University of Alberta

A role for nucleoside transport processes in the cytotoxicity of 2-chlorodeoxyadenosine in leukemic lymphoblasts from children with acute lymphoblastic leukemia

By

Adrienne M.P. Wright

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

Department of Pharmacology

Edmonton, Alberta
Fall 1997

The University of Alberta

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Degree	Doctor of Philosophy
Year this Degree Granted	1997

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Sept 19/1997

University of Alberta

Faculty of Graduate Studies and Research

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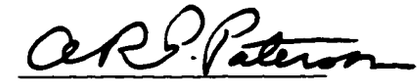

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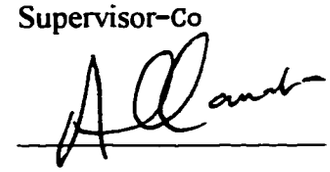

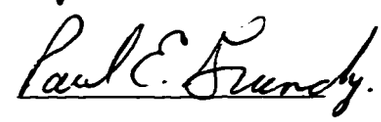

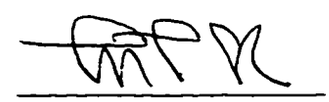

External Examiner

To my parents, who encouraged me

To my husband, who strengthened me

Abstract

A goal of this study has been evaluation of the cytotoxicity of the nucleoside analog, 2-chlorodeoxyadenosine (2-CdA), toward lymphoblasts from pediatric acute lymphoblastic leukemia (ALL) patients. Leukemic cells from previously untreated ALL patients were examined for sensitivity to 2-CdA and cytarabine (araC), a nucleoside analog used in induction and consolidation regimens in ALL treatment. A considerable interpatient variation in sensitivity to the nucleoside analogs was observed, with IC_{50} values ranging from 6 nM to > 5 μM for 2-CdA, and from 65 nM to > 5 μM for araC. Leukemic cells from pediatric ALL patients were more sensitive ($p < 0.005$) to 2-CdA ($n = 13$) than to araC ($n = 10$). Therefore, 2-CdA may be useful in treatment of some pediatric ALL patients, while for others it may be of little benefit.

Cytotoxicity of 2-CdA and araC is dependent on their conversion to triphosphate metabolites following entry via nucleoside transport (NT) processes into leukemic cells. The relationship between sensitivity to the nucleoside analogs and the cellular abundance of *es* nucleoside transporter elements, assayed by a flow cytometric technique that used the *es*-specific probe, 5-(SAENTA-x8)-fluorescein, was tested in lymphoblasts from patients. Cellular *es* abundance varied 14-fold among lymphoblasts from patient samples. While a linear relationship was not apparent between 2-CdA cytotoxicity (IC_{50}) and *es* site content (B_{max} value for 5-(SAENTA-x8)-fluorescein binding), a linear relationship, although not statistically significant ($p = 0.15$), was apparent between araC cytotoxicity and *es* site abundance.

The effect of the NT inhibitor, nitrobenzylthioinosine (NBMPR), on the cellular retention of 2-CdA and its metabolites was examined in four cultured lines of human ALL origin. When cultured lymphoblasts were "loaded" with 1 μM 2-CdA for 1 h and the medium level of 2-CdA was subsequently reduced by 10-fold (to initiate 2-CdA efflux), loss of 2-CdA and its metabolites followed a biexponential time course. In medium containing NBMPR, intracellular levels of 2-CdA and its metabolites were substantially increased ($p < 0.001$) compared with cells not exposed to NBMPR in the medium, and 2-CdA loss followed a monoexponential time course. These results suggest that nucleoside transport processes contribute to the efflux of 2-CdA from cultured lymphoblasts.

The cytotoxicity of 1-h exposure to 2-CdA of Reh-A2 and CCRF-CEM cells was enhanced 3-fold by subsequent exposure to 0.5 μM NBMPR relative to that of cells subjected to the same manipulations without NBMPR exposure ($p < 0.01$ in Reh-A2 and CCRF-CEM cells). It was demonstrated that, as expected, NBMPR inhibited transporter-mediated 2-CdA influx in Reh-A2 cells. This was evident as NBMPR "protection" of Reh-A2 cells against cytotoxicity during a 72-h interval of culture in 2-CdA-containing medium. Thus, NBMPR (and presumably other inhibitors of the *es* transporter, as well) may modulate 2-CdA cytotoxicity toward leukemic lymphoblasts in culture using a retention tactic to enhance toxicity or an exclusion tactic to achieve protection. In devising a clinical application for the *es* inhibitors as modulators of nucleoside analog cytotoxicity, the reversible nature of *es* fluxes must, of course, be a primary consideration.

ACKNOWLEDGMENTS

I am indebted to my supervisors Drs. A. R.P. Paterson and W.P. Gati for their support and encouragement throughout my Ph.D. program, for without them I would not have written this thesis. It was through their guidance and scientific insight that I have come to a greater understanding of the nucleoside transport field and science overall. Throughout my scientific career I know the values that they have taught me will continue to guide and influence me.

I would like to thank Drs. A.S. Clanachan and J.J. Akabutu for their helpful discussions, both scientific and personal, that made my stay at the University of Alberta fulfilling and at times challenging. I am indebted to Drs. J. J. Akabutu and P. Grundy for providing me with patient samples during the course of this project. I extend my gratitude to Drs. L.Y. Xue and S. Alessi-Severini for their companionship and advice throughout my Ph.D program. Thanks are also due to John Chan for his part in immunophenotyping the Reh and KM-3 cell lines and for bringing me patient samples, and to Deb Corby for teaching me most of what I know about cell culture. I extend my gratitude to everybody I knew in Dr. Paterson's and Dr. Gati's labs for their friendship and help with lab duties.

Finally I would like to thank my family and friends for their support and encouragement during the last five years. I owe special recognition to my husband Ralph who I'm sure could write a thesis on the joys and heartaches that are part of graduate school.

Financial support from the Alberta Heritage Foundation for Medical Research and the Alberta Cancer Board is gratefully acknowledged.

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A_{540}	absorbance measured at 540 nm
ADA	adenosine deaminase
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
araC	cytosine arabinoside; 1-β-D-arabinofuranosylcytosine
araCTP	araC 5'-triphosphate
bcl-2	tumor enhancer gene (inhibits apoptosis)
BDIS	Becton Dickinson Immunocytometry Systems
BM	bone marrow
2-CdA	2-chloro-2'-deoxyadenosine
2-CdATP	2-CdA 5'-triphosphate
CD	cluster of differentiation
cCD	cytoplasmic CD (cytoplasmic CD antigen expression)
cib	***c***oncentrative, ***i***nhibitor-insensitive nucleoside transporter with ***b***road substrate specifity
cif	***c***oncentrative, ***i***nhibitor-insensitive nucleoside transporter that accepts ***f***ormycin B as a substrate but not thymidine
cit	***c***oncentrative, ***i***nhibitor-insensitive nucleoside transporter that accepts ***t***hymidine as a substrate but not ***f***ormycin B
CLL	chronic lymphocytic leukemia
CML	chronic myelogenous leukemia
CR	complete response/remission
CNS	central nervous system
hCNT1	human concentrative nucleoside transporter 1
rCNT1	rat concentrative nucleoside transporter 1
mCNT2	mouse concentrative nucleoside transporter 2
cs	***c***oncentrative, inhibitor-***s***ensitive nucleoside transporter

CSF	cerebrospinal fluid
dAMP	2'-deoxyadenosine monophosphate
dADP	2'-deoxyadenosine diphosphate
dATP	2'-deoxyadenosine triphosphate
dCK	deoxycytidine kinase
dCyd	deoxycytidine
DNMG	Dulbecco's phosphate-buffered saline containing 5 mM glucose with N-methylglucammonium ion in place of Na^+
DPBS	Dulbecco's phosphate-buffered saline containing 5 mM glucose
DPM	dipyridamole; 2,6-bis(diethanolamino-4,8-diperidino)pyridimo[5,4-d]pyrimidine
hENT1	human equilibrative nucleoside transporter 1
ei	*e*quilibrative inhibitor-*i*nsensitive nucleoside transporter
es	*e*quilibrative inhibitor-*s*ensitive nucleoside transporter
FaraA	fludarabine; 2-fluoro-9-β-D-arabinofuranosyladenine
FB	formycin B
FBS	fetal bovine serum
FCM	flow cytometry
FITC	fluorescein isothiocyanate
Gy	Gray
h	hour
HCL	hairy cell leukemia
HD-MTX	high-dose methotrexate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HLA-DR	human leukocyte antigen-DR (class II)
HPLC	high-performance liquid chromatography
IC_{50}	concentration of drug(s) inhibiting the parameter measured in individual experiments by 50%.
Ig	immunoglobulin

cIg	cytoplasmic Ig
sIg	surface Ig
IT	intrathecal
i.v.	intravenous
mAb	monoclonal antibody
min	minute
MTD	maximum tolerated dose
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	methotrexate
NBMPR	nitrobenzylthioinosine; 6-[(4-nitrobenzyl)-thio]-9-β-D-ribofuranosylpurine
5'-NTase	5'-nucleotidase
NT	nucleoside transport
p53	tumor suppressor gene (induces apoptosis)
PE	phycoerythrin
PR	partial remission/response
Pre-B	precursor B
Ph^-	Philadelphia chromosome positive
RFI	relative fluorescence intensity
RH10F	RPMI 1640 medium containing 2 mM HEPES and 10% FBS
RH10FN	RH10F medium containing 0.5 μM NBMPR
SIA	stroma-supported immunocytometric assay
SAENTA	5'-*S*-(2-aminoethyl)-N^6-(4-nitrobenzyl)-5'-thioadenosine
5-(Sx8)-F	5-(SAENTA-x8)-fluorescein
SD	standard deviation
sec	second
SEM	standard error of the mean
SPNT	sodium-dependent purine nucleoside transporter
TCR	T-cell receptor

TK	thymidine kinase
TS	thymidylate synthase
T_{zero}	time-zero
UV	ultraviolet
VCR	vincristine
VM-26	teniposide
WBC	white blood cell

INTRODUCTION

1.1 Childhood acute lymphoblastic leukemia

1.1.1 Historical perspective

Acute lymphoblastic leukemia (ALL) is a clonal disease characterized by aberrant differentiation and proliferation of malignantly transformed lymphoblasts. ALL is the most common malignancy of childhood, accounting for approximately one quarter of newly diagnosed pediatric cancer cases (1). Prior to the development of the present poly-chemotherapeutic approach to the treatment of childhood ALL, this disease was fatal; the median duration of survival was only two to four months after diagnosis. Today, the outlook for children with ALL is more optimistic. With current polychemotherapeutic treatment, 95% of children with ALL achieve a successful remission and about two thirds of these will remain in continuous complete remission for five years or more (2). Progress in the treatment of childhood ALL can be attributed to the following factors: (a) more accurate diagnosis of ALL, (b) a greater understanding of the clinical and the biological basis of leukemia, (c) more effective drug combinations to achieve and maintain remission, (c) prophylactic therapy to prevent central nervous system (CNS) leukemia, (d) selected use of bone marrow transplantation, and (e) the tailoring of therapy to maximize long-term, event-free survival with minimum toxicity (2).

The discovery by Farber in 1948 (3) that treatment with aminopterin induced temporary remissions in childhood ALL was the first step toward improving the outcome of pediatric ALL. Treatment with the folic acid antagonist, aminopterin (3), and the

hypoxanthine analog, 6-mercaptopurine (4), introduced for the first time complete remissions (CR) that lasted approximately four months in 20% to 50% of patients (3). Attainment of CR, that is, the "complete" clinical and hematological disappearance of the disease, was found to be an important prognostic factor for survival (5). The recognition that ALL was a heterogenous disease (as defined by: (a) morphology, (b) cytogenetics, and (c) cell surface marker analysis, as well as clinically (6)), and the consequent introduction of combination chemotherapeutic protocols, further increased the number of patients achieving a CR (7). Despite CR rates of 20-50% it was noted that all patients eventually relapsed from remission.

Skipper *et al.* (8) demonstrated in exponentially growing rodent leukemia L1210 cells the log kill kinetic effect (that is, the number of cells killed is a linear, dose-dependent fraction of the number of cells present) of antileukemic agents. In the experiments of Skipper and colleagues, treatment failure was apparently due to an initial leukemic cell burden that was too high to allow eradication of the last leukemia cell. Although the growth of most human tumours appears to follow Gompertzian[1] kinetics, and thus will respond to cytotoxic drugs in a Gompertizan fashion (9), the findings of Skipper and colleagues apply, that is, cure theoretically represents elimination of all leukemia cells. It was realised that during clinical remission in pediatric ALL, the leukemic cell burden was still significant and if left untreated, would likely regrow, causing a relapse. Methotrexate (MTX), a folic acid antagonist introduced to ALL therapy in the 1950's was found to be

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[1]In Gompertzian "kinetics", the growth fraction of the tumour is not constant but decreases exponentially with time.

the best single agent for treatment during remission, especially when administered intermittently (10,11).

The incidence (40% to 70%) of meningeal leukemia rose as the CR rates increased. Leukemia often affects the CNS by infiltration of the meninges with leukemic cells (12). It appears that the CNS is a sanctuary for leukemic cells in that at conventional doses, antileukemic agents are essentially excluded from the CNS by the blood brain barrier (2). Since the mid-1970s, prophylactic treatment for CNS leukemia administered to newly diagnosed patients reduced the emergence of clinically overt CNS leukemia to between 4% and 10% (6,13,14). Unfortunately, certain groups of patients continued to have a high-risk of a relapse and failed to achieve long-term, event-free survival. Consequently, many contemporary protocols have included a so-called consolidation (intensification) phase of therapy that is administered immediately or soon after remission induction. This strategy has significantly improved the outcome for high-risk patients (15-17).

