F}$ = 27.2 Hz, C-4 *C*=O), 173.1 (COO). Anal. Calcd. for $C_{17}H_{20}FN_2O_9PCl_6$: C, 30.99; H, 3.04; N, 4.25. Found: C, 31.32; H, 2.81; N, 4.16.

4-14. *In Vitro* Cytotoxic Screening

The *in vitro* cytotoxic screen was performed by the United States National Cancer Institute (Skehan et al. 1990; Monks et al. 1991). Briefly, a total of 60 human tumor cell lines derived from nine major cancer types (leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, breast cancer) were used in this screen. A stock solution of each test compound was prepared in dimethyl sulfoxide (DMSO) together with the reference compounds FDU and FU. Each compound was tested at five concentrations (10-fold dilution with culture medium). The cells were plated in 96-well plates at densities between 5,000 to 40,000 cells/100 μl per well depending on the cell line and its growth characteristics. After pre-incubation for 24 hours, the tested compound was added. The cells were exposed to the test compound for 48 hours, and then the cells fixed with trichloroacetic acid were stained with sulforhodamine B (SRB) which binds to the basic amino acids of the cellular macromolecules. The unbound SRB was removed by washing with acetic acid (1%). The bound dye was solubilized in Tris buffer and measured spectrophotometrically on an automatic plate reader, followed by computerized data acquisition.

All of the growth inhibition versus dose data for the subpanels of the cancer cell lines were pooled according to their major types of cancer, consequently the average growth inhibition for each major cancer versus dose of each compound was obtained.

4-15. Effects of 3'-*O*-RFDU (17) and 3'-*O*-Bu-5'-PFDU (20) on Induction of Cell Differentiation in HL-60 APL cells

HL-60 APL cells were cultivated in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO). The cells were grown at 37 °C in a humidified air atmosphere with 5% CO_2. Cell density was determined by a Coulter counter (Coulter Electronics, Ltd.) and cell viability was estimated by trypan blue dye exclusion. The cells were harvested by centrifugation and resuspended in the growth medium at a density of 2×10^5 cells/mL, followed by dispensing 1 mL/well into 24-well culture plates. After 24 h of incubation, freshly prepared agents dissolved in DMSO were added with various concentrations; from 10^{-8} M to 10^{-5} M for compounds **(17)**, **(20)** and RA; from

10^{-6} M to 10^{-3} M for NaBu, in 10-fold increments. The final concentration of DMSO was less than 0.1% (v/v). Culture plates were then incubated for 7 days at 37 °C in a humidified air atmosphere with 5% CO_2 prior to morphological analysis using light microscopy. Differentiation was assessed morphologically by counting on Wright-stained cytospin preparations. Differential counts were always blind-scored on at least 200 cells per slide unless there were fewer than 200 cells on the plate, which occurred especially at high concentration of the agents. Representative pictures of HL-60 cells after exposure to the agents are shown in Figure 3-3-1, together with the blank control. The percentage differentiation versus dose for each agent is shown in Figure 3-3-2.

4-16. Effects of 3'-*O*-RFDU (17) and 3'-*O*-Bu-5'-PFDU (20) on Induction of Cell Death in HL-60 APL Cells

A stock solution of HO342 (0.5 mM) was prepared in distilled water. It was diluted to 0.1 mM with phosphate-buffered saline (Ca^{++} and Mg^{++} free) (PBS) just before use. Propidium iodide (0.03 mM) was freshly prepared in PBS. The fixative was a solution of 25% (v/v) ethanol in PBS.

HL-60 APL cells were cultivated in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO). Cells were grown at 37 °C in a humidified air atmosphere with 5% CO_2. The cell density was determined using a Coulter counter (Coulter Electronics, Ltd.) and cell viability was estimated by trypan blue dye exclusion. Cells were harvested by centrifugation and resuspended in the growth medium at a density of 2×10^5 cells/mL, followed by dispensing 1 mL/well into 24-well culture plates. After 24 hours incubation, the freshly prepared test compounds (17), (20) and RA dissolved in DMSO and test compounds FDU and NaBu dissolved in PBS were added at concentrations of 10^{-6} M and 10^{-5} M for compounds (17), (20), RA and FDU and 10^{-3} M for NaBu. The final concentration of DMSO was less than 0.1% (v/v). Culture plates were incubated for 1 day, 2 days and 7 days, respectively, at 37 °C in a humidified air atmosphere with 5% CO_2. Cells were then centrifuged and the medium was decanted. The cells were mixed with PI (100 μL) and placed on ice for 30 min. Following addition of fixative (1.9 mL) and vortex mixing for a few seconds, HO342 (0.1 mM, 50 μL) was

added and mixed by vortex for a few seconds. The sample was placed on ice for at least 30 min before the measurement of fluorescence intensity emitted from the DNA-HO342 or DNA-PI using a flow cytometer (Coulter Elite Inc.) at the Cross Cancer Institute, Edmonton, Alberta.

4-17. Responses of EMT-6 Murine Mammary Cancer Cells to RA and NaBu

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (50 mg) was dissolved in PBS (10 mL) to prepare a stock solution, which was diluted to 0.5 mg/mL with cell culture medium just prior to use. The solvent for dissolving reduced MTT was isopropanol (100 mL) acidified with HCl (0.3 mL).

EMT-6 murine mammary cancer cells were cultivated as monolayer in Waymouth medium supplemented with 13% FBS (GIBCO). Cells were grown at 37 °C in a humidified air atmosphere with 5% CO_2 and were subcultured twice a week. Cells were suspended by a 5 min incubation with 0.05% trypsin in Hanks' buffer (Ca^{++} and Mg^{++} free) at room temperature, followed by gentle pipetting. The cell density was determined using a Coulter counter and cell viability was estimated by trypan blue dye exclusion. The cells were harvested by centrifugation and resuspended in growth medium at a density from 1.47×10^5 cells/mL to 4,600 cells/mL in two-fold decrements, followed by dispensing 0.1 mL/well into a 96-well culture plate for detecting the linearity of the method. After incubation for 19 h, the culture medium was removed by inverting, flicking and blotting the plate. MTT (0.5 mg/mL, 100 μL) was added to the cells in each well, followed by incubation for 3 h at 37 °C in a humidified air atmosphere with 5% CO_2. The supernatant was removed by inverting and flicking. The reduced MTT was dissolved in acidified isopropanol (0.1 mL/well) and absorbance was measured at 540 nm using an automatic plate reader. The linearity in this experiment is shown in Figure 3-4-1.

EMT-6 cells were trypsinized, harvested by centrifugation and resuspended in growth medium at a density from 4.1×10^4 cells/mL, followed by dispersing 0.2 mL/well into 96-well culture plates. After incubation for 20 h, freshly prepared RA dissolved in DMSO and NaBu dissolved in PBS were added at various concentrations from 10^{-7} M to 10^{-4} M for RA and from 10^{-5} M to 10^{-2} M for NaBu, in 10-fold increments. The final

10^{-4} M for RA and from 10^{-5} M to 10^{-2} M for NaBu, in 10-fold increments. The final concentration of DMSO was less than 0.1% (v/v). Culture plates were incubated for 36 h and 80 h, respectively at 37 °C in a humidified air atmosphere with 5% CO_2. After removal of culture medium by inverting, flicking and blotting the plate, MTT (0.5 mg/mL, 100 μL) was added to the cells in each well, followed by incubation for 3 h at 37 °C in a humidified air atmosphere with 5% CO_2. The supernatant was removed by inverting and flicking and the reduced MTT was dissolved in acidified isopropanol (0.1 mL/well). The absorbance was measured at 540 nm on an automatic plate reader. The dose-response curves obtained are shown in Figure 3-4-2.

4-18. Dose Escalation and Drug Toxicity (Clinical Chemistry and Gross Pathological and Histopathological Postmortem Examination) Studies in Healthy BDF1 Female Mice

Test compounds, which included prodrugs (**17**) and (**20**) together with the reference agents RA, NaBu and FDU alone or in combination, were administered by intraperitoneal injection (i.p.) starting from the dosage regimen of 7 days × 0.0001 mmol/kg, in two-fold increment (i.e. 7 days × (0.0001 mmol/kg × 2^n), n = 0, 1, 2, 3, 4, 5, 6, 7), until the highest dosage (7 days × 0.013 mmol/kg) was reached. The test compounds were administered for 7 consecutive days to healthy female BDF1 mice (15-18 g). Three mice were used for each dose. The mice were observed to identify any acute or chronic toxicity, including restlessness, lethargy, inappropriate aggression, wobbling gait and weight loss, until euthanasia at the 60th day after dosing. Because inappropriate aggression was observed for the mice receiving RA either alone or in combination with FDU, probably due to toxic irritation of RA, the dose escalation study involving RA was suspended midway without completion. After euthanasia, the groups of mice that had received the dose (7 days × 0.013 mmol/kg) were subjected to clinical chemistry and gross pathological and histopathologic postmortem examinations at HSLAS. Briefly, the drug-treated female BDF1 mice, together with the controls, were euthanised with CO_2 and a cardiac bleeding was performed immediately. Blood was collected into a serum tube. After centrifugation, the serum was used for clinical chemistry examination. The clinical chemistry data are

shown in Table 3-5-1. A set of tissues including heart, lung, liver, spleen, kidney, brain, gonads, intestine and sternal bone marrow were obtained and immediately fixed in 10% neutral buffered formalin. The tissues were then processed into paraffin blocks, sectioned at 5 microns and stained with hematoxylin and eosin. The histopathologic examinations were performed on each of the tissue types.

4-19. Responses of EMT-6 Murine Mammary Tumor to 3'-*O*-RFDU (17) and 3'-*O*-Bu-5'-PFDU (20) Compared to RA, NaBu and FDU Alone or in Combination

EMT-6 murine mammary tumor cells were cultivated in monolayer in RPMI 1640 medium supplemented with 10% FBS (GIBCO). The cells were grown at 37 °C in a humidified air atmosphere with 5% CO_2. For the growth delay experiments, 1×10^6 EMT-6 tumor cells were implanted subcutaneously in the flanks of Balb/c female mice (17-21 g, age of 10-14 weeks).

FDU and NaBu were dissolved in Hanks' buffer. RA, 3'-*O*-RFDU (**17**) and 3'-*O*-Bu-5'-PFDU (**20**) were first dissolved in DMSO followed by dilution with 50% PEG 400 in Hanks' buffer. After tumor implantation for 24 h, the compounds (FDU, RA or NaBu alone or in combination) and the prodrugs (**17**) and (**20**) were injected i.p. at the doses of 0.0075 mmol/kg and 0.015 mmol/kg, respectively, for 7 consecutive days. Tumors were measured along 3 perpendicular diameters with a pair of calipers, usually at least once every other day from the seventh day after tumor implantation, until the tumor volume exceeded approximately 800 mm^3 or gross hemorrhage and tumor infiltration appeared. Tumor volumes were calculated using the volume formula for hemiellipsoids: $V=1/2(4\pi/3)(L/2)(W/2)(H)$, where L, W, and H are length, width and height of the tumor, respectively (Rockwell et al. 1972). The growth delay data are shown in Figures 3-6-1 to 3-6-5 and Table 3-6-1.

4-20. Pharmacokinetic Studies for 3'-*O*-RFDU (17)

4-20-1. Chemical Preparation

3'-*O*-RFDU (**17**) was dissolved in DMSO to prepare a stock solution, which was protected from air oxidation by purging storage vials with argon prior to storage at -80 °C

until use. The solution of (**17**) used for dosing was freshly prepared immediately prior to intravenous (i.v.) or oral (p.o.) administration. A stock solution of (**17**) was diluted to the required concentration with 30% Hanks' buffer in PEG 300 for the i.v. injection or diluted with PEG 300 alone for p.o. administration. The final solution contained 10% DMSO. The chemical purity of compound (**17**) in the dosing solution was checked by HPLC for the drug dose calibration. Because of the facile photo decomposition and oxidation of 3'-*O*-RFDU (**17**) and RA, all procedures involving compounds 3'-*O*-RFDU and RA were performed under dim fluorescent or yellow light. Exposure of 3'-*O*-RFDU or RA to air was minimized.

4-20-2. Cells and Animals

EMT-6 murine mammary tumor cells were cultivated as described in section **4-19**. For the pharmacokinetics experiment, 1×10^6 EMT-6 tumor cells were implanted subcutaneously into the flank of Balb/c female mice.

Female Balb/c mice were supplied by HSLAS. The mice were maintained with food and water available *ad libitum*. They weighed 19-21 g when used for the experiments.

Female Balb/c mice (HSLAS) for oral dosing were deprived of food for 24 hours, but with water *ad libitum*, prior to drug administration by gavage.

4-20-3. Animal Dosing and Sample Collection

3'-*O*-RFDU (**17**) was administered as either a single i.v. bolus dose (12.5 μmol/kg) via the tail vein, or p.o. (13.7 μmol/kg), when the tumors had grown to about 100 mm^3. Blood was collected from the mice via cardiac puncture with a heparinized syringe immediately following the euthanasia with carbon dioxide, and stored on ice in foil-wrapped containers prior to centrifugation. Plasma was prepared by centrifugation of the blood at 10,000 *g* for 15 min at 5 °C. The plasma was then transferred to a 0.6 mL Eppendorf tube and stored in the dark at -80 °C until further treatment for HPLC analysis. Tumor, liver, lung and kidney were collected and immediately placed in Eppendorf tubes on dry ice, and then stored at -80 °C in the dark till further treatment for HPLC analysis. Urine was collected on filter paper which was then put into a 50 mL round bottom flask.

Acetonitrile (15 mL) and the internal standard (ReAc, 2.5 μg) were added into the flask containing the filter paper, which were then stored in a dark room at 5 °C for 12 h before further treatment. Three mice, plus one mouse for the blank control, were used for each time point.

4-20-4. Sample Processing

1. Plasma (100 μL) containing internal standard (ReAc, 2.5 μg) was mixed with acetonitrile (5 mL). The mixture was centrifuged for 10 min at 300 *g* after 10 seconds vortex mixing. The supernatant was transferred to a 10 mL flask. After removal of the solvent *in vacuo*, ethyl acetate (250 μL) was added. The solution was transferred to an Eppendorf tube and stored at -20 °C overnight. The samples were centrifuged for 2 min at 10,000 *g* before 50 μL of clear supernatant was taken for HPLC injection.
2. Tissue samples (liver, kidney, lung, tumor and tail) were weighed before adding internal standard (2.5 μg) and 1M sodium acetate (100 μL). The samples were homogenized and extracted with acetonitrile (2 × 2.5 mL) at 5 °C. Two fractions of solution were collected and centrifuged for 10 min at 300 *g*. The supernatant was transferred to a 10 mL round bottom flask. After removal of the solvent *in vacuo*, ethyl acetate (250 μL) was added and the solution was stored in an Eppendorf tube at -20 °C overnight. The samples were centrifuged for 2 min at 10,000 *g* before 50 μL of clear supernatant was taken for HPLC injection.
3. Urine extract (10 mL) was transferred to a 25 mL round bottom flask. After removal of the solvent *in vacuo*, ethyl acetate (250 μL) was added and the solution was stored in an Eppendorf tube at -20 °C overnight. The samples were centrifuged for 2 min at 10,000 *g* before 50 μL of clear supernatant was taken for HPLC injection.

4-20-5. HPLC Conditions

A Waters reverse Radial-Pak cartridge column, 8C1810 (μBondapak, C18) or 8MBC1810 (Resolve, C18), preceded by a guard column was used. The mobile phase consisted of 3% acetonitrile in 1% of aqueous ammonium acetate (A) and acetonitrile (B) using the gradient elution profile shown in Table 4-20-5.

Table 4-20-5. Gradient composition of solvents A and B for separation of FDU, RA and 3'-*O*-RFDU (17) in biological samples

Time (min)	Flow (mL/min)	%A	%B	Crv*
0	0.8	100	0	
20	0.8	100	0	6
52	0.8	6.0	94	5
62	0.8	6.0	94	6
65	0.8	0	100	6
72	1.5	0	100	6
90	1.5	0	100	6
97	0.8	0	100	6
115	0.8	100	0	6
145	0.8	100	0	6

* In the Baseline 810 chromatography processing system, the Crv "6" program performs the linear change of the elution solvent gradient, and Crv "5" produces a non-linear change.

FDU was detected at a wavelength of 270 nm. RA, 3'-*O*-RFDU (**17**) and ReAc (internal standard) were detected at 350 nm. The wavelength was switched from 270 nm to 350 nm at about 25 ± 5 min during each run in order to facilitate the detection of FDU, RA, 3'-*O*-RFDU (**17**) and ReAc. Quantitation was performed using the internal standard method. FDU was determined only in the plasma, but not in the other biological samples, because of the low concentration of FDU and the high background absorbance of the tissue extracts at 270 nm.

4-20-6. Calibration Curves for 3'-*O*-RFDU (17), RA and FDU

The calibration curves were prepared for each tissue independently. The standard samples were prepared using the same procedure as used for unknown samples described in sections **4-20-4** and **4-20-5**. The calibration curves are shown in Figures 4-20-6-1 to 4-20-6-17.

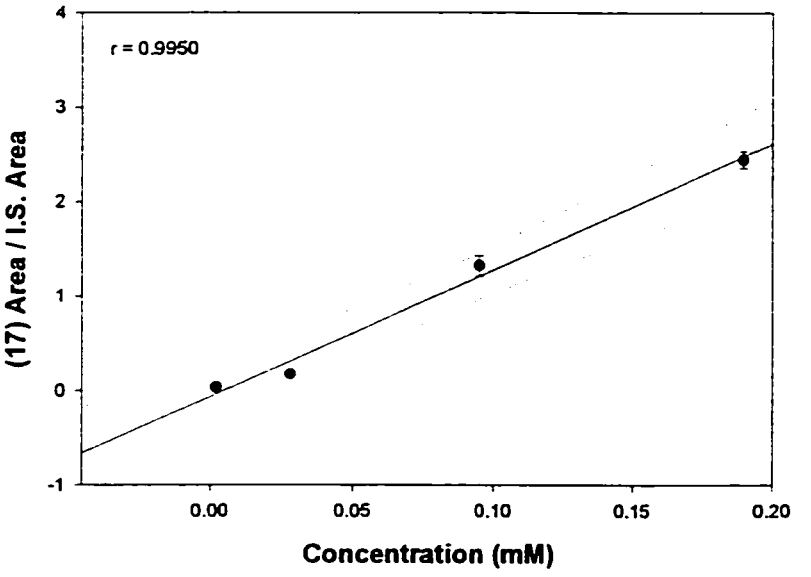


Figure 4-20-6-1. Calibration curve for 3'-*O*-RFDU (**17**) in mouse plasma, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

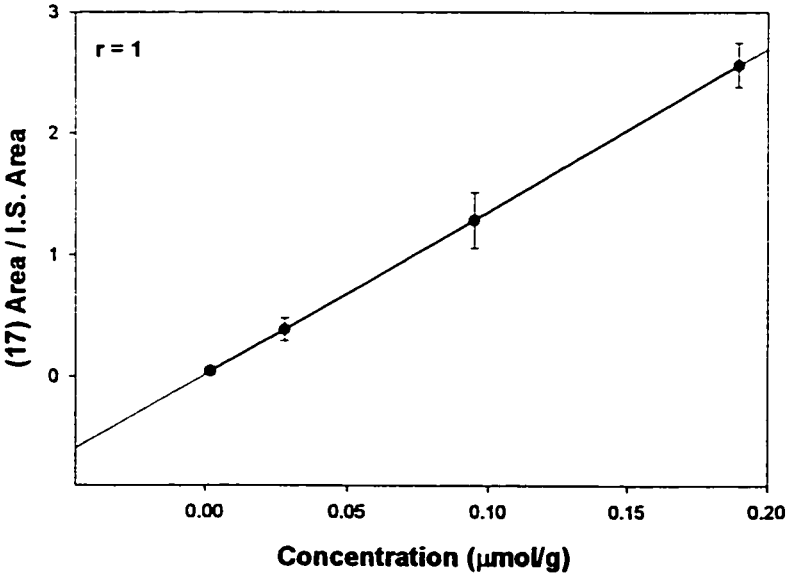


Figure 4-20-6-2. Calibration curve for 3'-*O*-RFDU (**17**) in mouse lung, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. Error bars represent SD.

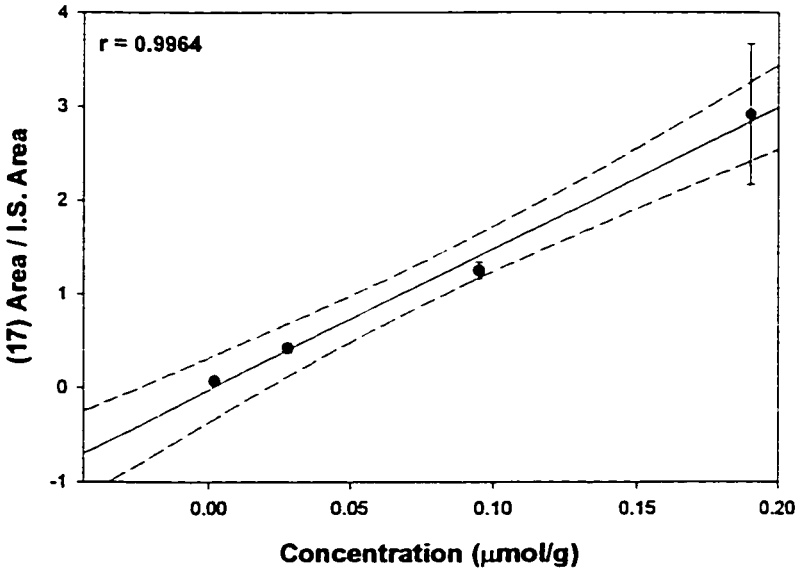


Figure 4-20-6-3. Calibration curve for 3'-*O*-RFDU (**17**) in mouse liver, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bar represents SD.