1.1.2 Classification of acute lymphoblastic leukemia

Childhood ALL is a heterogeneous disease characterized by morphologic, immunologic, histochemical and cytogenetic features of malignant lymphoblasts (2). Conventionally ALL is diagnosed when the content of lymphoblasts in the bone marrow (BM) is greater than 25%. Previously, classification of the acute leukemias has relied on the morphologic description of the predominant cell type present within the bone marrow, relating that to its presumed normal counterpart (18). In 1976, a group of French,

American and British (FAB) hematologists introduced a morphological classification system that subdivided ALL into three groups, L1, L2 and L3 (Table 1) (18). However, this classification system had problems of reproducibility and subjectivity, especially with the distinction between L1 and L2 blasts. Modifications in 1981 to this system (19) increased agreement among pediatric oncologists from 63% to 84%. Interobserver concordance was also increased when cytochemical analysis was added to the FAB morphological classification procedure (20).

Recent advances in immunologic phenotyping and molecular biology have improved the precision and the classification in diagnoses by providing information regarding both the lineage and stage of maturation of malignant cells (21). Currently, 98-99% of acute leukemias may now be reliably classified by immunological marker analysis (22). Greaves *et al.* (23,24) have observed that ALL evidently represents the neoplastic expansion of a clone of cells that express phenotypic features found in normal lymphoid cells of a particular lineage and maturational stage. Normal lymphoid cells express an array of cellular antigens[2] related to their lineage and stage of differentiation. Immunologic diagnosis and classification of ALL is based on the reactivity of the test cells with a panel of monoclonal antibodies (mAb) directed against antigens expressed on normal T- and B-lymphoblasts at various stages of differentiation.

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[2]Plasma membrane antigens recognized by several monoclonal antibodies are grouped into "cluster determinants" or "clusters of differentiation" (CDs). cCD refers to cytoplasmic antigen expression . A recent workshop on leukocyte antigens evaluated approximately 1,100 MAbs and 78 CDs (21).

Table 1

FAB classification of acute lymphoblastic leukemia[3]

Cytologic Features	L1	L2	L3
Cell size	Small cells predominate	Large[a], heterogeneous	Large and homogenous
Amount of cytoplasm	Scanty	Variable, moderately abundant	Moderately abundant[b]
Vacuolation	Variable	Variable	Prominent
Basophilia	Slight or moderate	Slight, deep in some	Very deep
Nucleoli	0-1, small/not visible	1+, prominent	1+, proniment
Nuclear chromatin	homogenous	Variable, heterogenous	Homogenous and finely stippled
Nuclear shape	Regular, occasional narrow clefts	Irregular, clefting and indentation common	Regular-oval to round
% L1 cells	90-100 %	74-90 %	≤ 74 %
Frequency (%)	82	17	1

[a] in a cell of "large" size, the diameter is understood to be about twice that of red blood cells

[b] ≥ 20% of surface area

[3]From Trigg (6), Mahoney (14), and Bennett *et al.*(18).

However, the identification of aberrant antigen expression (22) in some leukemic cells suggests that B- and T-ALL subtypes cannot be easily reconciled with the model of normal B- and T-cell differentiation (25-27). Based on this observation it was suggested that these leukemic blasts may not originate from phenotypically identical normal counterparts, but that they may possibly arise from the malignant proliferation of rare normal lymphoid cells not detectable with methods presently available (22).

Molecular biology studies of immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements have been expected to enhance the diagnostic accuracy of immunophenotyping. During early lymphoid development, the assembly of the variable regions of these genes is directed by DNA rearrangements that result in the production of a diverse array of proteins. The patterns of Ig heavy and light chain gene rearrangements, and the patterns of Ig protein expression (cytoplasmic (cIg) or surface (sIg)) are associated with different stages of B-cell development (28). The sequence of gene rearrangements is thought to occur as follows; heavy-chain (μ) gene rearrangements precede those of κ light-chain genes, and κ light-chain gene rearrangements precede those of λ light-chain genes (29). Similarly, TCR gene rearrangements and related protein expression changes are associated with different stages of T-cell development (28): two types of TCRs that have been identified are multimeric complexes comprised of a heterodimer isoform of the receptor ($\alpha\beta$ or $\gamma\delta$) and a glycoprotein (CD3) (30). However, due to aberrant gene rearrangements (30), the role of genotyping is most often confirmatory rather than diagnostic. Despite this, the presence of gene rearrangements may prove useful in confirming both the clonal origin of leukemic cells and completeness

of remission (31).

Studies of antigen expression and of Ig and TCR gene rearrangements have identified four to six subgroups of B-lineage ALL and three to four subgroups of T-lineage ALL. Criteria applied to define subgroups of B- and T-lineage ALL differ markedly between clinical studies, and a generally accepted classification system has not yet been established for immunophenotypic subgroups of ALL (22). However, although cytoplasmic CD3 (cCD3) and cCD22 are very reliable markers for the T- and B-cell lineages, respectively, most of the differentiation antigens found on T- and B-cells have also been identified on other cell types. Accordingly, it has been recommended that immunophenotypic classification of ALL should be based on a cellular pattern of lymphoblast reactivity with a panel of lineage-associated monoclonal antibodies, rather than on the presence or absence of a single antigen (32).

1.1.2.1 B-lineage ALL

B-lineage ALL accounts for 80% of newly diagnosed ALL cases. More than 20 different antigens have been identified on B-cells (21). Approximately 60% of children with ALL are assigned to an early Precursor B (Pre-B) lineage, the least differentiated B-lineage ALL. Early Pre-B lymphoblasts express HLA-DR, CD10[4], CD19, cCD22 and CD24; a subtype of this group is CD10-negative. Early Pre-B cases are often associated with μ gene rearrangements, but usually lack either cIg or sIg expression. Pre-B ALL, a

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[4]The CD10 antigen is also known as CALLA (common acute lymphoblastic leukemia antigen).

classification category distinguished from the preceding "early Pre-B ALL", which represents approximately 18% to 20% of childhood ALL (14), has immunological features similar to the early Pre-B ALL, except that cIg, CD20, CD21, and CD 22 antigens are expressed. In immature B-cell ALL (transitional Pre-B ALL), sIg(μ+) and μ, κ and λ gene rearrangements are evident. The most differentiated and rarest (2-3%) B-lineage ALL is B-cell ALL (14); this phenotype is postulated to be the leukemic phase of Burkitt's non-Hodgkin's lymphoma (33), and represents patients with an FAB L3 lymphoblast morphology. The lymphoblasts in this group are characterized by expression of sIg($\mu^{-}\kappa^{+}\lambda^{+}$) and CD23 and the majority are CD10-negative.

In 1-3% of children with ALL, neither lymphoid nor myeloid antigens are expressed on the leukemic blasts; these leukemias are classified as undifferentiated, null or stem-cell leukemias (14). Undifferentiated lymphoblasts usually express the progenitor cell antigen, CD34 (34) and HLA-DR. In many of these instances, the B-lineage origins have been recognised from the Ig heavy chain rearrangements (14).

1.1.2.2 T-lineage ALL

Fifteen to twenty-five percent of ALL occurrences in children are T-lineage leukemias. The conventional classification of pediatric T-cell ALL is based on the three proposed stages of normal thymic maturation (22,26,27) and accommodates 75% of cases, while the remainder are said to have ambiguous phenotypes (28). The early thymocyte phenotype (stage I) is characterized by the expression of CD2, CD5, CD7, and cCD3 antigens. Patients with an intermediate phenotype (stage II) express CD1, CD2,

CD5, CD7, cCD3 antigens, and cTCR; variable expression of CD4, CD8 and CD3 antigens is also found in this subgroup. Finally, those T-lineage acute lymphoblastic leukemias with a mature phenotype (stage III) also express sTCR and the CD3, CD5, CD7 and CD2 antigens. Mature T-cells are found in two distinct populations which are distinguished by the expression of either the CD4 or CD8 antigen (30). Controversy exists as to the relative proportions of each group. Reinherz and colleagues (26) state that the majority of T-ALL cases are of the early thymocyte phenotype, whereas, Roper and co-workers (27) found the majority of cases to be of the mature T-cell stage. T-lineage ALL has also been classified on the basis of rosetting[5] with sheep erythrocytes and CD2 reactivity, with early T-lineage ALL (pre-T ALL) cells being $CD7^{+}/CD2^{-}$/E-rosettes and T-lineage ALL cells being $CD7^{+}/CD2^{+}$/E-rosettes (22).

With the advent of comprehensive panels of monoclonal antibodies, it has been recognised that leukemic cells may coexpress antigens associated with more the one lineage; such leukemias are called biphenotypic. Leukemic blasts coexpressing combinations of B-, T- and myeloid-associated antigens have been reported (36,37). Myeloid-associated antigens have been reported in about 25% of childhood ALL cases (21,38). Bilineal or mixed lineage leukemia describes the presence of two distinct blast populations. Examples include the following: (a) mixed L1/L2 patterns, (b) the emergence of L2 type at the time of relapse of leukemia originally classified as L1, and (c)

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[5]Certain T-cell leukemias were identified based on the expression of a human T-cell marker, the sheep erythrocyte receptor (SER). Sheep erythrocytes form "rosettes" with lymphoblasts expressing the SER protein (35).

mixed ALL/AML patterns (2).

1.1.3 Prognostic factors

Although 95% of pediatric ALL patients will achieve remission with modern poly-chemotherapy, about a third of such patients will eventually relapse. Recognition that certain clinical features of ALL at presentation were related to treatment outcome has considerably changed strategies for ALL treatment. These prognostic features include initial white blood cell (WBC) count, age, sex, CNS leukemia at diagnosis, response to therapy, cytogenetics, and immunophenotype (1,2). Low-, intermediate- and high-risk categories, based on clinical features at presentation have become apparent; however, no universal definition of risk groups presently exists. In general, children with a low or standard risk of relapse are between the ages of 1 and 10 years, have a WBC count $\leq 10 \times 10^9$/L, lack evidence of a mediastinal mass or of CNS leukemia, and have a Pre-B ALL immunophenotype (39). The ability to predict which children have a high probability of treatment failure has permitted some tailoring of therapy.

1.1.3.1 Initial leukocyte count

Leucocyte count at diagnosis is one of the most important prognostic determinants for remission induction and event-free survival (40). An initial WBC count below 10×10^9/L is associated with a favourable outcome, whereas, children with an initial count above 50×10^9 cells/L have a less favourable prognosis. Hyperleukocytosis (WBC above 100×10^9/L) is a predictor of an unfavourable outcome. Hyperleukocytosis is often

associated with the following prognostic factors: age less than one year, mediastinal mass, T-cell ALL, CNS leukemia, massive organomegaly and deviations in leukemic cell ploidy, particularly, those with chromosome numbers of less than 50[6]. In addition, these factors are typically associated with a poor prognosis (2). Clinically, the initial WBC count is most commonly used to stratify patients into low-risk or high-risk categories (15,16,42).

1.1.3.2 Age and sex

The leukocyte count at diagnosis and the patient's age have proved to be the two most reliable predictors of response to therapy. Children between the ages of one and ten have the best prognosis. In pediatric ALL in remission, a long-term, event-free survival rate of 75% is expected with conventional treatment, whereas, the survival rate in infant ALL is between 20% to 55% (43). Possible reasons for this discrepancy between survival rates in childhood and infant ALL include the typical presenting features of hyperleukocytosis, CNS leukemia, hepatosplenomegaly, increased frequency of bone marrow and CNS relapse, and a high percentage of undifferentiated CD10-negative and mixed lineage leukemias in infant ALL patients (43). Also, an increased predominance of the translocation t(4;11)(q21;q23) in infant ALL has been correlated with poor prognosis (44,45). British investigators have highlighted the particularly poor prognosis for infants under 26 weeks of age (46).

ALL is the most common cancer in adolescents (47). This group of patients has not experienced the dramatic increases in long-term survival rates that have been achieved

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[6]Hyperdiploidy > 50 chromosomes is a favourable prognostic feature (41).

with modern chemotherapy in younger children. Adolescents are more likely to have a number of biological and phenotypic features associated with an adverse outcome. Typically, they present with high initial leukocyte counts (48) and, lymphoblasts with a T-ALL immunophenotype, L2 morphologic characteristics, a DNA index[7] less than 1.16 and with ploidy other than a hyperploidy greater than 50. Adolescents over 16 years of age have a significantly poorer outcome than adolescents in the 10- to 15-year-old group; the reason for this is unclear (48). In the past, the observation that females had a better prognosis than males was partially explained by testicular relapse and a higher incidence of T-ALL in males; however, with more effective therapy, the differences between males and females in prognosis have all but disappeared.

1.1.3.3 Immunophenotype

Children with B-lineage ALL have a poorer prognosis than other immuno-phenotype groups. This is due in part to a very aggressive characteristic of the disease, in that B-lineage ALL cells in these children have a doubling time of approximately 24 hours (14). T-ALL is also associated with a poor clinical outcome, but not independently of other factors (49). More than 40% of patients with T-ALL present with initial leukocyte counts greater than 100×10^9/L. Children with T-ALL also frequently present with the following characteristics: mediastinal masses, hepatosplenomegaly, adenopathy, CNS relapse, t(11;14)(q13;q11) translocations, a leukemic DNA index less than 1.16, and

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[7]The DNA index is the ratio of the DNA content in leukemic G_0/G_1 cells to that of normal cells.

pseudodiploidy with less than 50 chromosomes, all of which are factors with an adverse effect on event-free survival (14). Rivera and colleagues (15) found the prognosis was much better for CD10-positive T-ALL than for CD10-negative T-ALL. However, the relationship between prognosis and stage of T-cell differentiation remains controversial (1). Interestingly, a number of studies using intensive chemotherapy regimens found a T-cell immunophenotype to be a favourable prognostic factor (50,51).