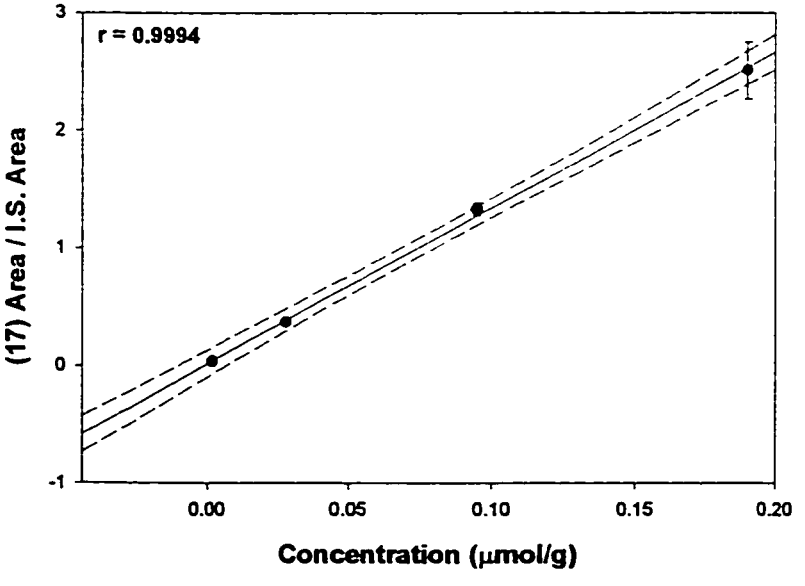


Figure 4-20-6-4. Calibration curve for 3'-*O*-RFDU (**17**) in mouse kidney, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

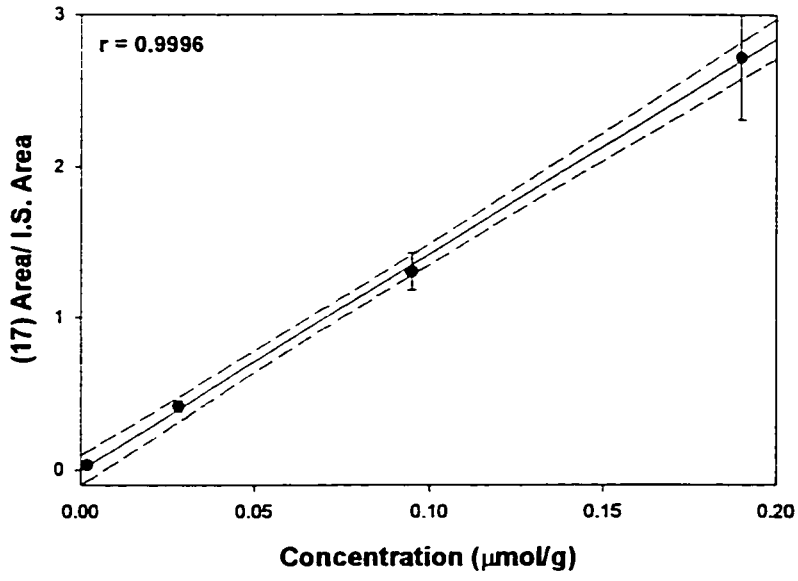


Figure 4-20-6-5. Calibration curve for 3'-*O*-RFDU (**17**) in mouse EMT-6 tumor, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

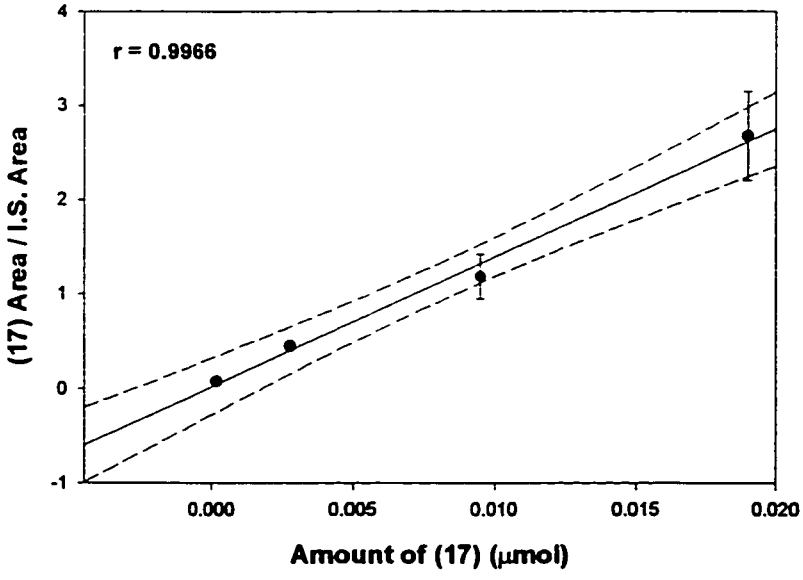


Figure 4-20-6-6. Calibration curve for 3'-*O*-RFDU (**17**) in mouse urine, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

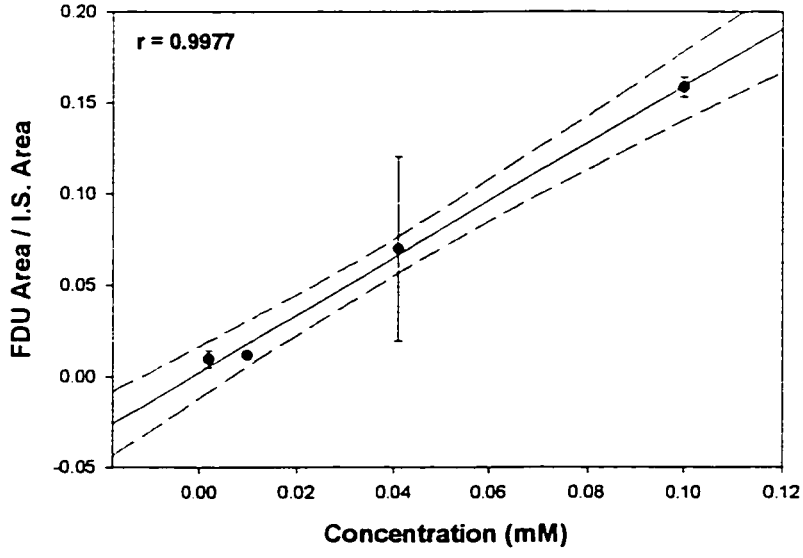


Figure 4-20-6-7. Calibration curve for FDU in mouse plasma, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bar represents SD.

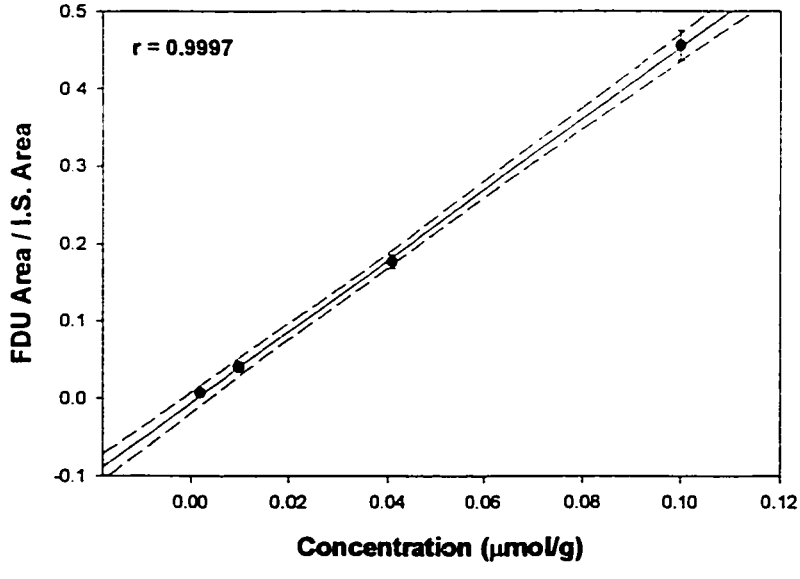


Figure 4-20-6-8. Calibration curve for FDU in mouse lung, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

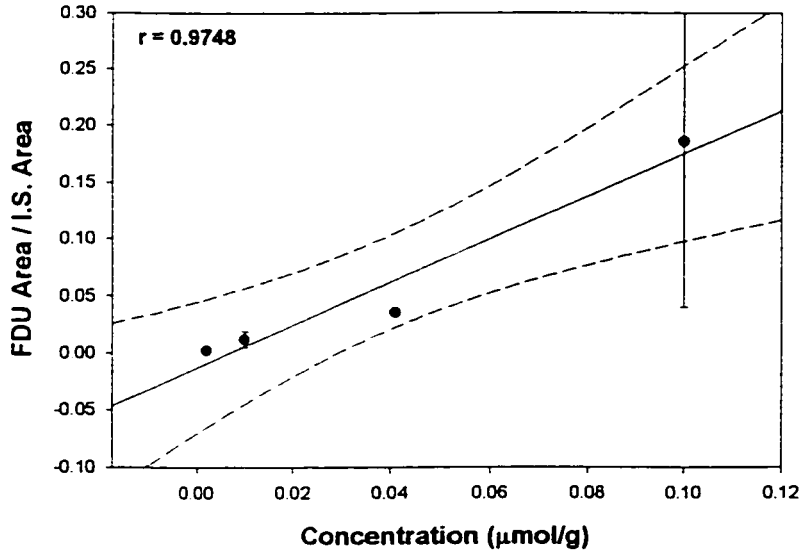


Figure 4-20-6-9. Calibration curve for FDU in mouse liver, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

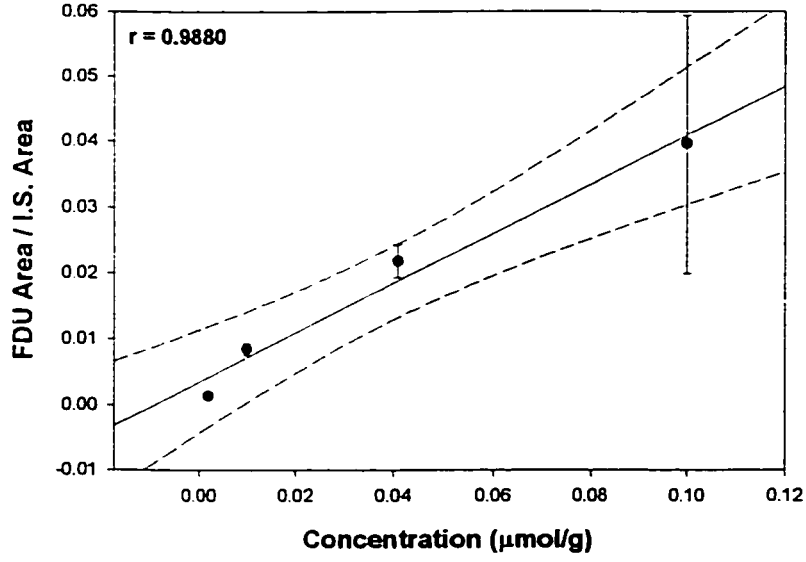


Figure 4-20-6-10. Calibration curve for FDU in mouse kidney. using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