Patients with early Pre-B ALL have higher rates of (a) attaining remission and (b) long-term, event-free survival than children with other types of ALL (23), and children with this disease are also more likely to have a low initial WBC count (below 20×10^9/L). Early Pre-B ALL patients, in whom leukemic blasts express CD10, fare better than children in whom the blasts do not express the CD10 antigen (52). Patients with Pre-B ALL are classified as low-, intermediate- or high-risk depending on the presence of other prognostic factors (39). The prognostic significance of myeloid-associated antigens in childhood ALL remains uncertain. Wiersma and colleagues found the expression of myeloid-associated antigens to be a predictor of poor outcome (38). However, others did not confirm this result (21,53). With the development of distinct chemotherapeutic regimens for each cell type, the importance of immunophenotype as a prognostic factor has declined. Despite this, the Pediatric Oncology Group (POG) continues to use separate treatment protocols for the different immunological subtypes of ALL (21).

1.1.3.4 Cytogenetics

Chromosomal abnormalities in leukemic lymphoblasts have also been shown to

have important prognostic value in pediatric ALL; such abnormalities have been identified in 80-90% of childhood ALLs (54). Chromosomal abnormalities are found as changes in chromosome number (ploidy) or chromosome structure (translocations, deletions or inversions) or both. Patients having a lymphoblast karyotype with hyperdiploidy of greater than 50 chromosomes and no structural changes in lymphoblast chromosomes generally have a more favourable outcome than other karyotype groups, while those with a hypodiploid karyotype usually have a particularly poor prognosis (55). Hyperdiploidy of greater than 50 chromosomes is associated with other favourable prognostic factors, including low initial WBC, early Pre-B ALL immunophenotype and an age between 1 and 10 years. A blast cell DNA index greater than 1.16 is associated with a 90% four-year, event-free survival rate (56).

The most common structural abnormalities in ALL lymphoblast chromosomes are translocations, which tend to be immunophenotype-specific (14). The t(8;14)(q24;q32.3) and t(8;22) are translocations found specifically in B-ALL and are associated with a poor prognosis (14,57). The t(9;22)(q34;q11) translocation (Philadelphia (Ph) chromosome) is found in approximately 6% of childhood ALL cases, 90% of which are of B-lineage origin (58). The presence of the Ph chromosome in an ALL karyotype is correlated with a poor prognosis if associated with a high initial leukocyte count, and an age of greater than 10 years (58,59). The t(4;11)(q21;q23) translocation occurs in ALL lymphoblasts in 5% of patients, mostly in infants, and is associated generally with adverse prognostic factors, including high initial leukocyte counts, hepatosplenomegaly, CD10-negative phenotype and mixed lineage immunophenotype (60,61). Improvements in therapy have reduced the

prognostic significance of several translocations that were once thought to be clinically important. However, the t(9;22), t(4;11) and the t(8;14) translocations remain predictors of poor outcome (2).

1.1.3.5 Initial response to treatment

Children with ALL who receive treatment and experience a marked reduction in leukemic cell burden (to less than 25% blasts in the bone marrow at day 14 of treatment or ≤ 1000 blasts/μl blood on day 8[8]) had a better prognosis than children who responded more slowly (6). For children who fail to achieve remission (less than 5% blasts in the bone marrow) within 4 to 6 weeks after initiation of therapy, the prognosis is considered to be dismal, and the expectation is that most of such patients will succumb to their disease. Fortunately, only 5% of children with ALL who undergo intensive induction regimens fail to achieve remission (62). The chance for long-term survival is small for ALL patients who achieve a remission with therapy and then suffer a marrow relapse; most patients in those circumstances fail to achieve long-term, event-free survival (2). Some studies have found that if marrow relapse occurs more than one year after the end of therapy the likelihood of achieving long-term remission remains reasonable (63).

1.1.4 Treatment of ALL

Two principles continue to dominate ALL therapy: (a) treatment should be

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[8]The Berlin-Frankfurt-Müster study group (50) defined poor response to therapy as a treatment outcome in which there was ≥1000/blasts/μl blood on day 8 of induction therapy.

designed with curative intent; and (b) toxicity and long-term morbidity should be limited without compromising treatment efficacy (6). The latter topic assumes importance as increasing numbers of children are cured. In general, contemporary therapeutic protocols include five basic phases: remission induction, consolidation or intensification, CNS prophylaxis, maintenance or continuation therapy, and treatment for relapsed patients.

1.1.4.1 Remission induction

The purpose of inductive therapy is to rapidly destroy as many leukemic cells as possible, while restoring normal hematopoesis. Based on the concepts of Skipper and associates (8) and of Goldie *et al.* (7), current treatment protocols use combination chemotherapy to rapidly achieve maximum cell kill of the ALL lymphoblast population. The purpose of this approach is to maximally reduce residual leukemic blasts, since it has been proved in animal systems that injection of a single cancer cell into a susceptible host can kill that animal (64). To achieve this goal, maximum tolerated doses of chemotherapeutic agents are administered as early as possible.

A complete remission (CR) is usually defined as the condition in which the marrow contains fewer than 5% lymphoblasts and peripheral blood cells are in the normal range. The majority of standard induction regimens use the following three drugs: prednisone, vincristine and L-asparaginase. The identification of different risk groups in pediatric ALL led to intensification of chemotherapy for high-risk groups. Intermediate-risk and high-risk patients were found to have better subsequent event-free survival when standard induction regimens were supplemented with anthracyclines (daunorubicin or

doxorubicin), cyclophosphamide, MTX, teniposide (VM-26) or cytosine arabinoside (araC) (65). Treatment with these combinations achieve complete remissions in 95% of children with ALL (62). This phase of treatment lasts from 4 to 6 weeks, depending on the risk category of the patient. Increased morbidity and mortality are associated with the intensified regimens; however, with improved supportive care, fewer than 2% of children die during the induction phase (2).

1.1.4.2 Consolidation

Most treatment failures in childhood ALL result from an inadequate reduction of the leukemic clone and the acquisition of drug resistance (15). A second phase of intensive treatment, termed consolidation treatment, has been administered to intermediate-risk and high-risk children, to reduce the leukemic cell burden and the emergence of drug-resistant clones (15,66). A recent trial by the Berlin-Frankfurt-Münster group has validated this approach (50). The latter study also showed that low-risk patients benefited from intensive consolidation therapy. Controversy exists about whether the greater morbidity associated with consolidation treatment with intensive multiagent chemotherapy is balanced by an enhanced survival rate in children in low-risk or intermediate-risk categories (67). Consolidation therapy may last from 4 to 30 weeks depending on the risk category of the child (6).

Several multiagent consolidation regimens have been investigated and have included certain combinations of these agents: cyclophosphamide, araC, 6-mercapto-purine, 6-thioguanine, L-asparaginase, MTX and high-dose MTX (HD-MTX) with

leucovorin rescue. Because of serious complications associated with multiagent regimens, rotational combination chemotherapy[9] has been evaluated as an alternative option (15,66). Rivera and colleagues (15) observed improved responses in certain high-risk patients treated with intensified induction regimens followed by rotational combination chemotherapy. At St. Jude Children's Research Hospital, a regimen that consisted of rapidly rotated drug combinations, started in the induction phase and continued for 2 years through the maintenance phase, was administered to children presenting with late hematological relapses (1). This trial produced results similar to those achieved in patients with newly diagnosed ALL. Treatment intensification has also been achieved by increasing the dosage of a single agent in an otherwise standard regimen. A classic example of this has been the administration of HD-MTX (68). In the late 1960s, Djerassi and colleagues (69) demonstrated that by increasing the conventional dosage of MTX, remissions were induced in patients resistant to this drug. Wang *et al.* (70) showed that by administering *i.v.* HD-MTX in combination with intrathecal (IT) MTX, higher levels of MTX were maintained in the CSF for a 24-h period than if IT MTX was administered alone. HD-MTX has the additional advantage of "hitting" leukemic cells in pharmacological sanctuaries such as the testis or the ovaries (70). However, use of HD-MTX is not without hazard, as this approach is associated with toxicity, including mucositis, neutropenia, seizures, fever and skin rash (68).

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[9]Treatment consists of rapidly alternating, non-cross resistant drugs, commencing early in the induction phase and continuing throughout the maintenance period.

1.1.4.3 Central nervous system prophylaxis

As improved chemotherapy has increased the numbers of children with ALL that achieve complete remission, the incidence of CNS leukemia has also risen substantially, from 4% in 1947 to 40% in 1960 (12). In patients who achieve remission, microscopic foci of leukemia in the CNS may progress to become a frank CNS leukemia. The realization that in the majority of ALL children, microscopic foci of leukemia were present in the CNS at the time of diagnosis, led to the introduction of specific CNS prophylactic therapy during the induction and consolidation phases of therapy. As with the induction and consolidation phases of therapy, the methods of CNS prophylaxis vary widely among treatment centres.

Investigators at St. Jude Children's Research Hospital were the first to demonstrate that craniospinal irradiation, or cranial irradiation (24 Gy) with IT MTX, was effective in preventing CNS leukemic relapse (71). A CNS relapse rate of only 4% was experienced with the implementation of these treatment protocols. These regimens remain the standard against which other treatment protocols for CNS prophylaxis are evaluated (13). Later studies showed that lower radiation doses (18 Gy) combined with IT therapy to be equally effective and less toxic relative to the St Jude protocol (72). Nesbit *et al.* (72) also reported equivalent disease control in low-risk patients treated with IT MTX alone. Due to the delayed effects on CNS function associated with craniospinal and cranial irradiation, alternative treatment schedules that exclude irradiation are of continuing interest. This is especially important in the treatment of ALL in infants of less than one year of age who tolerate irradiation to the CNS poorly (73).

There is a general move away from cranial irradiation to IT chemotherapy for the treatment of low-risk and intermediate-risk patients with ALL (73). MTX, hydrocortisone and araC in combination ("triple therapy") are commonly used in IT protocols. Some centres do not include hydrocortisone in IT therapy because this agent has been shown to reduce the uptake of MTX by leukemic cells (74). The Dutch Childhood Leukemia Study Group found the substitution of prednisone by dexamethasone in conventional induction and consolidation regimens to be highly effective in preventing CNS relapse (75), presumably because dexamethasone penetrates the CSF to a greater extent than prednisone. IT therapy is usually administered for one year, during the induction and consolidation phases of treatment, and given at least every eight weeks (1). The duration of treatment is adjusted according to the patient's risk category.

High-risk patients with B-cell leukemias and lymphomas have also been treated successfully (achieving long-term remissions in 50-80% of cases) with IT therapy combined with systemic MTX and araC (76-78). Wang *et al.* (70) and Freeman and colleagues (79) used systemic HD-MTX and intermediate dose MTX, respectively, to achieve high plasma concentrations adequate for MTX penetration into the cerebrospinal fluid (CSF). It was hypothesized that high doses of MTX would have systemic antileukemic effects as well. However, the results of these studies revealed no significant advantage of HD-MTX in intermediate-risk patients, and among high-risk patients this strategy did not prevent CNS relapse or improve overall disease control. At present, the majority of high-risk patients are treated with cranial irradiation in addition to IT and systemic treatment modalities (73,80). CNS irradiation is recommended for ALL patients

who present with overt CNS leukemia at diagnosis (67). Craniospinal irradiation is currently reserved for treated ALL patients who suffer a CNS relapse (1).

We should continue to seek improvement in CNS prophylaxis, as the incidence of CNS relapse is hovering at approximately 5%. The prognosis is poor for treated children with ALL who suffer CNS relapse because only 20% to 50% of these patients will achieve long-term, disease-free survival (6). The refractory nature of relapsed ALL in the CNS is not understood, but its basis may include drug resistance or the inability of IT drugs to achieve adequate concentration in the meninges (81). Steinherz and colleagues (81) have attempted to overcome these problems by administering intraventricular chemotherapy via an Ommaya reservoir in combination with low dose craniospinal irradiation (9 Gy). They observed an increase in the duration of CNS remission in children with ALL, but only 32% of treated patients achieved long-term, event-free survival. Prevention of CNS leukemia is a critical goal in the treatment of ALL and it is important that efforts be continued to find effective, nontoxic regimens to eliminate CNS relapse as a complication of ALL treatment.

1.1.4.4 Maintenance therapy

ALL is unique among human malignancies in requiring chemotherapeutic treatment of 2 to 3 years duration (82). B-lineage ALL is exceptional among ALL subtypes in that this disease is usually treated with short-term (< 7 months), intensive therapy, consisting of cyclophosphamide, araC and MTX in association with IT chemotherapy (82). The goal of maintenance or continuation therapy is to kill remaining

leukemic cells that have survived the chemotherapeutic treatment because of a drug-resistant genotype that has, in effect, been "selected" from the treated leukemic cell population. The present inability to detect minimal residual disease, that is, to detect "the last leukemic cell" or to recognize "zero percent blasts" has resulted in controversy over the effectiveness of the agents used in maintenance treatment and the duration of that treatment (82). Conventionally, a treated ALL patient is deemed to be in remission if the percentage of blasts in the marrow is less than 5%; however, this criterion may still include a large leukemic cell burden. Morphologic, enzymatic, genetic and immunologic methods are currently being tested for their ability to detect residual leukemic blasts (83). The majority of ALL maintenance protocols consist of weekly MTX, daily 6-mercaptopurine, with monthly or quarterly doses of vincristine and prednisone. It is interesting that little information exists regarding the effects of MTX on the initial leukemic cell burden, or on its usefulness as an induction agent (67). Children in high-risk categories are often treated with more complex multiagent regimens than the standard maintenance protocols described above. These complex regimens usually include the standard treatment, combined with araC, VM-26, L-asparaginase, cyclophosphamide and doxorubicin given as separate agents or in combinations. The efficacy of such combinations remains to be confirmed (14). The conventional duration of maintenance treatment has been 3 years (82); an increment in disease-free survival has not been demonstrated with longer treatment periods. However, as therapy has been intensified, and as knowledge of the long-term effects of combined chemotherapy and radiotherapy of the CNS has increased, shortening the length of therapy has become a desirable option, especially for low-risk and

intermediate-risk groups. Most children with ALL now undergo between 2 and 3 years of therapy (6).