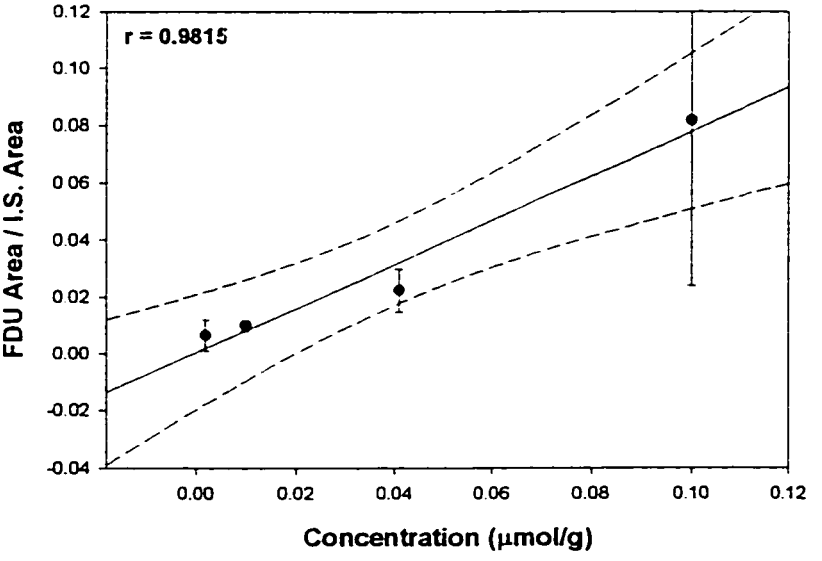


Figure 4-20-6-11. Calibration curve for FDU in mouse EMT-6 tumor, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

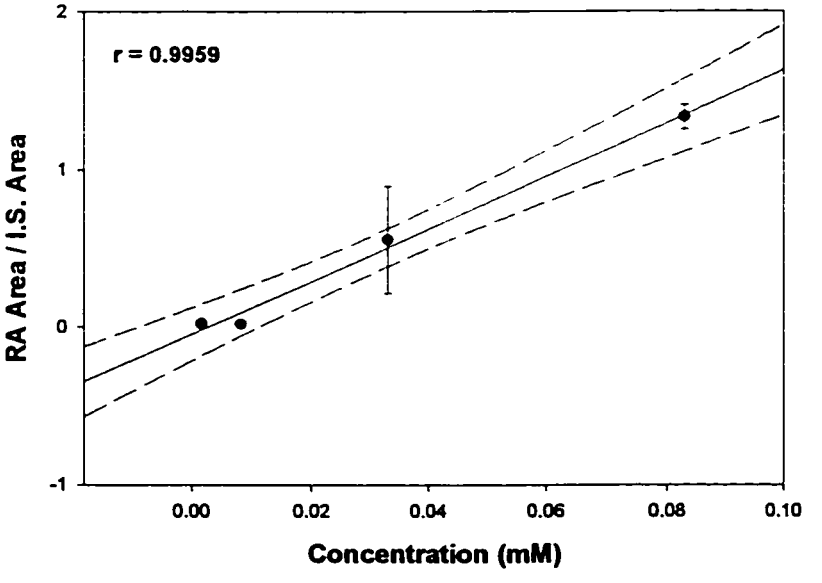


Figure 4-20-6-12. Calibration curve for RA in mouse plasma, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

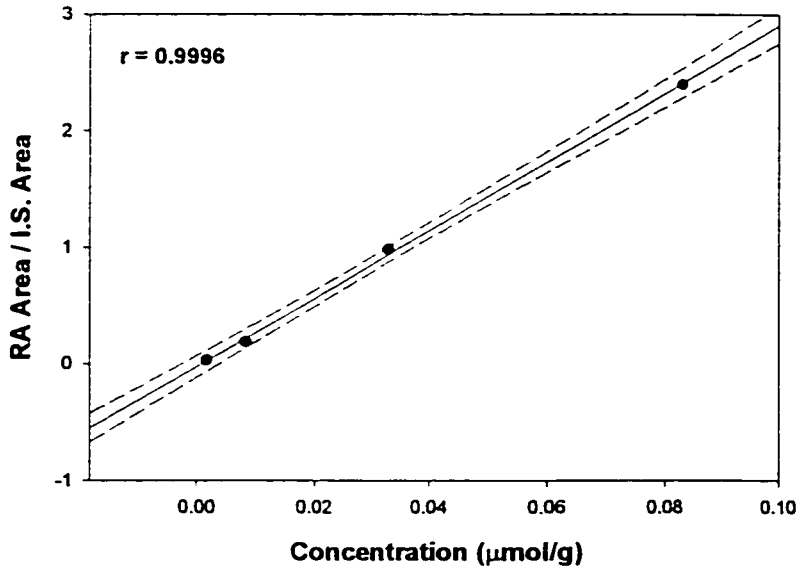


Figure 4-20-6-13. Calibration curve for RA in mouse lung, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

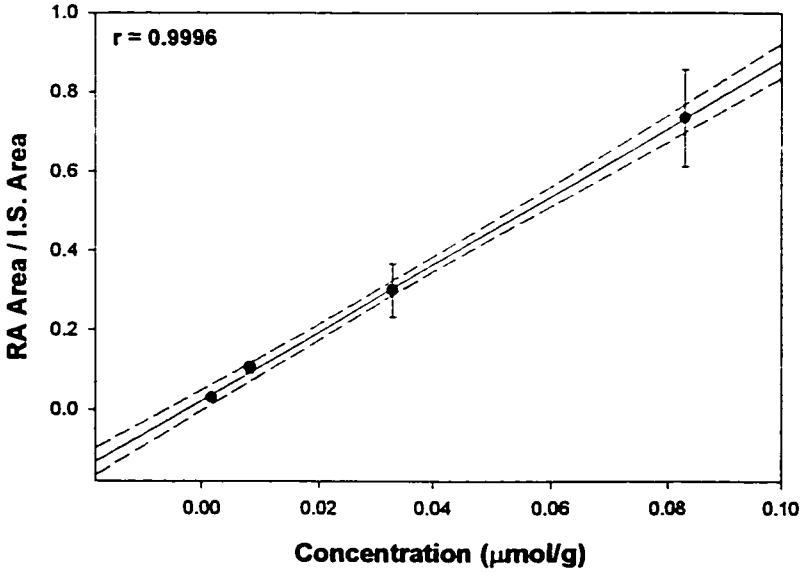


Figure 4-20-6-14. Calibration curve for RA in mouse liver, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

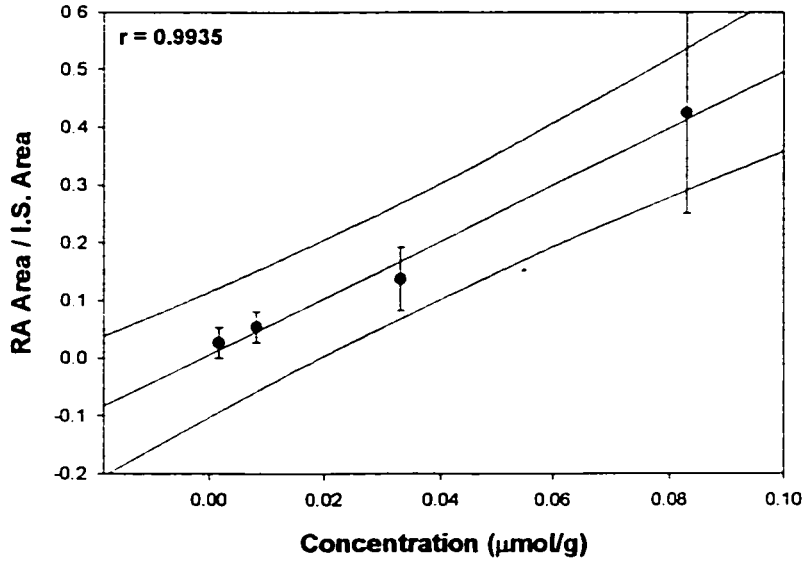


Figure 4-20-6-15. Calibration curve for RA in mouse kidney, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

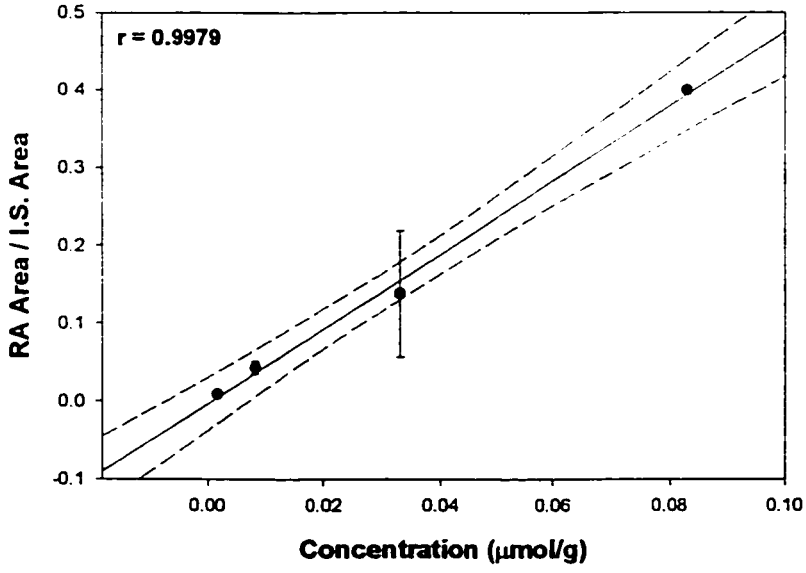


Figure 4-20-6-16. Calibration curve for RA in mouse EMT-6 tumor, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

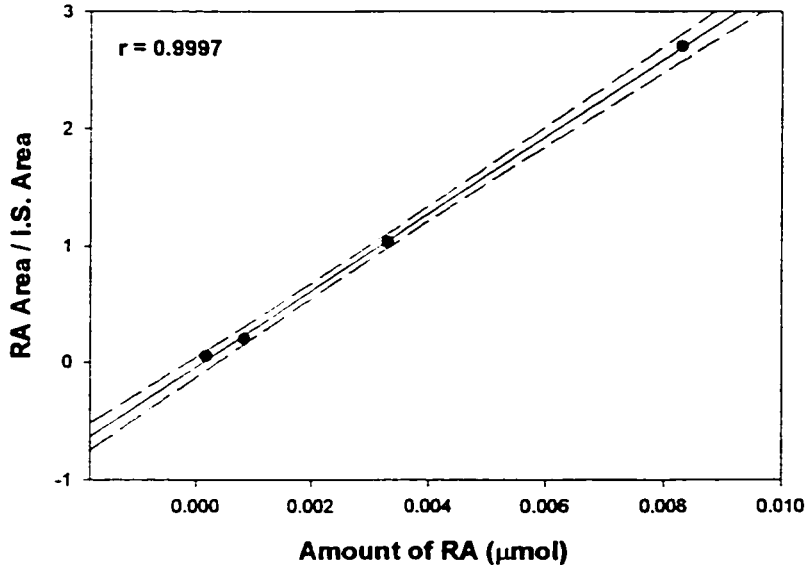


Figure 4-20-6-17. Calibration curve for RA in mouse urine, using ReAc as internal standard. The solid straight line represents the linear regression of the calibration line. The dash lines represent the 95% confidence interval. Error bars represent SD.