1.1.4.5 Treatment of relapsed ALL

Despite the achievement of CR in 95% of children with ALL who undergo induction therapy, 25% to 30% of treated children with ALL relapse within 5 years of diagnosis (2,14,84). The long-term, disease-free survival rate for children treated after relapse ranges between 30% and 65% (13). The prognosis is better for patients who experience only extramedullary relapse, and is better for patients who relapse 6 months or more after the cessation of therapy (13). Response to treatment differs with the following factors: the site of relapse, length of time to relapse, previous treatment and the number of relapses.

For children with bone marrow relapse, the likelihood of long-term, disease-free survival is small, especially in instances where relapse occurs during treatment (85). Controversy exists as to the optimal treatment for relapsed ALL (65). Chemotherapy that repeats earlier protocols may not be the treatment of choice for relapsed pediatric ALL, because those patients may have disease resistant to chemotherapy agents used in induction and consolidation treatment. Therefore, intensive chemotherapy different from that employed initially, is recommended for children with late marrow relapses (82,85). Approximately 40% of patients that achieve extended first remissions also achieve a sustained second remission when treated with intensive chemotherapy (82). Multidrug regimens incorporating araC, VM-26 and cyclophosphamide are used to treat patients

resistant to MTX and 6-mercaptopurine-based treatment protocols (65). CNS prophylactic therapy is essential in treating ALL in marrow relapse because the effect of the initial prophylactic CNS therapy is abolished by the relapse. The use of cranial irradiation in late hematological relapse is controversial because of the potential for severe neurotoxicity (85).

Allogeneic bone marrow transplantation is usually recommended for patients who relapse early while undergoing therapy, or for patients in their second or third remission, and for children with Ph-positive ALL (86). However, data concerning allogeneic bone marrow transplantation for infants have not been published (65). Experimental approaches to the treatment of marrow relapse include; HD-araC followed by L-asparaginase (87); and treatment with MTX and VM-26 prior to conventional reinduction therapy (1).

The CNS and the testes are the two most common sites of extramedullary relapse. At present, the treatment of choice for CNS relapse is a combination of IT MTX and craniospinal radiation. However, treatment protocols for CNS relapse have included IT araC, IT "triple therapy" (Section 1.1.4.3), intraventricular MTX, HD-MTX, or combinations of these therapies. New experimental therapies include; ventriculolumbar cerebrospinal perfusion; IT gold; frequent IT administration of low dose MTX; IT diazoquinine, thiothepa, 6-mercaptopurine, mafosfamide or etoposide (2).

While testicular relapse occurs at rates of 1% to 6%, the prognosis for such patients is generally good. Boys who relapse 6 months or more after the end of therapy have a much higher disease-free survival (80%) than those who relapse while on therapy

(2). The frequency of testicular relapse is decreasing as the efficacy of ALL therapy improves. (2). Patients presenting with testicular leukemia usually receive radiation (24 Gy) to the testes, followed by aggressive systemic chemotherapy.

Although the fraction of pediatric ALL patients achieving CR has risen dramatically in the last 10 years, contemporary therapy fails in approximately 25% of patients. This fact emphasises the need for further research into the biology of ALL and for critical re-evaluation of treatment protocols. A major challenge is development of effective therapy for patients who relapse on intensive protocols. Identification of antileukemic agents capable of killing resistant blast cells, which are a major cause of relapse, would probably improve the outcome for these patients. However, few drugs with unique mechanisms of action have been introduced for the treatment of ALL since the 1970s.

Drugs investigated in exploratory clinical trials include taxol, topotecan, 2-deoxycoformycin and 2-chlorodeoxyadenosine (cladribine, 2-CdA) (65). 2-CdA is a purine analog with important cytotoxic activity in both resting and proliferating lymphocytes (88). 2-CdA is in current use in the treatment of a number of indolent lymphoid malignancies (89-96), and has also shown notable antileukemic activity in previously untreated pediatric acute myeloid leukemia (AML) and in relapsed AML (97,98). The success of 2-CdA in the treatment of these lymphoid malignancies has made this agent a candidate for evaluation of cytotoxicity towards lymphoblasts from pediatric ALL patients.

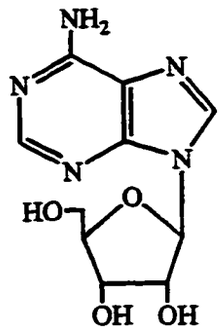
1.2 2-Chlorodeoxyadenosine

The understanding that in intact animals, adenosine deaminase (ADA) deficiencies with genetic or pharmacologic bases result in enhanced levels of plasma deoxyadenosine and of its phosphate metabolites in blood cells, and that these circumstances led to lymphoid cell death, prompted a search for ADA inhibitors and for deoxyadenosine congeners resistant to ADA (99). Carson *et al.* (99) were the first to explore the lymphocytotoxic potential of 2-CdA, a deoxyadenosine analog that is a poor substrate for ADA. Christensen *et al.* (100) had earlier reported the inhibition of growth of L1210 cells by 2-CdA. 2-CdA (Fig. 1) is resistant to ADA because the substitution of chlorine for hydrogen in the 2-position of the purine ring prevents deamination at the 6-position of the purine ring (101).

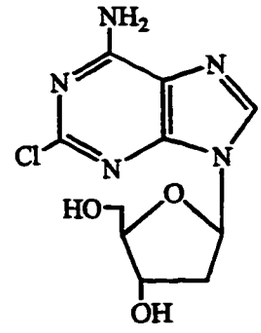
1.2.1 Mechanism of action of 2-CdA

2-CdA is cytotoxic to both resting and proliferating lymphocytes (88), a finding that may account for its impressive activity in patients with indolent lymphoid neoplasms; the latter respond poorly to antimetabolites. The cytotoxic activity of 2-CdA requires transporter-mediated (102,103) entry into leukemia cells and sequential phosphorylation by deoxycytidine kinase (dCK) and purine nucleotide kinases, for conversion to its putative active form, the 5'-triphosphate ester (2-CdATP) (104).

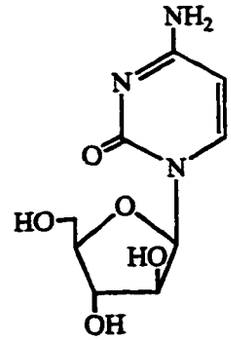
Deoxycytidine kinase (dCK) and cytoplasmic 5'-nucleotidase (5'-NTase) are the principal enzymes that phosphorylate 2-CdA and dephosphorylate 2-CdA monophosphate (2-CdAMP), respectively (105). Controversy exists over the relative importance of the



Adenosine



2-Chlorodeoxyadenosine



Cytosine arabinoside

Figure 1. Structural formulae for adenosine , 2-chlorodeoxyadenosine (2-CdA) and cytosine arabinoside (araC).

two enzymes in the cytotoxicity of 2-CdA. Some researchers have showed that the antileukemic effects of 2-CdA correlate to the ratio of dCK activity to 5'-NTase activity (88,105). A recent study demonstrated an increase in sensitivity to 2-CdA of cell lines transfected with the retroviral vector, LNPO, which includes cDNA sequences that encode dCK (106). Hapke and colleagues (106) suggest that dCK gene transfer may prove useful in the treatment of malignancies responsive to nucleoside analogs. However, Saven *et al.*(107) found no correlation between cell sensitivity to 2-CdA and the activity of dCK and 5'-NTase.

It has recently been shown that phosphorylation of dCyd was more efficient when UTP was used as a phosphate donor instead of ATP (108). Based on this information, Shewach and colleagues (109) suggested that increasing intracellular UTP levels would facilitate phosphorylation of araC and 2',2'-difluoro-2'-deoxycytidine (dFdC). Therefore, increasing intracellular UTP levels as an adjuvant therapeutic approach may also be useful in 2-CdA therapy (110).

The mechanism by which 2-CdA kills cells is not yet fully understood and it is possible that the mechanism may differ with cell type. In proliferating cells, incorporation of a single 2-CdAMP residue into DNA by DNA polymerase β or incorporation of several consecutive residues by polymerase α results in chain termination (111). Significant intracellular concentrations (0.1-0.3 μM) of 2-CdATP may also inhibit ribonucleotide reductase, resulting in an imbalance in deoxyribonucleotide triphosphate pools, with subsequent impairment of DNA synthesis and repair (112,113). Ribonucleotide reductase is active in dividing cells, as it is S-phase specific. A decrease in intracellular dATP levels

also facilitates the incorporation of 2-CdATP into DNA through reduction of dATP competition for DNA polymerases (112).

In resting cells, the accumulation of 2-CdATP is believed to inhibit DNA repair because of inhibition of DNA polymerase β. Unrepaired breaks in DNA strands appear to activate poly(ADP)ribose synthetase, resulting in cellular depletion of NAD and ATP, and initiation of apoptosis (112,114). Recently, Gartenhaus *et al.* (115) demonstrated the induction of p53 and its target WAF1/CIP1 in apoptotic leukemic cells from chronic lymphocytic leukemia (CLL) patients treated with 2-CdA. p53-Dependent apoptosis has been implicated as a critical modulator in the cytotoxicity of a number of DNA damaging agents (116) and WAF1/CIP1 has recently been identified as a down-stream target of p53 in regulating cell cycle progression through a G_1 checkpoint. Another study by Petersen *et al.* (117) demonstrated downregulation of bcl-2 expression and an increase in apoptosis in cells from CLL patients exposed to 2-CdA.

1.2.2 Pharmacokinetics of 2-CdA

Relatively few pharmacokinetic studies with 2-CdA have been conducted. 2-CdA has a two-phase plasma clearance in humans, with a $t_{1/2\alpha}$ of approximately 35 minutes, and a $t_{1/2\beta}$ of approximately 7 h (118). This agent is weakly bound to plasma proteins (119). 2-CdA has also been shown to enter the CSF with a plasma/CSF ratio of 4 (120).

Prolonged exposure of leukemic cells to 2-CdA was found to be necessary for cytotoxic effects (88). On the basis of these studies, daily 2-h intravenous infusions over 5 or 7 days are the foundation of current protocols for 2-CdA treatment (121,122). The

long terminal half-life results in a trough level of 2-CdA in the plasma that is only slightly lower than the steady-state level given during continuous infusion (118). Hence, the subcutaneous and oral administration of 2-CdA would seem feasible. Liliemark *et al.* (123) have reported a bioavailability of 100% following subcutaneous administration of 2-CdA and a bioavailability of 55% following oral administration. A clinical study using oral 2-CdA as the primary therapy for patients with B-lineage CLL was encouraging (124) in that oral 2-CdA treatment induced durable remissions in 75% of the patients and oral administration of the drug appeared as effective as that given by the intravenous route. However, as the N-glycosidic linkage of 2-CdA is acid-labile and the nucleoside moiety is degraded rapidly by bacterial nucleoside phosphorylases, a substantial part of orally administered 2-CdA is degraded before absorption (124). Accordingly, in the latter study, oral doses were twice those by the intravenous route (124).

Currently, interest continues in strategies to reduce the degradation of the 2-halopurine nucleosides. 2-Chloro-2'-arabinofluoro-2'-deoxyadenosine (2-CAFdA) is an analog of 2-CdA that is more acid-stable than 2-CdA and is not cleaved by bacterial phosphorylases, and, therefore, 2-CAFdA may be more suitable for oral administration than 2-CdA (125,126)..

1.2.3 Phase I studies

Intravenous infusions of 2-CdA have been shown to cause cumulative lymphopenia and reversible, but profound thrombocytopenia and neutropenia (127). At low 2-CdA doses (0.1 mg/kg/day for 5-7 days in adult patients; 6.2 mg/m^2/day for 5 days in pediatric

patients[10]), minor non-hematological toxicity was observed, including nausea, diarrhea, skin toxicity, elevated serum hepatic transaminase levels and fever. The fever may have been attributable to cytokine release from dying tumour cells, because (a) fever onset coincided with the rapid disappearance of circulating hairy leukemia cells in patients treated for hairy cell leukemia (HCL), and (b) most febrile episodes were not associated with systemic infections (128). A decrease in the number of CD4- and CD8-expressing lymphocytes occurred in all patients, indicating that 2-CdA is also immunosuppressive. As a result, fatal systemic bacterial and fungal infections have occurred in adult and pediatric patients treated with this drug (129,130).

At high doses (5 to 10 times above therapeutic dosages), 2-CdA treatment may cause renal and CNS toxicities (129,130). In adult AML patients receiving high doses of 2-CdA (for example, 21 mg/m^2/day), degenerative neuropathic disorders were the dose-limiting toxicities (131). However, the role of 2-CdA in these toxicities is difficult to determine as these patients had previously received other therapies known to be either nephrotoxic or neurotoxic (130,132).

1.2.4 Phase II studies

Treatment with 2-CdA has shown remarkable efficacy in phase II trials of HCL therapy (133,134). In the United States, 2-CdA is licensed by the FDA for sale for the treatment of HCL. Patients with CLL have obtained encouraging therapeutic results with

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[10]To convert human dosages in mg/kg of body weight to mg/m^2 of surface area, multiply by 40 (9)

2-CdA (92,135,136). This agent has been particularly useful in the treatment of CLL patients refractory to 2-fluoro-9-β-D-arabinofuranosyladenine (fludarabine, FaraA) (135,136). In this context, the remarkable and profound delayed neurotoxicity of FaraA, another 2-halopurine nucleoside, may be noted (137). The use of 2-CdA to treat CLL patients resistant to FaraA is also associated with myelosuppression and immunosupression (138). Patients with low-grade lymphomas also respond to 2-CdA (94), and partial responses have been observed in AIDS-associated lymphomas and high-grade lymphomas (129). Encouraging results have been observed in cutaneous T-cell lymphoma patients resistant to conventional therapies (95) and in Waldenström's macroglobulinaemia (96). Saven *et al.* (95) observed a CR rate of 20% and a PR rate of 27% in 15 patients treated with 2-CdA for cutaneous T-cell lymphoma. In 29 patients treated with 2-CdA for Waldenström macroglobulinaemia, 59% achieved a response (50% reduction of IgM synthesis), of whom one achieved a CR (96).

Although 2-CdA cytotoxicity was originally believed to be directed toward lymphoid cells, 2-CdA has shown clinical utility in the treatment of chronic myelogenous leukemia (CML) (139) and AML (97,98). The activity of 2-CdA in the treatment of CML is noteworthy because CML is regarded as a difficult disease to treat (140). Recently, Santana *et al.* (97,98) evaluated a protocol for the use of 2-CdA in treating children with AML, and reported a CR and partial response (PR) rate of 47% and 12%, respectively in relapsed pediatric AML patients (97). In newly-diagnosed pediatric AML, 27% of patients treated with 2-CdA experienced a CR and in 32% partial remissions were obtained (98). In 4 of 6 AML patients tested, 2-CdA eliminated leukemic cells from the

cerebrospinal fluid. Santana and colleagues have proposed that 2-CdA should be evaluated in new regimens of combination chemotherapy in treatment of pediatric AML (98). The activity of 2-CdA in treatment of adult AML has been disappointing relative to responses in pediatric AML patients; Kornblau *et al.* (142) concluded that as a single agent, 2-CdA at the maximum tolerated dose (MTD) was a cytoreductive agent, but was not sufficiently active to achieve a complete response in adult patients with relapsed AML. However, Vahdat *et al.* (131) reported that at doses considerably higher than the pediatric MTD, 2-CdA was a promising agent for treatment of adult patients with AML. Those authors also suggested that, because of the immunosuppressive activity of 2-CdA, that agent may have utility in conditioning regimens for patients undergoing bone marrow transplantation.

Dimopoulos *et al.* (143) evaluated treatment with 2-CdA in 10 patients with multiple myeloma; 2-CdA treatment did not affect myeloma protein levels or bone marrow plasmacytosis. Poor responses to 2-CdA treatment were also seen in other multiple myeloma studies (129).

1.2.5 2-CdA activity in pediatric ALL

Few reports of the clinical use of 2-CdA in ALL patients have been published. Beutler (129) treated one adult ALL patient with 2-CdA and reported an excellent, but brief response. Phase I and Phase II clinical trials by Santana *et al.* (97,130) reported the antileukemic activity of 2-CdA in treatment of children with refractory ALL and with relapsed ALL. However, the numbers of patients (13 in the Phase I trial, and 7 in the

Phase II trial) in these trials were small, and some patients, being in their second or third relapse, were poor candidates for reinduction with any single agent. In one patient with CSF leukemic infiltration, the CSF was free of detectable leukemia after a single course of 2-CdA therapy and that patient achieved complete remission. The CNS is a primary site of relapse in this disease. In this group of patients, 2-CdA concentrations of 6.54 nM were achieved in the CSF with a well-tolerated, conventional regimen, suggesting a potential role for systemic 2-CdA in the treatment of meningeal leukemia. It is possible that the lack of activity of 2-CdA in pediatric ALL patients may be attributed to inadequate dose levels. Vahdat *et al.* (131) reported that children tolerate substantially higher doses of 2-CdA than those previously administered. To explore the potential of 2-CdA in the treatment of ALL, additional clinical studies with large numbers of patients without previous treatment are clearly needed (97).

The bases of the differences between AML and ALL sensitivity to 2-CdA are not understood (97). *Ex vivo* studies have shown that lymphoid cell lines were as sensitive to 2-CdA as myeloid cell lines (144), and that cultured lymphoblasts were sensitive to 2-CdA at the same concentrations as those found in the plasma of pediatric leukemia patients. Interestingly, phosphorylation of 2-CdA in the cultured cells did not correlate with their sensitivities to 2-CdA, a finding similar to the observation reported previously for Phase I and Phase II clinical trials of 2-CdA in pediatric ALL (97,130). An *in vitro* study using stroma-supported cultures of fresh ALL cells showed 2-CdA at a concentration of 100 nM to be cytotoxic to leukemic cells in most of the ALL samples tested (145). In fresh ALL cells, sensitivity to 2-CdA appeared to be independent of a high-risk karyotypic

abnormality or of a previous relapse history, factors often predictive of an unfavourable response to chemotherapy. The cytotoxicity expressed towards cells from relapsed pediatric ALL patients after multiagent therapy suggested the possible use of 2-CdA in patients with high-risk ALL (145).

1.2.6 Combination protocols with 2-CdA

In clinical studies that have evaluated 2-CdA treatment in leukemia therapy, most have used the drug as a single agent. Recently, the possibility of using 2-CdA in combination with other drugs has been investigated. Kristensen *et al.* (146) observed additive and synergistic interactions when 2-CdA was combined with either araC or daunorubicin, in *ex vivo* experiments with fresh cells from AML patients. 2-CdATP is believed to augment araCTP levels in the target cells by activation of deoxycytidine kinase (dCK) (147). Mechanistic studies have suggested that inhibition of ribonucleotide reductase by 2-CdATP may allosterically enhance the activity of dCK through reduction of cellular levels of dCTP (148).

Modulation of araC metabolism in AML myeloblasts has been attempted with 2-CdA. In clinical studies, daily infusions of araC have been administered 1 day after starting a 5-day continuous infusion of 2-CdA (142,147). The rationale for this approach was to allow at least 24 h for 2-CdA to augument dCK activity before administration of araC. The clinical application of 2-CdA modulation of araCTP levels in heavily pre-treated, relapsed adult AML patients was not encouraging because the experimental regimen (described above) did not appear to show any improvement over existing

modalities (142). In that study, 2 of the 17 patients treated with the experimental regimen achieved a CR, but relapsed with 17 weeks of treatment. However, this patient group may not have been appropriate for this study, because the patients were already resistant to a wide variety of agents and all had relapsed within one year of treatment, or had never achieved remission. It is possible, however, that 2-CdA modulation of araCTP levels may have utility in induction regimens in previously untreated patients. This strategy may also prove useful in treatment of pediatric patients with ALL because Suki *et al.* (149) have shown FaraA and araC to be an effective and relatively safe antileukemic combination in refractory or relapsed adult ALL patients. As well, 2-CdATP appears to be a more potent inhibitor of ribonucleotide reductase than FaraATP (147).

1.3 Cytosine arabinoside

An aim of the present study was to compare the cytotoxic effects of 2-CdA and araC as separate agents toward leukemic lymphoblasts from previously untreated pediatric ALL patients, using an *in vitro* tetrazolium dye (MTT) reduction assay to measure cell viability after intervals of exposure to these agents. AraC is important in this context because (a) it is a nucleoside analog (Fig. 1), and (b) because of its current use in remission induction and consolidation protocols in ALL therapy (1,2).

1.3.1 Pharmacology of araC

Following oral administration, araC is rapidly deaminated by cytidine deaminase to the presumed inactive metabolite 1-β-D-arabinofuranosyluracil (araU) (150). Therefore,

araC is generally administered as a continuous intravenous infusion (100 mg/m^2/day for 4-5 days; resulting in plasma levels of 0.06-0.8 μM), or by frequent *i.v.* administration of high doses (0.5-3.0 g/m^2 every 12 h for 6 days; resulting in plasma levels of approximately 100-200 μM) (156). A prerequisite for araC cytotoxicity is its transport into cells which takes place because araC is a substrate for NT systems (151). The cytotoxic activity of araC is further dependent on phosphorylations by dCK and nucleotide kinases to convert intracellular araC to the 5'-triphosphate ester, araCTP. White *et al.* (151) have shown that at concentrations below 1 μM, the rate of araC uptake was determined primarily by membrane transport. However, when concentrations exceeded 10 μM, phosphorylation capacity was shown to be the principal determinant of the uptake rate.

1.3.2 Mechanism of action of araC

Although the mechanism by which araC causes cell death remains unclear, its cytotoxicity has been shown to correlate with araCMP incorporation into DNA (152). AraCMP incorporation into DNA, results in eventual termination of DNA elongation (153). AraCTP also acts as a DNA polymerase inhibitor by competing with the binding of dCTP to that enzyme (154). AraC has its greatest cytotoxic effect during S-phase, presumably because it requires incorporation into DNA for cytotoxic activity, and S-phase is associated with greater activity of anabolic enzymes compared to other phases of the cell-cycle (155). Other biochemical actions of araC include inhibition of ribonucleotide reductase and formation of araCDP-choline, an analog of cytidine 5'-diphosphocholine that inhibits synthesis of membrane glycoproteins (155).

1.3.3 Clinical activity of araC

AraC is among the most effective drugs used in the treatment of pediatric and adult AML (2). Most remission induction regimens in AML therapy include combinations of araC with an anthracycline (daunorubicin or doxorubicin), with or without 6-thioguanine or etoposide (2,82). With these regimens, remission induction rates approach 80% (2). New approaches to increase the long-term survival rate in pediatric AML have included the use of HD-araC in consolidation and postinduction therapy (2,156). Approximately half of all pediatric patients diagnosed with AML fail to achieve an initial remission, or alternatively patients develop resistant disease and relaspe (2). Treatment of relapsed AML with high-dose araC (HD-araC) in combination with 6-mercaptopurine or mitoxantrone has produced remission reinduction rates of approximately 35-65% (2), however, these regimens are associated with severe dose-limiting toxicities (156).

As described in Section 1.1.4, araC is commonly used in remission induction and consolidation protocols in pediatric ALL therapy. AraC is also an important agent in the prophylactic treatment of CNS leukemia (156). The penetration of araC through the blood brain barrier is limited, but is significantly increased if high plasma levels are maintained during HD-araC administration (156). AraC may also be administered intrathecally, with the result that concentrations above the threshold for cytotoxicity (0.4 μM) are maintained for at least 24 h (155).

The dose-limiting toxicities of araC are myelosupression and gastrointestinal epithelial injury. HD-araC is also associated with pulmonary toxicity, including noncardiogenic pulmonary edema. IT administration of araC is associated with seizures

occurring within 24 h of administration (155). Acute toxicity of HD-araC includes nausea and vomiting in most patients and drug-induced fever in about 50% of patients (156).

1.4 Nucleoside transport

The physiological nucleosides and many analogs thereof do not diffuse across the plasma membranes of animal cells, but these molecules enter cells principally as substrates for a group of nucleoside-specific transport processes. The first step in expression of the cytotoxicity of 2-CdA and araC is the mediated entry into target cells via one or more of the nucleoside transport (NT) processes that are known (157,158). However, the role of membrane transport as a determinant of the efficacy of the nucleoside analogs as antileukemic drugs has not been extensively addressed. The present study has examined the relationship between *in vitro* sensitivity to 2-CdA and uptake of this agent by ALL cells.

The current classification of NT processes is based on pharmacological and functional characteristics of membrane fluxes (158). At present, seven distinct nucleoside transporter subclasses are recognized. Nucleoside transport processes are divided into two distinct classes: (a) the equilibrative or "facilitated diffusion" transporters that catalyze both the influx and efflux of nucleosides, and (b) the Na^+-dependent transporters that mediate the inward, Na^+-dependent co-transport of nucleosides. The latter processes may be concentrative. Nucleoside fluxes in the outward direction mediated by the Na^+-dependent cotransport systems have not been reported. Transporter-independent permeation of nucleosides has been shown to be a minor process in cultured mammalian

cells, by the resistance to cytotoxic nucleoside analogs in cells that are incapable of transporting nucleoside analogs as a consequence of mutations or of treatment with NT inhibitors (102,103,159,160,161). However, certain nucleoside analogs (for example, 3'-azido-3'-deoxythymidine and 2',3'-dideoxythymidine) have been shown to diffuse through the lipid bilayer at a significant rate (162,163). The ability of certain nucleoside analogs to enter cells by simple diffusion has been related to the absence of the 3'-OH group, and to an increased lipophilicity relative to nucleosides and the majority of their analogs (163). A number of antiviral agents, including 2',3'-dideoxyguanosine and acyclic nucleosides such as acyclovir and ganciclovir, have been shown to enter cells by means of nucleobase transport processes (164-166).

1.4.1 Measurement of nucleoside fluxes

The uptake of nucleosides by mammalian cells is rapid, and the participation of membrane transporters in the uptake process becomes evident through transport rate saturability, permeant competition, inhibition by specific agents, and stereoselectivity (157). Metabolism of the nucleoside permeants occurs rapidly following permeation across the plasma membrane (167); nucleosides are phosphorylated by a number of nucleoside kinases, a process that traps the permeating species intracellularly by removing them from transport equilibria. These metabolic transformations complicate time courses of nucleoside accumulation in cells and have been associated with the erroneous measurements of the initial rates of transport of nucleosides into cells (168,169). The initial rate of permeant uptake in cells is a measure of the inward flux of the permeating

species. Unidirectional, inward fluxes of nucleosides are measured as initial rates of cellular uptake from time courses (of a few seconds) after initiation of their accumulation. Rapid sampling techniques are required to obtain time courses that clearly define initial rates, allowing measurement of the unidirectional, inward flux of a permeant. Rapid sampling techniques include rapid centrifugal pelleting of cells under oil-layers to rapidly remove cells from permeant-containing medium, and the use of transport inhibitors to rapidly end intervals of permeant uptake, with use of short interval techniques or quenched-flow technology (170). As with most techniques, these methods are associated with particular limitations that require careful evaluation of their applicability to one cell type or another (170).