4-20-7. Recoveries of 3'-*O*-RFDU (17), RA, FDU and ReAc in Biological Samples

Two concentrations of 3'-*O*-RFDU (3 × 1 μg/g (or μg/mL)) and 3 × 100 μg/g (or μg/mL)), RA (3 × 0.5 μg/g (or μg/mL) and 3 × 25 μg/g (or μg/mL)) and FDU (3 × 2.5 μg/g (or μg/mL) and 3 × 25 μg/g (or μg/mL)) and one concentration of ReAc (6 × 250 μg/g (or μg/mL)) were added in the blank biological tissues (except urine). These samples were treated and analyzed by HPLC as described in sections **4-20-4** and **4-20-5**. In addition, two concentrations of the compounds (**17**), RA, FDU and ReAc (standards) were used for direct injection for HPLC analysis. Percentage recovery for the extraction procedure was determined for each tissue by comparing detector response to standards with the response to extracted standards from the spiked tissues.

4-20-8. Assay Precision and Accuracy for 3'-*O*-RFDU (17), RA and FDU in Biological Samples

Three standard samples (blank samples containing known amounts of 3'-*O*-RFDU (17), FDU, RA and ReAc) for each tissue were taken on different days in order to determine the precision and accuracy of the method. The samples were processed using the same procedures used for unknown samples (see sections **4-20-4** and **4-20-5**). The measured concentrations of each compound by HPLC were compared to the amount of the compound added (theoretical concentration). The accuracy was presented as the percentage difference between measured concentration and the theoretical concentration. The precision was described as standard deviation of the three measurements for each tissue.

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Appendix 1. *in Vivo* Anticancer Screening Data

The negative $\log_{10}$ GI50, TGI and LC50 values, respectively are listed as follows for each compound with the individual cell line identifiers listed after the tumor-type subpanel identifier (I, leukemia; II, non-small cell lung cancer; III, colon cancer; IV, CNS cancer; V, melanoma; VI, ovarian cancer; VII, renal cancer; VIII, prostate cancer; IX, breast cancer).

3'-*O*-Butanoyl-5-fluoro-2'-deoxyuridine: [I]: CCRF-CEM (7.66, >5.00, >5.00), HL-60 (TB) (6.89, >5.00, >5.00), K562 (5.94, >5.00, >5.00), MOLT-4 (5.32, >5.00, >5.00), RPMI-8226 (6.81, >5.00, >5.00), SR (7.21, >5.00, >5.00). [II]: A549/ATCC (7.32, >5.00, >5.00), EKVX (>5.00, >5.00, >5.00), HOP-62 (7.17, >5.00, >5.00), NCI-H226 (>5.00, >5.00, >5.00), NCI-H23 (7.14, 6.30, >5.00), NCI-H322M (5.35, >5.00, >5.00), NCI-H460 (8.29, >5.00, >5.00). [III]: COLO 205 (5.20, >5.00, >5.00), HCC-2998 (8.93, >5.00, >5.00), HCT-116 (6.32, >5.00, >5.00), HCT-15 (5.23, >5.00, >5.00), HT29 (6.66, >5.00, >5.00), KM12 (>5.00, >5.00, >5.00), SW-620 (>5.00, >5.00, >5.00). [IV]: SF-268 (7.34, >5.00, >5.00), SF-295 (6.62, >5.00, >5.00), SF-539 (7.98, 5.94, >5.00), SNB-19 (>5.00, >5.00, >5.00), SNB-75 (5.76, 5.18, >5.00), U251 (5.56, >5.00, >5.00). [V]: LOX IMVI (7.82, >5.00, >5.00), MALME-3M (5.11, >5.00, >5.00), M14 (6.56, 5.45, >5.00), SK-MEL-2 (>5.00, >5.00, >5.00), SK-MEL-28 (>5.00, >5.00, >5.00), SK-MEL-5 (6.41, 5.51, >5.00), UACC-257 (>5.00, >5.00, >5.00), UACC-62 (7.49, >5.00, >5.00). [VI]: IGROV1 (5.46, >5.00, >5.00), OVCAR-3 (5.61, >5.00, >5.00), OVCAR-4 (>5.00, >5.00, >5.00), OVCAR-5 (>5.00, >5.00, >5.00), OVCAR-8 (7.05, >5.00, >5.00), SK-OV-3 (5.60, >5.00, >5.00). [VII]: 786-0 (6.71, >5.00, >5.00), A498 (5.95, >5.00, >5.00), ACHN (7.38, >5.00, >5.00), CAKI-1 (6.85, >5.00, >5.00), RXF-393 (5.47, >5.00, >5.00), SN12C (6.02, >5.00, >5.00), TK-10 (>5.00, >5.00, >5.00), UO-31 (7.15, >5.00, >5.00). [VIII]: PC-3 (5.81, >5.00, >5.00), DU-145 (6.60, >5.00, >5.00). [IX]: MCF7 (7.59, >5.00, >5.00), MCF7/ADR-RES (6.19, >5.00, >5.00), MDA-MB-231/ATCC (>5.00, >5.00, >5.00), HS 578T (>5.00, >5.00, >5.00), MDA-MB-435 (5.91, >5.00, >5.00), MDA-N (5.85, >5.00, >5.00), BT-549 (6.14, >5.00, >5.00), T-47D (>5.00, >5.00, >5.00).

5'-*O*-Butanoyl-5'-fluoro-2'-deoxyuridine: [I]: CCRF-CEM (7.52, >5.00, >5.00), HL-60 (TB) (6.71, >5.00, >5.00), K562 (5.69, >5.00, >5.00), MOLT-4 (>5.00, >5.00, >5.00), RPMI-8226 (7.39, >5.00, >5.00), SR (7.44, >5.00, >5.00). [II]: A549/ATCC (7.43, >5.00, >5.00), EKVX (>5.00, >5.00, >5.00), HOP-62 (7.06, >5.00, >5.00), HOP-92 (5.96, >5.00, >5.00), NCI-H226 (5.24, >5.00, >5.00), NCI-H23 (6.60, 5.58, >5.00), NCI-H322M (5.37, >5.00, >5.00), NCI-H460 (8.40, >5.00, >5.00). [III]: COLO 205 (5.98, >5.00, >5.00), HCC-2998 (8.72, 6.06, >5.00), HCT-116 (6.40, >5.00, >5.00), HCT-15 (5.25, >5.00, >5.00), HT29 (6.50, >5.00, >5.00), KM12 (>5.00, >5.00, >5.00), SW-620 (>5.00, >5.00, >5.00). [IV]: SF-268 (7.34, >5.00, >5.00), SF-295 (6.97, 6.04, >5.00), SF-539 (8.39, 6.03, >5.00), SNB-19 (>5.00, >5.00, >5.00), U251 (5.69, >5.00, >5.00). [V]: LOX IMVI (7.13, >5.00, >5.00), MALME-3M (5.59, >5.00, >5.00), M14 (6.34, >5.00, >5.00), SK-MEL-2 (5.03, >5.00, >5.00), SK-MEL-28 (5.34, >5.00, >5.00), SK-MEL-5 (6.60, 5.79, >5.00), UACC-257 (5.18, >5.00, >5.00), UACC-62 (7.25, >5.00, >5.00). [VI]: IGROV1 (5.18, >5.00, >5.00), OVCAR-3 (6.35, >5.00, >5.00), OVCAR-4 (>5.00, >5.00, >5.00), OVCAR-5 (5.18, >5.00, >5.00), OVCAR-8 (6.85, >5.00, >5.00), SK-OV-3 (>5.00, >5.00, >5.00). [VII]: 786-0 (6.63, >5.00, >5.00), A498 (6.08, >5.00, >5.00), ACHN (7.47, >5.00, >5.00), CAKI-1 (7.40, >5.00, >5.00), RXF-393 (>5.00, >5.00, >5.00), SN12C (6.41, >5.00, >5.00), TK-10 (5.90, >5.00, >5.00), UO-31 (7.14, >5.00, >5.00). [VIII]: PC-3 (5.90, >5.00, >5.00), DU-145 (6.49, >5.00, >5.00). [IX]: MCF7 (8.33, >5.00, >5.00), MCF7/ADR-RES (6.40, >5.00, >5.00), MDA-MB-231/ATCC (>5.00, >5.00, >5.00), HS 578T (5.39, >5.00, >5.00), MDA-MB-435 (5.20, >5.00, >5.00), MDA-N (5.95, >5.00, >5.00), BT-549 (6.03, >5.00, >5.00), T-47D (5.12, >5.00, >5.00).

3',5'-Di-*O*-Butanoyl-5-fluoro-2'-deoxyuridine: [I]: CCRF-CEM (7.80, >5.00, >5.00), HL-60 (TB) (7.09, >5.00, >5.00), K562 (5.89, >5.00, >5.00), MOLT-4 (5.44, >5.00, >5.00), RPMI-8226 (6.85, >5.00, >5.00), SR (7.91, >5.00, >5.00). [II]: A549/ATCC (7.36, >5.00, >5.00), EKVX (>5.00, >5.00, >5.00), HOP-62 (7.27, >5.00, >5.00), HOP-92 (7.29, >5.00, >5.00), NCI-H226 (5.21, >5.00, >5.00), NCI-H23 (6.54, 5.26, >5.00), NCI-H322M (5.76, >5.00, >5.00), NCI-H460 (8.27, >5.00, >5.00). [III]: COLO 205 (5.37, >5.00, >5.00), HCC-2998 (8.55, >5.00, >5.00), HCT-116 (6.50, >5.00,

>5.00), HCT-15 (5.71, >5.00, >5.00), HT29 (6.82, >5.00, >5.00), KM12 (5.60, >5.00, >5.00), SW-620 (>5.00, >5.00, >5.00). [IV]: SF-268 (7.31, >5.00, >5.00), SF-295 (6.87, >5.00, >5.00), SF-539 (8.31, 5.95, >5.00), SNB-19 (>5.00, >5.00, >5.00), U251 (5.79, >5.00, >5.00). [V]: LOX IMVI (7.49, >5.00, >5.00), MALME-3M (5.38, >5.00, >5.00), M14 (6.53, >5.00, >5.00), SK-MEL-2 (>5.00, >5.00, >5.00), SK-MEL-28 (>5.00, >5.00, >5.00), SK-MEL-5 (6.66, 5.49, >5.00), UACC-257 (5.55, >5.00, >5.00), UACC-62 (7.36, >5.00, >5.00). [VI]: IGROV1 (>5.00, >5.00, >5.00), OVCAR-3 (5.35, >5.00, >5.00), OVCAR-4 (>5.00, >5.00, >5.00), OVCAR-5 (>5.00, >5.00, >5.00), OVCAR-8 (6.93, >5.00, >5.00), SK-OV-3 (>5.00, >5.00, >5.00). [VII]: 786-0 (6.58, >5.00, >5.00), A498 (7.07, 5.07, >5.00), ACHN (7.38, >5.00, >5.00), CAKI-1 (6.84, >5.00, >5.00), RXF-393 (5.07, >5.00, >5.00), SN12C (6.32, >5.00, >5.00), TK-10 (5.81, >5.00, >5.00), UO-31 (7.21, >5.00, >5.00). [VIII]: PC-3 (6.03, >5.00, >5.00), DU-145 (6.62, >5.00, >5.00). [IX]: MCF7 (8.24, >5.00, >5.00), MCF7/ADR-RES (6.45, >5.00, >5.00), MDA-MB-231/ATCC (>5.00, >5.00, >5.00), HS 578T (5.70, >5.00, >5.00), MDA-MB-435 (5.48, >5.00, >5.00), MDA-N (6.04, >5.00, >5.00), BT-549 (6.42, >5.00, >5.00), T-47D (5.42, >5.00, >5.00).