Kinetic analyses of nucleoside transport can be accomplished more simply in cells that lack phosphorylation capabilities than in cells that convert transported nucleosides to their mono-, di- and triphosphate derivatives. For example, human erythrocytes lack kinases and phosphorylases that accept uridine and thymidine as substrates and, accordingly, uridine and thymidine are not metabolised by human erythrocytes. However, human erythrocytes do express a NT system of the facilitated diffusion type that accepts thymidine and uridine as substrates and thus these nucleosides are "non-metabolized substrates" for the erythrocytic *es* nucleoside transporter (171). This system has been of fundamental importance in the exploration of the NT process. Other approaches that have been used to avoid metabolism are; (a) the depletion of ATP in cells, (b) the use of appropriate inhibitors (for example, deoxycoformycin) to block metabolic transformations, and (c) the use of formycin B (FB), a poorly-metabolized analog of inosine as a

transporter substrate (169,172).

1.4.2 Equilibrative nucleoside transport processes

The facilitated diffusion NT systems (also termed "equilibrative" nucleoside transporters) were the first NT processes identified and are divided into two types on the basis of their differential sensitivity to 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (NBMPR). The *e*quilibrative, NBMPR-*s*ensitive (***es***) transporters are sensitive to nanomolar concentrations of NBMPR and bind NBMPR reversibly with high affinity (K_D ≈0.1 to 1 nM), while *e*quilibrative, NBMPR-*i*nsensitive transporters (***ei***) are insensitive to nanomolar concentrations of NBMPR (157), since they lack high affinity NBMPR-binding sites (173). The equilibrative NT systems are widely distributed among mammalian cells, and the ***es*** transporter distribution is virtually ubiquitious (158). The NBMPR-binding site is believed to be located on the extracellular face of the plasma membrane and appears to coincide with the nucleoside permeation site (174,175). This site appears to be exposed to the aqueous microenvironment, since large, impermeant derivatives of N^6-(4-nitrobenzyl)adenosine, like the SAENTA fluoresceins, are potent inhibitors of the ***es*** transport system (158, 176). The closely related ***es*** transporter probe, NBMPR, has a high affinity for the ***es*** transporter sites, and occupancy of those sites by NBMPR has been correlated with inhibition of NT (177).

1.4.2.1 Inhibitors of equilibrative NT processes

NBMPR and related S^6-derivatives of 6-thiopurine nucleosides are potent

inhibitors of the *es* transporter (178). The 2',3'-hydroxyl groups appear to be critical to the binding of NBMPR to its high-affinity binding site (179), a result consistent with an earlier study that found the 2'- and 3'-hydroxyl groups of nucleosides to be important in nucleoside substrate recognition by this carrier (180). Both *es* and *ei* transporters are inhibited by the non-nucleoside NT inhibitors, dipyridamole (DPM) and dilazep, compounds initially recognized as vasodilatory and antiplatelet agents (157). In general, the *ei* transporter appears to be less sensitive to DPM and dilazep than the *es* transporter (181,182). However, this is not the case in cells and tissues from the rat (181), where DPM and dilazep are similarly weak inhibitors of both *es* and *ei*. Similarly, in Ehrlich ascites cells of mouse origin, DPM was unable to distinguish between the two systems, since DPM has a low affinity for the rat *es* transporter relative to that of mouse and human cells (183). Both DPM and dilazep bind at membrane sites that appear to overlap with the NBMPR-binding site (184,185). In addition, dilazep, along with other inhibitors, has been shown to bind to a second low affinity inhibitor binding site (185) that may be linked allosterically to the high affinity permeation site. Interestingly, the UASJ-2.9 clone of the Novikoff hepatoma cell line, has been shown to bind NBMPR with high affinity while being insensitive to NBMPR, suggesting that in this clone, the NBMPR-binding site may not be functionally coupled to the permeation site (186).

Among the many compounds that inhibit the equilibrative nucleoside transporters are lidoflazine and its congeners. As with other *es* inhibitors (for example, with DPM and dilazep), the potencies (IC_{50}) of lidoflazine and its analogs in inhibition of *es* processes in cells, depend on the species of origin of the cells under study. It is a further complication

that the *es* transporter is generally more sensitive than the *ei* transporter to this group of agents. Soluflazine is an exception, in that it is a more potent inhibitor of the *ei* carrier in rat erythrocytes than the *es* transporter (187). Interaction of benzodiazepines with the *es* transporter has also been investigated by Hammond *et al.* (188), who have demonstrated that the affinities of benzodiazepines for the *es* NT system in fresh human erythrocytes were about 1000-fold lower than those of NBMPR or DPM. It appears that benzodiazepines inhibit both nucleoside transport function and NBMPR binding to the *es* protein by competing with NBMPR for the permeant site (186).

Thiol groups may be essential for activity of both equilibrative NT proteins. For example, the NBMPR-sensitive uptake of uridine was inhibited by the thiol reagent p-chloromercuribenzene sulfonate (pCMBS) in inside-out vesicles prepared from human erythrocytes (189), and the NBMPR-insensitive uptake of uridine in Walker 256 rat carcinosarcoma cells was inhibited by pCMBS (190). These studies indicated that the thiol groups involved in the *es* and *ei* transport process differ in their orientation within the membrane. The thiol groups of the *es* protein appear to be located within the plasma membrane or in its cytoplasmic face, whereas the thiol groups of the *ei* protein appear to be accessible from the extracellular surface of the plasma membrane. However, a later study (191) identified a Na^+-dependent transport process in Walker 256 rat carcinosarcoma cells. Therefore, conclusions made concerning the location of the thiol groups in the *ei* NT protein in these cells may be erroneous.

1.4.2.2 Substrate specificity of equilibrative transporters

Both equilibrative systems transport a broad array of purine and pyrimidine nucleosides with low to moderate affinity (K_m for influx about 2 μM to 2-4 mM at 22-25°C). The affinity of substrates for both the ***es*** and ***ei*** carriers appears to be both species-dependent and cell type-dependent (181). Of the naturally occurring nucleosides, adenosine appears to have the greatest affinity for the equilibrative transporters, with K_m values for influx of 2-50 μM at 22-25°C (181). The ***ei*** transporter generally has a lower affinity for substrates than the ***es*** transporter (181). Transport of a nucleoside or its analog via the ***es*** transporter appears to be dependent on the presence of a hydroxyl group at the 3'-position (180); as well, the 5'-hydroxyl group appears to be a minor determinant in recognition of ***es*** transporter substrates (163). In several cell types, the enantiomeric configuration at C-1 has been shown to be a determinant of permeant tranportability. For example, the L-enantiomers of uridine, thymidine and adenosine are poor permeants for the ***es*** NT system in S49 mouse lymphoma cells, whereas, the D-enantiomers are accepted as transporter substrates (157). In mouse erythrocytes and L1210 cells, L-adenosine fluxes were only partially inhibited by NBMPR (192).

1.4.2.3 SAENTA-fluorescein: A flow cytometric probe for the es transporter

The site-specific binding of [^{3}H]NBMPR at equilibrium is often used to enumerate NBMPR-sensitive sites on mammalian cells and is considered to be the standard method for enumeration of ***es*** sites in suspended cells (173,193,194). However, this procedure is time-consuming and requires a large number of cells (about 10^8 cells). A fluorescent

ligand for the *es* transporter was recently synthesized by reacting a fluorescein derivative with 5'-*S*-(2-aminoethyl)-N^6-(4-nitrobenzyl)-5'-thioadenosine (SAENTA) to produce 5-(SAENTA-x8)-fluorescein (5-(Sx8)-F) (176). SAENTA is structurally related to NBMPR and has proven to be a useful probe for the *es* nucleoside transporter. In 5-(SAENTA-x8)-fluorescein, the 5'-carbon of the SAENTA moiety is linked to the C-5 position of fluorescein through the linkage -CONH$(CH_2)_5$CO-, producing a fluorescent ligand that is tightly bound to plasma membrane sites associated with the *es* transporter (176). Several SAENTA-fluorescein conjugates have been developed, differing in the linkage structure and point of attachment of SAENTA to the fluorescein moiety (Fig. 2). Buolamwini *et al.* (176) found 5-(Sx8)-F, which had a 12-atom linkage[11], to bind most tightly in a set of seven SAENTA-fluorescein conjugates to the *es* transport protein in mouse leukemia L1210/B23.1 cells. This high-affinity ligand for the *es* transporter is a suitable reagent for flow cytometric quantitation of *es* expression (195,196). Flow cytometric enumeration of *es* sites requires 10-fold fewer cells than the conventional [^{3}H]NBMPR binding assay, and measurements of *es* site content by this method can be conducted more easily than by determining B_{max} for the equilibrium binding of [^{3}H]NBMPR.

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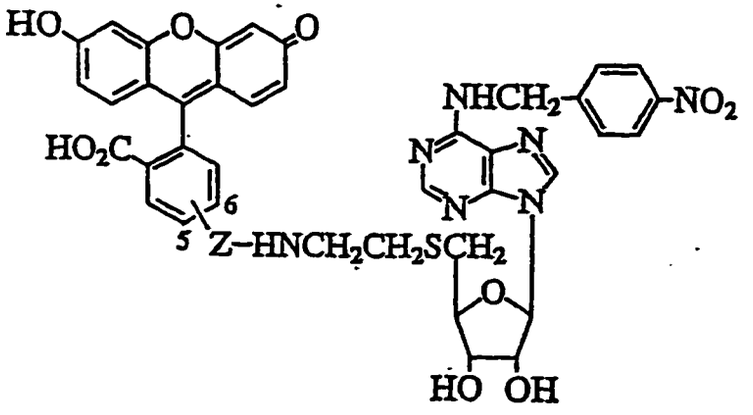
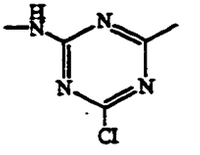
[11]Linkage between the 5'-carbon of SAENTA and the fluorescein 5-(or 6-) ring atoms (see Fig. 2).



Compound	Linkage
1,2	-NHCS-
3,4	-COH(CH$_2$)$_5$CO-
5,6	-CO
7	

Compounds 1-7

Nomenclature:

Compound	1	5-Sx2-F	2	6-Sx2-F
Compound	3	5-Sx8-F	4	6-Sx8-F
Compound	5	5-Sx1-F	6	6-Sx1-F
Compound	7	5-Sx4-F		

* Group Z was linked to the fluorescein C-5 position except in Compounds 2, 4, and 6, in which Z was linked to C-6.

From Buolamwini *et al.* (176).

Figure 2. Structures of SAENTA-fluorescein conjugates

1.4.2.4 Molecular properties of the facilitated diffusion transport systems

In the initial purification of the *es* nucleoside transporter, human erythrocyte ghosts were employed as the source material because the *es* protein is the only NT process expressed in these cells (157,197). Isolation of the *es* protein was a challenge because the *es* transporter may represent approximately 0.1% of the membrane protein in human erythrocytes, and, as well, the glucose transporter co-migrates with the *es* glycoprotein on SDS polyacrylamide gel electrophoretograms (157). Both the glucose and the nucleoside transporters were identified in band 4.5 preparations of human erythrocyte membranes.

To identify the *es* protein, photoaffinity ligands for the *es* site, [^{3}H]N^6-(*p*-azidobenzyl)adenosine and [^{3}H]NBMPR, were used to covalently photolabel[12] plasma membrane proteins in the band 4.5 polypeptides (198). Radiolabelling was inhibited by nucleosides and NT inhibitors, and did not occur in membranes from cells lacking the *es* transporter, suggesting that the radiolabelled protein was, indeed, the *es* transporter (199). The erythrocyte *es* transporter was identified as an integral membrane glycoprotein of apparent M_r 45,000 to 66,000 (200). Photoaffinity studies revealed considerable variation in the size of the *es* transporter from different cell types (M_r 45,000-87,000). Polyclonal antibodies raised against the human erythrocyte *es* transport protein cross-reacted with the *es* transporter purified from rabbit and pig erythrocytes and from rat liver, indicating that these mammalian *es* transporters share common epitopes (181). Radiation inactivation studies have indicated that the human erythrocyte *es* carrier may exist *in situ*

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[12]Photoaffinity labelling procedures resulted in covalent linkages of the labelled ligands to the transport protein upon exposure to UV light.

as a dimer (201).

Griffiths *et al.* (202) have recently reported that the first human equilibrative nucleoside transporter (hENT1) was cloned from a human placental cDNA library (202). hENT1 shows no sequence similarity to the other cloned NT proteins including CNT1 (concentrative nucleoside transporter 1) and SPNT (sodium-dependent purine nucleoside transporter). In order to determine the functional properties of hENT1, recombinant hENTI was expressed in *Xenopus oocytes*, which lack endogenous NT activity. Uridine uptake by the transfected oocytes was inhibited in the presence of 2 mM naturally occuring nucleosides, a result consistent with the broad substrate specificity associated with ***es*** transporters. Uptake of uridine was not dependent on sodium ions and was almost completely inhibited by 1 μM MBMPR, providing evidence of ***es*** transport activity. hENT1 was also shown to transport a number of anticancer nucleoside analogs, including 2-CdA and araC (202).