3'-*O*-Butanoyl-2',5-difluoro-2'-deoxyuridine: [I]: CCRF-CEM (5.41, >4.00, >4.00), HL-60 (TB) (4.09, >4.00, >4.00), MOLT-4 (>4.00, >4.00, >4.00), RPMI-8226 (5.80, >4.00, >4.00), SR (5.27, >4.00, >4.00). [II]: A549/ATCC (>4.00, >4.00, >4.00), EKVX (4.17, >4.00, >4.00), HOP-62 (5.35, >4.00, >4.00), HOP-92 (>4.00, >4.00, >4.00), NCI-H226 (>4.00, >4.00, >4.00), NCI-H23 (>4.00, >4.00, >4.00), NCI-H322M (4.51, >4.00, >4.00), NCI-H522 (>4.00, >4.00, >4.00). [III]: COLO 205 (>4.00, >4.00, >4.00), HCT-116 (4.65, >4.00, >4.00), HCT-15 (>4.00, >4.00, >4.00), HT29 (4.26, >4.00, >4.00), KM12 (>4.00, >4.00, >4.00), SW-620 (>4.00, >4.00, >4.00). [IV]: SF-268 (-, >4.00, >4.00), SF-295 (4.09, >4.00, >4.00), SF-539 (5.78, >4.00, >4.00), SNB-75 (5.23, >4.00, >4.00), U251 (5.13, >5.00, >5.00). [V]: LOX IMVI (>4.00, >4.00, >4.00), MALME-3M (4.37, >4.00, >4.00), SK-MEL-2 (>4.00, >4.00, >4.00), SK-MEL-28 (>4.00, >4.00, >4.00), SK-MEL-5 (4.27, >4.00, >4.00), UACC-257 (>4.00, >4.00, >4.00), UACC-62 (4.62, >4.00, >4.00). [VI]: IGROV1 (4.02, >4.00, >4.00), OVCAR-3 (4.26, >4.00, >4.00), OVCAR-4 (4.22, >4.00, >4.00), OVCAR-5 (>4.00, >4.00, >4.00),

OVCAR-8 (4.14, >4.00, >4.00), SK-OV-3 (>4.00, >4.00, >4.00). [VII]: 786-0 (4.42, >4.00, >4.00), A498 (5.25, >4.00, >4.00), ACHN (4.36, >4.00, >4.00), CAKI-1 (4.30, >4.00, >4.00), RXF-393 (>4.00, >4.00, >4.00), SN12C (4.81, >4.00, >4.00), TK-10 (>4.00, >4.00, >4.00), UO-31 (>4.00, >4.00, >4.00). [VIII]: PC-3 (4.03, >4.00, >4.00), DU-145 (4.75, >4.00, >4.00). [IX]: MCF7 (6.22, >4.00, >4.00), MCF7/ADR-RES (4.95, >4.00, >4.00), HS 578T (4.27, >4.00, >4.00), MDA-MB-435 (4.23, >4.00, >4.00), BT-549 (4.94, >4.00, >4.00), T-47D (>4.00, >4.00, >4.00).

5'-*O*-Butanoyl-2',5-difluoro-2'-deoxyuridine: [I]: CCRF-CEM (5.65, >4.00, >4.00), HL-60 (TB) (>4.00, >4.00, >4.00), K-562 (5.22, >4.00, >4.00), MOLT-4 (4.90, >4.00, >4.00), RPMI-8226 (4.85, >4.00, >4.00), SR (>4.00, >4.00, >4.00). [II]: A549/ATCC (5.23, >4.00, >4.00), EKVX (>4.00, >4.00, >4.00), HOP-62 (5.30, >4.00, >4.00), NCI-H226 (>4.00, >4.00, >4.00), NCI-H23 (4.10, >4.00, >4.00), NCI-H322M (4.47, >4.00, >4.00), NCI-460 (5.55, >4.00, >4.00), NCI-H522 (4.25, >4.00, >4.00). [III]: COLO 205 (4.72, >4.00, >4.00), HCC-2998 (>4.00, >4.00, >4.00), HCT-116 (5.30, >4.00, >4.00), HCT-15 (4.15, >4.00, >4.00), HT29 (4.13, >4.00, >4.00), KM12 (>4.00, >4.00, >4.00), SW-620 (>4.00, >4.00, >4.00). [IV]: SF-268 (5.16, >4.00, >4.00), SF-295 (5.12, >4.00, >4.00), SF-539 (6.26, >4.00, >4.00), SNB-19 (4.11, >4.00, >4.00), SNB-75 (4.52, >4.00, >4.00), U251 (4.79, >4.00, >4.00). [V]: LOX IMVI (5.07, >4.00, >4.00), MALME-3M (>4.00, >4.00, >4.00), M14 (4.73, >4.00, >4.00), SK-MEL-2 (>4.00, >4.00, >4.00), SK-MEL-28 (>4.00, >4.00, >4.00), SK-MEL-5 (4.79, >4.00, >4.00), UACC-257 (4.45, >4.00, >4.00), UACC-62 (5.00, >4.00, >4.00). [VI]: IGROV1 (4.23, >4.00, >4.00), OVCAR-3 (4.46, >4.00, >4.00), OVCAR-4 (>4.00, >4.00, >4.00), OVCAR-5 (>4.00, >4.00, >4.00), OVCAR-8 (4.83, >4.00, >4.00), SK-OV-3 (4.26, >4.00, >4.00). [VII]: 786-0 (4.58, >4.00, >4.00), ACHN (4.96, >4.00, >4.00), RXF-393 (>4.00, >4.00, >4.00), SN12C (4.16, >4.00, >4.00), TK-10 (>4.00, >4.00, >4.00), UO-31 (4.11, >4.00, >4.00). [VIII]: PC-3 (>4.00, >4.00, >4.00), DU-145 (5.56, 4.35, >4.00). [IX]: MCF7 (6.16, >4.00, >4.00), MCF7/ADR-RES (4.20, >4.00, >4.00), HS 578T (4.01, >4.00, >4.00), MDA-MB-435 (4.20, >4.00, >4.00), MDA-MB-231/ATCC (4.42, >4.00, >4.00), MDA-N (4.83, >4.00, >4.00), BT-549 (4.14, >4.00, >4.00),

3',5'-Di-*O*-Butanoyl-2',5-difluoro-2'-deoxyuridine: [I]: CCRF-CEM (5.12, >4.00, >4.00), HL-60 (TB) (>4.00, >4.00, >4.00), K-562 (4.25, >4.00, >4.00), MOLT-4 (>4.00, >4.00, >4.00), RPMI-8226 (5.03, >4.00, >4.00), SR (5.01, >4.00, >4.00). [II]: A549/ATCC (5.00, >4.00, >4.00), EKVX (4.30, >4.00, >4.00), HOP-62 (5.39, >4.00, >4.00), HOP-92 (4.76, >4.00, >4.00), NCI-H226 (>4.00, >4.00, >4.00), NCI-H23 (4.45, >4.00, >4.00), NCI-H322M (4.23, >4.00, >4.00), NCI-460 (5.42, >4.00, >4.00), NCI-H522 (>4.00, >4.00, >4.00). [III]: COLO 205 (4.44, >4.00, >4.00), HCT-116 (4.67, >4.00, >4.00), HCT-15 (>4.00, >4.00, >4.00), HT29 (4.34, >4.00, >4.00), KM12 (>4.00, >4.00, >4.00), SW-620 (4.27, >4.00, >4.00). [IV]: SF-268 (5.07, >4.00, >4.00), SF-295 (4.31, >4.00, >4.00), SF-539 (5.79, 4.56, >4.00), SNB-75 (4.36, >4.00, >4.00), U251 (4.96, >4.00, >4.00). [V]: LOX IMVI (4.32, >4.00, >4.00), MALME-3M (4.44, >4.00, >4.00), M14 (4.44, >4.00, >4.00), SK-MEL-2 (>4.00, >4.00, >4.00), SK-MEL-28 (>4.00, >4.00, >4.00), SK-MEL-5 (4.43, >4.00, >4.00), UACC-257 (4.07, >4.00, >4.00), UACC-62 (4.59, >4.00, >4.00). [VI]: IGROV1 (4.23, >4.00, >4.00), OVCAR-3 (4.38, >4.00, >4.00), OVCAR-4 (5.25, >4.00, >4.00), OVCAR-5 (4.02, >4.00, >4.00), OVCAR-8 (4.71, >4.00, >4.00), SK-OV-3 (>4.00, >4.00, >4.00). [VII]: 786-0 (4.74, >4.00, >4.00), A498 (5.20, >4.00, >4.00), ACHN (4.59, >4.00, >4.00), CAKI-1 (4.64, >4.00, >4.00), RXF-393 (4.26, >4.00, >4.00), SN12C (4.72, >4.00, >4.00), TK-10 (4.31, >4.00, >4.00), UO-31 (4.16, >4.00, >4.00). [VIII]: PC-3 (>4.00, >4.00, >4.00), DU-145 (4.82, >4.00, >4.00). [IX]: MCF7 (6.22, >4.00, >4.00), MCF7/ADR-RES (5.13, >4.00, >4.00), HS-578T (4.58, >4.00, >4.00), MDA-MB-435 (4.29, >4.00, >4.00), MDA-MB-231/ATCC (>4.00, >4.00, >4.00), MDA-N (>4.00, >4.00, >4.00), BT-549 (4.86, >4.00, >4.00), T-47D (>4.00, >4.00, >4.00).

3'-*O*-Retinoyl-5-fluoro-2'-deoxyuridine: [I]: CCRF-CEM (5.46, 4.48, >4.00), HL-60 (TB) (6.52, 5.58, 4.48), K-562 (6.18, 4.71, >4.00), MOLT-4 (6.61, 4.76, >4.00), RPMI-8226 (6.47, 4.60, >4.00), SR (6.55, 4.65, >4.00). [II]: A549/ATCC (6.97, 4.74, 4.10), EKVX (4.90, 4.45, >4.00), HOP-62 (6.91, 4.95, 4.45), HOP-92 (4.95, 4.44, >4.00), NCI-H226 (4.64, 4.30, >4.00), NCI-H23 (5.61, 4.74, 4.37), NCI-460 (7.30, 4.85, 4.28), NCI-H522 (5.93, 4.94, 4.38). [III]: COLO 205 (5.42, 4.66, 4.25), HCT-116 (6.00, 4.77, 4.32), HCT-15 (5.46, 4.65, 4.23), HT29 (5.47, 4.71, 4.34), KM12 (4.96, 4.55,

4.13), SW-620 (4.96, 4.61, 4.26). [IV]: SF-268 (6.36, 4.62, 4.12), SF-295 (7.24, 4.94, 4.34), SF-539 (7.24, 6.27, >4.00), SNB-75 (6.04, 4.76, 4.32), U251 (6.16, 4.78, 4.37). [V]: LOX IMVI (4.97, 4.64, 4.32), MALME-3M (4.92, 4.50, 4.07), M14 (5.74, 4.84, 4.31), SK-MEL-2 (4.75, 4.44, 4.13), SK-MEL-28 (5.29, 4.67, 4.30), SK-MEL-5 (5.91, 4.90, 4.44), UACC-257 (4.87, 4.50, 4.13), UACC-62 (6.72, 5.64, 4.56). [VI]: IGROV1 (5.40, 4.65, 4.24), OVCAR-3 (5.77, 4.74, 4.33), OVCAR-4 (5.45, 4.72, 4.25), OVCAR-5 (4.80, 4.47, 4.13), OVCAR-8 (6.26, 4.69, 4.06), SK-OV-3 (5.82, 4.71, 4.35). [VII]: 786-0 (6.34, 4.84, 4.42), ACHN (6.98, 4.87, 4.42), CAKI-1 (6.34, 4.74, 4.23), RXF-393 (5.50, 4.65, 4.21), SN12C (5.33, 4.61, 4.18), TK-10 (5.07, 4.67, 4.33), UO-31 (6.44, 4.87, 4.38). [VIII]: PC-3 (5.92, 4.77, 4.39), DU-145 (6.54, 4.94, 4.46). [IX]: MCF7 (7.24, 4.71, 4.12), MCF7/ADR-RES (5.97, 4.65, 4.08), HS-578T (5.77, 4.50, >4.00), MDA-MB-435 (5.67, 4.61, 4.18), MDA-MB-231/ATCC (5.25, 4.61, 4.15), MDA-N (5.35, 4.70, 4.33), BT-549 (6.80, 4.81, 4.34), T-47D (5.80, 4.90, 4.20).

3',5'-Di-*O*-Retinoyl-5-fluoro-2'-deoxyuridine: [I]: CCRF-CEM (6.05, 5.08, >4.00), HL-60 (TB) (5.84, >4.00, >4.00), K-562 (5.40, >4.00, >4.00), MOLT-4 (5.87, >4.00, >4.00), RPMI-8226 (6.28, 4.35, >4.00), SR (6.13, >4.00, >4.00). [II]: A549/ATCC (6.42, 4.63, >4.00), EKVX (4.53, >4.00, >4.00), HOP-62 (6.50, 5.11, 4.10), HOP-92 (5.90, 4.43, >4.00), NCI-H226 (4.71, 4.32, >4.00), NCI-H23 (4.82, 4.09, >4.00), NCI-460 (7.25, 5.15, 4.13), NCI-H522 (5.09, 4.55, 4.06). [III]: COLO 205 (4.93, 4.44, >4.00), HCT-116 (5.48, 4.44, >4.00), HCT-15 (4.91, 4.49, 4.06), HT29 (4.92, >4.00, >4.00), KM12 (4.71, 4.25, >4.00), SW-620 (4.92, 4.56, 4.19). [IV]: SF-268 (6.18, 4.14, >4.00), SF-295 (6.81, 4.79, >4.00), SF-539 (6.50, >4.00, >4.00), SNB-19 (5.41, >4.00, >4.00), SNB-75 (5.01, >4.00, >4.00), U251 (5.32, 4.53, >4.00). [V]: LOX IMVI (4.87, 4.53, 4.19), MALME-3M (5.13, >4.00, >4.00), M14 (5.30, 4.63, 4.07), SK-MEL-2 (4.23, >4.00, >4.00), SK-MEL-28 (4.78, 4.22, >4.00), SK-MEL-5 (5.52, 4.82, 4.32), UACC-257 (4.76, 4.07, >4.00), UACC-62 (6.33, 5.25, >4.00). [VI]: IGROV1 (5.40, 4.40, >4.00), OVCAR-3 (5.78, 4.81, >4.00), OVCAR-4 (4.99, 4.17, >4.00), OVCAR-5 (4.69, 4.34, >4.00), OVCAR-8 (5.72, 4.35, >4.00), SK-OV-3 (4.94, >4.00, >4.00). [VII]: 786-0 (6.02, 4.67, >4.00), ACHN (6.35, 4.63, >4.00), CAKI-1 (5.90, >4.00, >4.00), RXF-393 (4.71, >4.00, >4.00), SN12C (5.26, >4.00, >4.00), TK-10 (4.89, 4.47, 4.05),

UO-31 (6.33, 5.16, >4.00). [VIII]: PC-3 (5.24, 4.50, >4.00), DU-145 (6.18, 4.67, >4.00). [IX]: MCF7 (6.60, 4.73, >4.00), MCF7/ADR-RES (5.65, >4.00, >4.00), HS-578T (5.08, 4.33, >4.00), MDA-MB-435 (4.92, 4.36, >4.00), MDA-MB-231/ATCC (4.76, 4.12, >4.00), MDA-N (5.24, 4.63, 4.19), BT-549 (4.26, >4.00, >4.00), T-47D (4.96, 4.38, >4.00).

5'-*O-Bis*(2,2,2-trichloroethyl)phosphoryl-5-fluoro-2'-deoxyuridine: [I]: CCRF-CEM (6.71, >4.00, >4.00), HL-60 (TB) (5.23, 4.29, >4.00), K-562 (5.39, >4.00, >4.00), MOLT-4 (6.02, >4.00, >4.00), RPMI-8226 (4.83, >4.00, >4.00), SR (6.46, >4.00, >4.00). [II]: A549/ATCC (6.84, >4.00, >4.00), EKVX (>4.00, >4.00, >4.00), HOP-62 (6.92, 6.12, >4.00), NCI-H226 (4.02, >4.00, >4.00), NCI-H23 (4.89, >4.00, >4.00), NCI-H460 (7.29, >4.00, >4.00), NCI-H522 (5.55, 4.92, 4.22). [III]: COLO 205 (4.63, >4.00, >4.00), HCT-116 (5.70, >4.00, >4.00), HCT-15 (4.50, >4.00, >4.00), HT29 (4.61, >4.00, >4.00), KM12 (>4.00, >4.00, >4.00), SW-620 (>4.00, >4.00, >4.00). [IV]: SF-268 (6.06, >4.00, >4.00), SF-295 (6.70, >4.00, >4.00), SNB-19 (5.74, >4.00, >4.00), SNB-75 (5.21, >4.00, >4.00), U251 (5.92, >4.00, >4.00). [V]: LOX IMVI (4.91, >4.00, >4.00), MALME-3M (5.01, >4.00, >4.00), M14 (5.92, >4.00, >4.00), SK-MEL-2 (>4.00, >4.00, >4.00), SK-MEL-28 (>4.00, >4.00, >4.00), SK-MEL-5 (5.74, >4.00, >4.00), UACC-257 (>4.00, >4.00, >4.00), UACC-62 (6.40, >4.00, >4.00). [VI]: IGROV1 (4.37, >4.00, >4.00), OVCAR-3 (4.70, >4.00, >4.00), OVCAR-4 (>4.00, >4.00, >4.00), OVCAR-5 (4.05, >4.00, >4.00), OVCAR-8 (6.22, >4.00, >4.00), SK-OV-3 (4.63, >4.00, >4.00). [VII]: 786-0 (6.68, >4.00, >4.00), ACHN (7.63, >4.00, >4.00), CAKI-1 (6.67, >4.00, >4.00), SN12C (4.96, >4.00, >4.00), TK-10 (>4.00, >4.00, >4.00), UO-31 (6.04, >4.00, >4.00). [VIII]: PC-3 (5.26, >4.00, >4.00), DU-145 (6.02, >4.00, >4.00). [IX]: MCF7 (7.28, >4.00, >4.00), MCF7/ADR-RES (5.81, >4.00, >4.00), HS-578T (4.28, >4.00, >4.00), MDA-MB-435 (>4.00, >4.00, >4.00), MDA-MB-231/ATCC (4.66, >4.00, >4.00), MDA-N (5.17, >4.00, >4.00), T-47D (4.59, >4.00, >4.00).

5'-*O-Bis*(2,2,2-trichloroethyl)phosphoryl-3'-*O*-butanoyl-5-fluoro-2'-deoxyuridine: [I]: CCRF-CEM (6.43, 4.03, >4.00), HL-60 (TB) (4.97, 4.39, >4.00), MOLT-4 (5.50, 4.41, >4.00), RPMI-8226 (5.26, 4.06, >4.00), SR (6.26, >4.00, >4.00). [II]: A549/ATCC (6.48, >4.00, >4.00), EKVX (4.36, >4.00, >4.00), HOP-62 (7.16, >4.00, >4.00), NCI-

H226 (4.19, >4.00, >4.00), NCI-H23 (5.08, >4.00, >4.00), NCI-H460 (7.84, 4.61, >4.00), NCI-H522 (5.87, 5.37, >4.00). [III]: COLO 205 (7.21, 4.23, >4.00), HCT-116 (5.49, >4.00, >4.00), HCT-15 (4.63, >4.00, >4.00), HT29 (4.77, 4.23, >4.00), KM12 (4.74, >4.00, >4.00), SW-620 (4.69, >4.00, >4.00), [IV]: SF-268 (5.93, >4.00, >4.00), SF-295 (6.53, 4.72, 4.10), SNB-19 (6.03, >4.00, >4.00), SNB-75 (5.10, 4.12, >4.00), U251 (5.84, 4.21, >4.00). [V]: LOX IMVI (4.96, 4.31, >4.00), MALME-3M (5.56, >4.00, >4.00), M14 (5.87, >4.00, >4.00), SK-MEL-2 (4.45, >4.00, >4.00), SK-MEL-28 (4.84, >4.00, >4.00), SK-MEL-5 (5.69, >4.00, >4.00), UACC-257 (4.36, >4.00, >4.00), UACC-62 (5.69, >4.00, >4.00). [VI]: IGROV1 (4.66, >4.00, >4.00), OVCAR-3 (5.33, 4.21, >4.00), OVCAR-4 (4.84, >4.00, >4.00), OVCAR-5 (4.69, >4.00, >4.00), OVCAR-8 (6.00, >4.00, >4.00), SK-OV-3 (4.81, >4.00, >4.00). [VII]: 786-0 (6.67, >4.00, >4.00), ACHN (7.46, 4.04, >4.00), CAKI-1 (6.87, >4.00, >4.00), SN12C (4.58, >4.00, >4.00), TK-10 (>4.59, >4.00, >4.00), UO-31 (6.30, >4.00, >4.00). [VIII]: PC-3 (5.15, >4.00, >4.00), DU-145 (6.16, 4.34, >4.00). [IX]: MCF7 (7.16, >4.00, >4.00), MCF7/ADR-RES (6.18, >4.00, >4.00), HS-578T (4.86, >4.00, >4.00), MDA-MB-435 (4.74, >4.00, >4.00), MDA-MB-231/ATCC (4.67, >4.00, >4.00), MDA-N (5.14, >4.00, >4.00), T-47D (4.37, >4.00, >4.00).