The present lack of a potent inhibitor for the ***ei*** transporter has probably hindered development in our knowledge of the molecular and functional properties of the ***ei*** transporter. Hammond and colleagues have functionally reconstituted the ***ei*** protein into liposomal membranes (203). Recently, this elusive transporter was cloned by Crawford *et al.*(204). A 2.4 kb cDNA isolated from a HeLa cell library, conferred uridine transport activity when transfected into a transport-deficient clone of the CCRF-CEM human T-lymphoblast line. This human cDNA imparted ***ei***-like NT characteristics to transfected cells in which uridine uptake was shown to be insensitive to 1 μM NBMPR and sodium-independent, propertities consistent with published reports of ***ei*** activity (205). Uridine

uptake by the transfected cells was also inhibited by 10 μM DPM. The cloning of the *ei* transporter will allow investigators to gain a greater understanding of the role this transport protein plays in chemotherapeutic responses to nucleoside analogs.

1.4.3 Concentrative, Na^+-linked NT processes

In addition to the equilibrative nucleoside transporters, concentrative NT systems have been identified in mammalian plasma membranes (157,158). These systems mediate the movement of nucleosides across the plasma membrane by sodium/nucleoside cotransport mechanisms, that is, by inwardly directed nucleoside-specific transport processes coupled to the inward movement of sodium down a concentration gradient (206). The requirement for sodium is specific, with only a single study reporting the ability of Li^+ to replace Na^+ ions (207). Lee *et al.* (208) demonstrated that uridine uptake in rat renal brush border membrane vesicles was mediated by a K^+-linked process and additionally by a Na^+-dependent transport system. The Na^+: uridine and K^+: uridine coupling stoichiometries were found to be 1:1 and 3:2, respectively, supporting the idea that Na^+- and K^+-dependent uridine uptake processes represent separate transport systems. The physiological significance of the K^+-dependent transport is not clear, but it may be involved in the secretion of nucleosides in the renal tubule, as the authors have speculated (208).

Substrate specificity and functional characteristics have enabled five distinct concentrative NT systems to be distinguished. Two systems of nomenclature are in use, the first of which involves numerical designations (N1-N5) that signify the order of

discovery (Table 2) (158). The second nomenclature scheme, that of Belt and colleagues (209), distinguished between several major NT processes on the basis of their functional properties, including substrate selectivity; (a) ***cif*** refers to a ***c***oncentrative, Na^+-linked process that is ***i***nsensitive to NBMPR and accepts ***f***ormycin B (an inosine analog) as a substrate, but not thymidine, (b) ***cit*** refers to a ***c***oncentrative, Na^+-linked process that is ***i***nsensitive to NBMPR and accepts ***t***hymidine as a substrate, but not formycin B, (c) ***cib*** refers to a ***c***oncentrative, Na^+-linked process that is ***i***nsensitive to NBMPR and has a ***b***road permeant selectivity, and (d) ***cs*** refers to a ***c***oncentrative, Na^+-linked process that is ***s***ensitive to low concentrations of NBMPR. The permeant selectivity for the ***cs*** transporter remains to be determined, as this NT process has only been identified in fresh leukemic cells from patients (210). At present, adenosine, 2-CdA, FaraA and FB are known to be substrates for the ***cs*** transporter (210).

The sodium:nucleoside stoichiometry (Table 2) for the ***cif*** (N1) and ***cit*** (N2 and N4) systems studied so far, have been estimated as 1:1, and are consistent with a co-transporter in which binding of Na^+ precedes that of the permeant (211-213). Sodium ions behaves as a competitive activator in the ***cif*** and ***cit*** systems, because an increase in extracellular sodium levels increases the affinity of the nucleoside for the transporter without affecting the maximum velocity of influx (211,213). In contrast, a stoichiometry of 2:1 (Table 2) was observed between Na^+ and guanosine, uridine and thymidine for the ***cib*** (N3) process in slices of rabbit choroid plexus (204). The sodium-dependent transport systems in general, have a higher affinity for nucleoside permeants than the equilibrative systems, with affinity constants ranging from 1 to 40 μM at 22°C (181).

Table 2

Nucleoside transporter subclasses

	Equilibrative		Concentrative				
	es	***ei***	***cif***	***cit***		***cib***	***cs***
Propertities			N1	N2	N4	N3	N5
Inhibited by							
NBMPR	+	-	-	-	-	-	+
DPM	+	+	-	-	-	-	+
Na^+-dependent	-	-	+	+	+	+	+
Stoichiometry:							
Na^+/n'side ratio	-	-	1:1	1:1	1:1	2:1	nd
Permeants							
Adenosine	+	+	+	+	+	+	+
Uridine	+	+	+	+	+	+	nd
Guanosine	+	+	+	-	+	+	nd
Inosine	+	+	+	-	-	+	nd
FB	+	+	+	-	-	+	+
Thymidine	+	+	-	+	+	+	nd

nd, not determined.

From Cass, C.E. (158).

Dagnino *et al.* (211) showed that substitution at the C-2 position of a purine nucleoside may be a determinant of the interaction of this agent with the ***cif*** transporter. Those authors also showed that the ***cif*** transporter distinguished between the D- and L-enantiomers of adenosine, the latter being a virtual non-permeant. Dagnino and colleagues (211) also found that thiol groups of the transporter may be important in the ***cif*** sodium-linked permeation process, either in the recognition of substrates by the permeation site and/or in the permeation translocation step. Interestingly, the ***cit*** transporter appears to be resistant to the sulfydryl-reactive agent, N-ethylmaleimide (214).

The concentrative nucleoside transporters have been found predominantly, but not exclusively, in normal epithelial tissues (209). The ***cif*** (N1) transporter is widely distributed, being found in a large number of normal cells (158,215), as well as in various lines of cultured neoplastic cells, including L1210 murine leukemia (211,216,217) and Walker 256 rat cariocarcinoma (191). The ***cit*** (N2) transporter has been identified in intestine and kidney epithelia (158). A ***cit***-like transporter (N4) which accepts guanosine is found in human renal brush border membrane vesicles (212). The ***cib*** (N3) transporter is found in rabbit choroid plexus and rat jejunum, as well as in HL-60 human cultured promyelocytic leukemia cells, in CaCo human cultured colorectal carcinoma cells (158), in human myeloid leukemic cells, and possibly in freshly isolated myeloblasts (218). The ***cs*** (N5) NT process has been identified in freshly isolated leukemic cells from CLL, AML and ALL patients (210).

In cells that express both the ***es*** and concentrative NT systems, accumulation of cellular nucleoside permeants above equilibrium levels has been observed in the presence

of either DPM or NBMPR (216,217,219). The accumulation of free nucleosides in the presence of these inhibitors is apparently due to (a) inhibition of bidirectional nucleoside movement via the ***es*** NT system, and (b) progressive nucleoside uptake via inhibitor-insensitive, sodium-dependent systems (216). The level of outward ***ei*** transport activity that would compromise concentrative uptake of nucleosides in the presence of NBMPR or DPM in this situation is not yet known. Dagnino *et al.* (217) showed an increase in the sensitivity of L1210/C2 cells to 9-β-D-arabinofuranosyladenine (araA) in the presence of DPM. This effect was due in part to an increase in araATP accumulation and retention, caused by blockade of araA efflux via the ***es*** transport system.

1.4.3.1 Molecular properties of Na^+-dependent NT systems

Hitherto, the lack of specific inhibitors or immunological probes for the concentrative NT systems may have slowed the purification and characterisation of sodium-dependent transport proteins. To date, cDNAs encoding three different sodium-dependent proteins have been cloned and expressed in oocytes of *Xenopus laevis* (220-222). cDNA encoding SNST1 (Na^+-nucleoside cotransporter) was isolated from a rabbit renal cDNA library by hybridization with the rabbit renal SGLT1 (Na^+-glucose transporter) cDNA (220). Expression of recombinant SNST1 in *Xenopus* oocytes resulted in a 2-fold increase in sodium-dependent nucleoside transport by a process apparently similar to ***cib***-type activity (220). Hydropathy plots predict a protein with 12 transmembrane domains.

Expression cloning was used to isolate a cDNA from rat jejunal epithelium

encoding a pyrimidine-selective, sodium-dependent nucleoside transporter, cNT1 (concentrative nucleoside transporter 1), or rCNT1 (rat concentrative nucleoside transporter 1) (221). Messenger RNA transcripts isolated from rat intestinal mucosa were fractionated by chromatography and microinjected into *Xenopus* oocytes, and rCNT1 was identified by screening those fractions for sodium-dependent uridine uptake. Expression of rCNT1 in *Xenopus* oocytes resulted in a 10,000 fold increase in sodium-dependent uridine uptake by these cells, in a process with characteristics of the ***cit*** transporter (221). The encoded amino acid sequence analysis showed little similarity to SNST1, suggesting they are members of different gene families. Hydropathy plots predict a protein with 14 transmembrane domains. A cDNA for a human homolog of rCNT1 has been isolated from human kidney and designated hCNT1 (223). When cDNA for hCNT1 was expressed in oocytes, it exhibited characteristics of a Na^+-dependent transporter with selectivity for pyrimidine nucleosides. hCNT1 was 83% identical to rCNT1 in the encoded amino acid sequence indicating they are most likely members of the same gene family.

A third cDNA encoding a sodium-dependent purine nucleoside transporter (SPNT) was isolated from rat liver (222). SPNT is 64% identical to rCNT1 in amino acid sequence, suggesting that these two sodium-dependent nucleoside transporters are members of a single gene superfamily that also includes bacterial nucleoside transporters. SPNT and rCNT1 differ in tissue distribution as well as in substrate specificity. Northern blot analysis detected SPNT expression in the liver, jejunum, spleen and heart, whereas rCNT1 was detected only in intestine and kidney. The most divergent regions between the two transporters are the N- and C-terminal domains, which may be important in tissue-

specific regulation (222). There is no significant homology between SPNT and SNST1 (222). Hydropathy plots predict a protein with 14 transmembrane domains.

Huang *et al.* (224) have functionally expressed mRNA from rat jejunum in *Xenopus* oocytes and identified two sodium-dependent NT components, one component consistent with ***cif*** activity and a second, consistent with ***cib*** activity. A mouse homolog of rSPNT (mCNT2) was isolated from a mouse spleen cDNA library (225). When mCNT2 was expressed in COS cells, transport of uridine was consistent with ***cif*** activity. mCNT2 was 93% and 61% identical in amino acid sequence to rSPNT and rCNT1, respectively. It is expected that in the near future, many more transport proteins will be cloned by hybridization screening of cDNA libraries with rCNT1, hCNT1, rSPNT and mCNT2 probes.

1.4.4 Regulation of nucleoside transporter expression

Until recently, regulation of NT activity was not recognised. It is now known that changes in nucleoside transporter expression correlate with the proliferation rates of cell populations (158). Changes in NT activity may include (a) an increase in a particular transporter activity, for example, in ***es*** (226), or (b) a change in the proportions of the NT processes expressed (227). However, controversy exists as to whether or not ***es*** NT expression is correlated with proliferative rates in myeloblasts from AML patients (228). In contradiction of studies in myeloblasts from peripheral blood by Wiley *et al.* (226), Powell *et al.* (228) were unable to identify a correlation between ***es*** expression and the proliferative rate in myeloblasts from AML bone marrow samples. It is possible that the

discrepancy in these results reflects a difference in NT activity between peripheral blood and bone marrow myeloblasts (229). A later study by Wiley and colleagues (196) observed an increase in *es* expression on myeloblasts from relapsed or refractory AML patients after *in vivo* administration of GM-CSF; it was postulated that this increase in *es* expression was related to enhanced proliferation of marrow myeloblasts *in vivo*. A study by Petersen *et al.* (117) noted that *in vitro* exposure of fresh CLL cells to 2-CdA and FaraA resulted in an increase in both the level of *es* transporter expression and the percentage of cells in S-phase.

It was recently shown that neoplastic transformation may also modulate the NT activity of rat fibroblasts (230). In addition, when HL-60 cells were induced to differentiate along either the monocytic or the granulocytic pathways, a marked decrease in *es* transport activity coupled with an increase in sodium-dependent NT (*cib*) activity was observed (231,232). When HL-60 cells were induced by phorbol 12-myristate 13-acetate to differentiate along the monocytic pathway, protein kinase C activity was induced, as were apparent conformational changes in the substrate-binding/transport site of the *es* transporter; those changes may have been responsible for the reduction in transport activity by the *es* carrier that was a feature of the differentiation (231).

There is some evidence that thymidine kinase (TK) and *es* NT expression may have a common regulatory mechanism that is sensitive to endogenous dTTP pools (233). Thymidylate synthase (TS) inhibitors block the formation of dTMP and consequently impair dTTP levels so that proliferating cells rely on salvage of preformed dThd from the extracellular mileau to replenish dTMP levels. Hedley and colleagues (233) observed an

increase in both TK activity and *es* NT expression in MGH-U1 cells (a human bladder cancer cell line) when culture proceeded in the presence of the TS inhibitors, D1694 and AG-331. These authors speculate that TK activity and nucleoside transporter expression may have a common regulatory mechanism that is sensitive to dTTP pools, and that nucleoside salvage pathways are a complex system of kinases coordinated with the transport of nucleosides (233).

Therefore, an understanding of the regulation of nucleoside transporter expression by agents such as the TS inhibitors may have important applications in chemotherapy, since a number of cytotoxic nucleosides have anticancer applications. The transport of nucleosides and their analogs across the plasma membrane is complex, and the characterization of NT processes present in normal and neoplastic cell types may help to refine anticancer therapy, by exploiting differences between these cell types. Besides conventional permeant flux studies, development of cDNA probes for various nucleoside transporters and the development of immune reagents directed against epitopes in the transporters should aid in the identification of NT processes present in tumour samples.

A number of studies have examined the role of membrane transport processes in the cellular accumulation of araC. It has been reported that the number of *es* sites on human leukemia cells correlates with both araC influx and levels of araCTP formed (234,235). Recent work by Gati *et al.* (236) has indicated a correlation between *es* site abundance and chemosensitivity to araC in leukemic cells. This demonstration suggests that NT levels may contribute to differences in sensitivity to araC and other nucleoside analogs, including 2-CdA. Determination of transporter abundance in leukemic cells from

patients may allow prediction of resistance to nucleoside analogs.