Appendix 2. Data of Pharmacokinetics in Balb/c Mice Bearing EMT-6 Murine Mammary Tumors

Table 1. Time *vs.* concentrations (mean ± SD) of 3'-*O*-RFDU, RA and FDU in plasma after a single i.v. bolus of 3'-*O*-RFDU (17) (12.5 μmol/kg) determined by HPLC

Time (min)	3'-*O*-RFDU (μM)[#]	$SD_{3'\text{-}O\text{-}RFDU}$	RA (μM)	SD_{RA}	FDU (μM)	SD_{FDU}
1.00	31.05	3.67	0.46	0.09	ND*	
3.00	14.46	0.76	0.56	0.21	1.23	0.71
5.00	13.29	1.41	0.76	0.20	1.49	0.40
15.00	5.21	0.44	1.49	0.39	9.56	1.00
30.00	2.95	1.03	1.40	0.11	10.76	3.13
60.00	1.90	0.55	1.84	0.38	2.36	1.07
120.00	0.77	0.09	0.38	0.10	1.49	0.98
240.00	0.49	0.06	ND		ND	
480.00	0.35	0.09	ND		ND	

Table 2. Time *vs.* concentrations (mean ± SD) of 3'-*O*-RFDU, RA and FDU in plasma after a single p.o. dose of 3'-*O*-RFDU (17) (13.7 μmol/kg) determined by HPLC

Time (min)	3'-*O*-RFDU (μM)	$SD_{3'\text{-}O\text{-}RFDU}$	RA (μM)	SD_{RA}	FDU (μM)	SD_{FDU}
3.00	0.49	0.13	0.27	0.21	ND*	
5.00	0.42	0.08	0.29	0.25	ND	
15.00	0.70	0.11	0.49	0.05	2.22	1.26
30.00	1.03	0.16	0.41	0.05	0.31	0.29
60.00	1.50	0.34	0.54	0.11	0.01	0.01
120.00	1.46	0.90	0.49	0.20	ND	
240.00	1.06	0.74	0.20	0.05	ND	
480.00	0.78	0.37	0.20	0.05	ND	
720.00	0.36	0.02	0.28	0.11	ND	

Table 3. Time *vs.* concentrations (mean ± SD) of 3'-*O*-RFDU and RA in kidney after a single i.v. bolus of 3'-*O*-RFDU (17) (12.5 μmol/kg) determined by HPLC

Time (min)	3'-*O*-RFDU (μM)	$SD_{3'\text{-}O\text{-}RFDU}$	RA (μM)	SD_{RA}
1.00	19.65	3.41	0.65	1.12
3.00	15.23	1.76	2.21	0.29
5.00	15.12	1.72	2.12	0.28
15.00	14.75	3.88	2.27	2.12
30.00	15.07	2.57	3.72	3.19
60.00	14.70	3.13	4.29	1.28
120.00	6.70	1.72	0.68	0.59
240.00	2.79	1.63	ND*	
480.00	0.66	0.59	ND	

Table 4. Time *vs.* concentrations (mean ± SD) of 3'-*O*-RFDU and RA in liver after a single i.v. bolus of 3'-*O*-RFDU (17) (12.5 μmol/kg) determined by HPLC

Time (min)	3'-*O*-RFDU (μM)	$SD_{3'\text{-}O\text{-}RFDU}$	RA (μM)	SD_{RA}
1.00	106.06	10.67	ND*	
3.00	40.75	13.79	7.73	6.72
5.00	40.63	5.74	6.57	1.51
15.00	40.48	3.80	2.36	1.16
30.00	38.57	5.70	ND	
60.00	7.03	0.48	ND	
120.00	3.29	0.35	ND	
240.00	1.40	0.57	ND	
480.00	1.24	0.33	ND	

Table 5. Time *vs.* concentrations (mean ± SD) of 3'-*O*-RFDU and RA in lung after a single i.v. bolus of 3'-*O*-RFDU (17) (12.5 μmol/kg) determined by HPLC

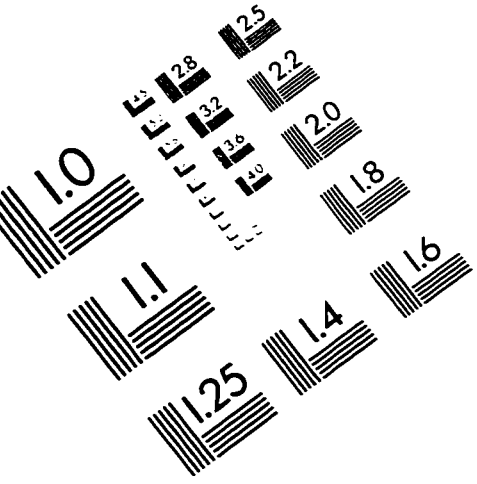
Time (min)	3'-*O*-RFDU (μM)	$SD_{3'\text{-}O\text{-}RFDU}$	RA (μM)	SD_{RA}
1.00	193.16	16.85	0.41	0.62
3.00	84.38	10.08	0.61	0.20
5.00	72.80	15.14	0.82	0.07
15.00	25.69	5.12	0.92	0.49
30.00	16.37	5.07	0.43	0.34
60.00	7.86	0.72	0.37	0.16
120.00	3.14	1.33	0.10	0.11
240.00	1.14	1.22	ND*	
480.00	0.97	0.74	ND	

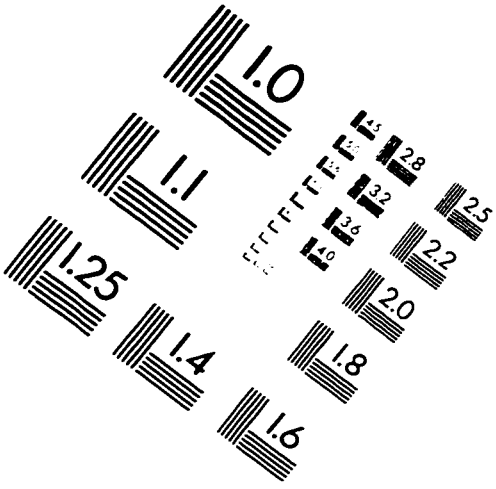
Table 6. Time *vs.* concentrations (mean ± SD) of 3'-*O*-RFDU and RA in tumor after a single i.v. bolus of 3'-*O*-RFDU (17) (12.5 μmol/kg) determined by HPLC

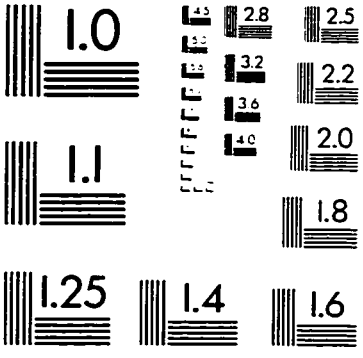
Time (min)	3'-*O*-RFDU (μM)	$SD_{3'\text{-}O\text{-}RFDU}$	RA (μM)	SD_{RA}
1.00	1.22	0.37	ND*	
3.00	4.16	3.56	0.65	0.51
5.00	2.72	0.17	0.30	0.05
15.00	2.13	0.76	0.42	0.18
30.00	2.21	0.77	0.77	0.94
60.00	2.47	1.04	1.95	1.64
120.00	1.99	0.41	1.06	0.33
240.00	1.37	0.63	0.79	0.93
480.00	4.55	4.33	0.44	0.24

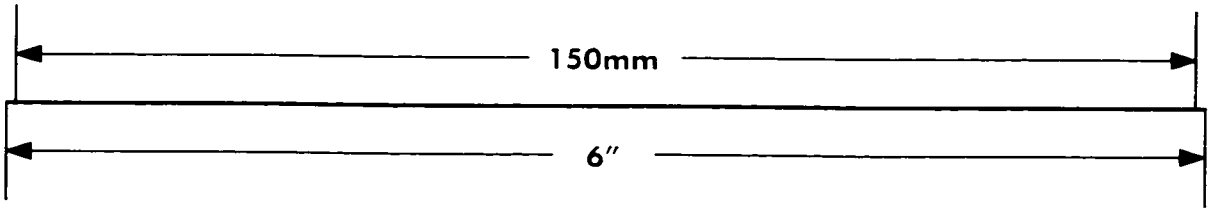
μM : μmol/L for plasma; μmol/kg for kidney, liver, lung and tumor.
* ND: Not detectable.

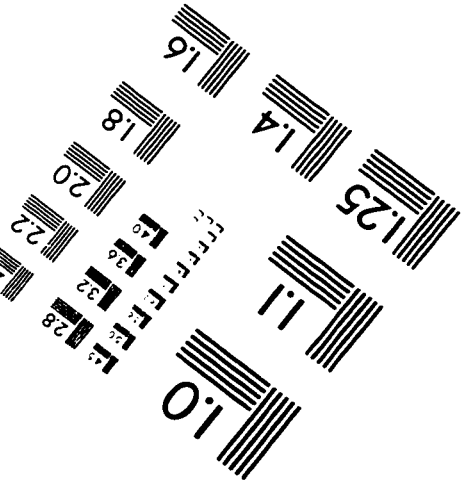
IMAGE EVALUATION TEST TARGET (QA-3)

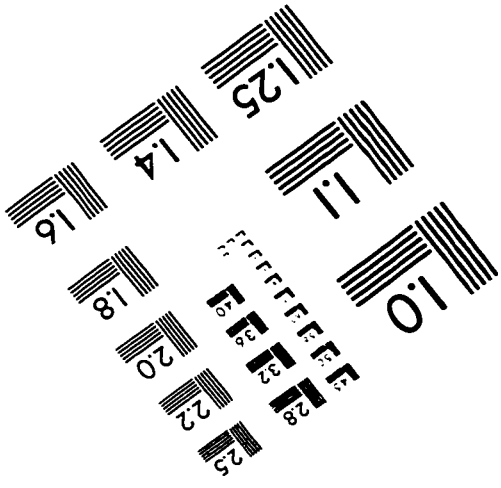












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