1.4.5 Modulation of antimetabolite agents with NT inhibitors

Knowledge of the NT processes expressed in leukemic cells from individual patients may allow the use of NT inhibitors to modulate the activity of antimetabolites including nucleoside analogs. For example, it is conceivable that cells expressing only the *es* transporter might be sensitive to a combination of NBMPR and a cytotoxic inhibitor of either *de novo* pyrimidine nucleotide or purine nucleotide biosynthesis; it is reasoned that the NT inhibitor would impair "salvage" of endogenous nucleosides from the circulation. This approach would be unlikely to succeed against leukemic cells expressing NBMPR-insensitive transporters. The level of NBMPR-insensitive transport activity that would compromise such a therapeutic approach has not yet been defined (209).

Alternatively, NT inhibitors may be useful in protecting dose-limiting tissues from the toxic effects of nucleoside analogs. For example, NBMPR-P (NBMPR 5'-phosphate, a prodrug form of NBMPR) has been used in mice to protect against lethal doses of tubercidin, a toxic adenosine analog (237). The applicability of this tactic requires dose-limiting tissues to express NBMPR-sensitive NT processes and target cells to express NBMPR-insensitive systems (238). A reversal of this situation may lead to increase cytotoxicity in dose-limiting tissues.

1.5 Research Objectives

Current therapy for childhood ALL fails in approximately 25% of patients; those failures are due primarily to relapses of the disease, that is, recurrence of the disease in patients in remission. Recurrence mostly represents the emergence of drug-resistant clones in the treated leukemia cell population. Identification of agents capable of bypassing mechanisms of multidrug resistance may improve the outcome of patients with a high risk of relapse. 2-CdA is an agent that has shown important activity in the treatment of indolent lymphoid malignancies, but as yet has not been extensively tested in ALL patients.

A goal of this study has been evaluation of the nucleoside analog, 2-CdA, as an agent with cytotoxicity toward malignant lymphoblasts from pediatric ALL patients. The experimental approach was to evaluate leukemic cells from previously untreated pediatric ALL patients for sensitivity to 2-CdA (using the *in vitro* MTT dye reduction assay to measure cytotoxicity), and to compare 2-CdA with araC, another nucleoside drug used in induction and consolidation regimens in treatment of childhood ALL. Cytotoxicity of both nucleoside analogs is a consequence of analog conversion to the 5'-triphosphate metabolites (2-CdATP and araCTP), following entry via nucleoside transport processes into leukemic cells. This study examined the relationship between sensitivity to 2-CdA and araC, and the cellular content of the *es* nucleoside transporter, that is, to the expression of the *es* transporter protein in the cell surface. A flow cytometric assay, employing an *es*-specific ligand, 5-(Sx8)-F, was used to determine the cellular abundance of the *es* transporter protein. It is hypothesized that the cytotoxicity of both nucleoside

analogs is correlated with the cellular abundance of the *es* transporter.

Bone marrow samples from pediatric ALL patients are of a "once-only" nature and the samples are typically small. For this reason, cultured cells of human ALL origin were used as model systems for (a) evaluation of nucleoside analog cytotoxicity and (b) for kinetic and mechanistic studies of NT processes in ALL cells. Another aim of this study was to determine if cultured ALL lymphoblasts of human origin were adequate models for lymphoblasts from pediatric ALL patients.

Rustum and Preisler (239) showed a significant correlation between *in vitro* retention of araCTP by acute myelocytic leukemia cells and the duration of remission of patients receiving araC. A later study by Kufe *et al.* (240) demonstrated a relationship between the cellular levels of araCTP, incorporation of araCTP into DNA and the cytotoxic potential of this agent. Similarly, it is hypothesized that inhibition of mediated 2-CdA efflux by NT inhibitors will tend to sustain cellular pools of 2-CdA and its metabolites, and should thereby enhance cytotoxicity of this agent. The present study aimed at (a) determining the effect of supplementary NBMPR and DPM on the cellular retention of 2-CdA and its metabolites in ALL cell lines, and (b) determining if the cytotoxicity of 2-CdA in cultured human lymphoblasts is significantly enhanced by subsequent exposure to NBMPR.

MATERIALS and METHODS

2.1 Chemicals

[^{3}H]2-CdA, [^{3}H]NBMPR, and [^{14}C]sucrose were purchased from Moravek Biochemicals (Brea, CA, USA). For metabolism experiments, [^{3}H]2-CdA samples (in 40% methanol) were dried at 22°C under a stream of nitrogen and then redissolved in the experimental medium. [^{3}H]Polyethylene glycol and [^{3}H]H_2O were purchased from Du Pont Canada Inc. (Lachine, QC, Canada) and ICN Radiochemicals (Irvine, CA, USA), respectively. 2-CdA was prepared (241) by Dr. J.S. Wilson (Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, Canada). FaraA, araC, 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DPM were obtained from Sigma Chemical Co. (St Louis, MO, USA). NBMPR and 5-(Sx8)-F were obtained from Alberta Nucleoside Therapeutics, Department of Pharmacology, University of Alberta, Edmonton, AB Canada. Ficoll-Paque was obtained from Pharmacia Biotech (Baie d'Urfé, QC, Canada). The mouse monoclonal antibodies specific for the surface antigens, CD5/CD7, CD3, CD19, CD13, CD38, HLA-DR, CD33, CD34, CD10 were from Becton Dickinson Immunocytometry Systems (BDIS), and were generous gifts from Dr. J.J. Akabutu (Department of Pediatrics, University of Alberta, Edmonton, AB, Canada). "Transport oil" was composed of a mixture of Dow Corning 550 silicone oil (Dow Corning, Mississauga, ON, Canada) and light paraffin oil (Fisher Scientific, Fair lawn, NJ, USA) adjusted to a density of 1.03 g/ml at 37°C (205). Cell culture materials were purchased from Gibco BRL (Burlington, ON, Canada).

Various other chemicals used in this project were obtained from the Sigma Chemical Co. (St Louis, MO, USA).

2.1.1 Purification of radiochemicals

Tritium-labelled 2-CdA and NBMPR were purified[13] after storage by high-performance liquid chromatography on a preparative C_{18} reversed-phase column (Whatman Partisil 10 ODS (9 × 250 mm); Whatman, Clifton, NJ, USA). Nucleosides were eluted at ambient temperature with a linear gradient of methanol-water solutions at a flow rate of 2 ml/min. 2-CdA eluate absorbance was monitored at 264 nm and NBMPR eluate absorbance was monitored at 290 nm.

2.1.2 Media and other solutions

Phosphate-buffered saline (PBS, pH 7.4): 137 mmol/L NaCl, 8.1 mmol/L $Na_2HPO_4 \cdot 7H_2O$, 2.7 mmol/L KCl , and 1.1 mmol/L KH_2PO_4 (153).

Dulbecco's PBS (DPBS, pH 7.4): 137 mmol/L NaCl, 8.1 mmol/L $Na_2HPO_4 \cdot 7H_2O$, 2.7 mmol/L KCl , 1.1 mmol/L KH_2PO_4, 1 mmol/L $CaCl_2$ (anhydrous), 0.5 mmol/L $MgCl_2 \cdot 6H_2O$, and 5 mmol/L glucose (153).

Li^+ medium (modified from DPBS, pH 7.4): 137 mmol/L LiCl, 1.1 mmol/L KH_2PO_4, 1 mmol/L $CaCl_2$ (anhydrous), 0.5 mmol/L $MgCl_2.6H_2O$, 8 mmol/L K_2HPO_4 and 5 mmol/L glucose.

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[13]Radiochemicals were purified by HPLC procedures developed in this laboratory.

Choline medium (pH 7.4): 137 mmol/L choline chloride, 2.7 mmol/L KCl, 1.1 mmol/L KH_2PO_4, 1 mmol/L $CaCl_2$ (anhydrous), 0.5 mmol/L $MgCl_2 \cdot 6H_2O$, 8 mmol/L K_2HPO_4 and 5 mmol/L glucose.

N-methylglucammonium medium (DNMG , pH 7.4):137 mmol/L N-methylglucammonium, 2.7 mmol/L KCl, 1.1 mmol/L KH_2PO_4,, 1 mmol/L $CaCl_2$ (anhydrous), 0.5 mmol/L $MgCl_2 \cdot 6H_2O$, 8 mmol/L K_2HPO_4 and 5 mmol/L glucose. The osmolality of all media was 300 ± 20 mOmol. Media were sterilized by filtration (0.22 μm Millipore filter) and stored at 4°C for up to two months.

NBMPR Stock solutions of NBMPR were saturated aqueous solutions prepared at 22°C and filtered with a 0.22 μm Millipore filter. The concentration of stock solutions was determined (ϵ^{290}, 25.1×10^3) on a Gilford 240 spectrophotometer (Oberlin, OH, USA). The NBMPR stock solutions were diluted to appropriate concentrations in DPBS for the nucleoside flux assays, and in PBS for the flow cytometry assays.

DPM Stock solutions of DPM were prepared in methanol solution and were protected from light and stored at 4°C. For use, the stock solution was diluted 100-fold in distilled water and the concentration was determined (ϵ^{293}, 30.9×10^3) on a Gilford 240 spectrophotometer. Immediately before use, the DPM stock solution was diluted to the appropriate concentration in DPBS.

2.2 Cell culture procedures

2.2.1 Ficoll-Paque separation of leukemic cells from bone marrow samples from ALL patients

Cells were obtained from bone marrow (BM) aspirates from 19 pediatric ALL patients and from one peripheral blood sample. All patients were newly diagnosed and immunophenotyping[14] was performed at both the Department of Laboratory Medicine, University of Alberta Hospital, Edmonton, AB, Canada and at the Flow Cytometry Laboratory, The John Hopkins School of Medicine, Baltimore, MD, USA. Leukemic cells were isolated using a conventional, density-gradient procedure in which the heparinized BM aspirate was diluted 1:1 with RPMI 1640 medium containing 2 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffer and 10% FBS (RH10F medium). The cell suspension was layered on a Ficoll-Paque "cushion" and centrifuged (30 min, 400 x g). No more than 20 ml of the cell suspension was layered on 12 ml of Ficoll-Paque in a 50 ml sterile tube. Cells from the Ficoll-Paque interface were washed twice (10 min, 150 x g) with RH10F medium and resuspended (a) in RH10F medium at a concentration of 1×10^7 cells/ml for the colorimetric MTT cytotoxicity assay, (b) in PBS at a concentration of 6×10^6 cells/ml for the flow cytometric 5-(Sx8)-F binding assay, or (c) in DPBS and DNMG media at a concentration of 2×10^7 cells/ml for the

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[14]Immunophenotyping of patient samples by flow cytometry was performed at these institutes using antibodies for CD3, CD5, CD7, CD10, CD 13, CD19, CD21, CD22 and HLA-DR labelled with FITC and PE.

nucleoside flux assay.

In this study, the use of leukemia cells from patients was limited by a low patient accrual rate in the Edmonton area, and by small numbers of cells in the "once-only" patient samples that were obtained. For these reasons ALL cell lines were used as model systems to undertake kinetic and mechanistic studies of NT processes in cultured leukemic lymphoblasts.

2.2.2 Cell lines

This study used four lines of cells that were of human pediatric ALL origin (242-245). These lines were maintained in culture in RPMI 1640 medium with 10% fetal bovine serum (RPMI-10% FBS). Cell concentrations in the cell cultures were maintained between 0.5 x 10^5 cells/ml and 5 x 10^5 cells/ml to ensure exponential growth. A Pre-B ALL clonal line, Reh-A2, and a T-ALL clonal line, KM3-2B, were established as part of this project by limiting dilution method (246) from Reh and KM-3 "wild-type" (242,243) cell lines obtained through the generosity of Dr. Diane Cole (National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA). CCRF-SB (hereafter termed SB), a B-ALL cell line (244), was purchased from the American Type Culture Collection (Rockville, MD, USA) and CCRF-CEM (hereafter termed CEM), a T-ALL cell line (245), was originally provided by Dr. J.A. Belt (St Jude Children's Research Hospital, Memphis, TN, USA) and maintained in this laboratory for several years before the present project. Stocks of these lines were frozen in RPMI-20% FBS growth media supplemented

with 10% dimethylsulfoxide were stored under liquid nitrogen. Cultures from the frozen stocks were passaged for no more than 30 culture generations.

For nucleoside flux experiments, cell stocks were harvested centrifugally (5 min, 200 x g), washed twice in the appropriate buffer with centrifugal pelleting (5 min, 200 x g), and resuspended in Na^+-replete or Na^+-deficient medium at a concentration of 1 x 10^7 cells/ml. Cell concentrations were determined using an electronic particle counter (Coulter Counter Model Z_F, Coulter Electronics Inc., Hialeah, FL, USA). For the MTT assay, cells were harvested and resuspended in RH10F medium. The cell concentrations in the microcultures in the 96-well plates were adjusted to accord with the population doubling times in the plates of the cell lines used and with the intended incubation period for the MTT assay (Table 3). The intent of this adjustment was to assure exponential proliferation of cells during the incubation period.

2.2.3 Immunophenotyping of KM-3 and Reh cell lines

The immunophenotypes of the KM-3 and Reh cells and their clones were determined by flow cytometry. Cells were stained with mouse IgG monoclonal antibodies (BDIS), San Jose, CA, USA) specific for particular human CD antigens (Table 4); these antibodies were directly derivatized with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) by the manufacturer. The appropriate monoclonal antibody (10 µl; protein concentration was 0.09 mg/ml) was added to 0.1 ml of cell suspensions (5 x 10^6 cells/ml) and incubated in the dark at 4°C for 20 min. Afterwards, cells were washed with

Table 3

Cell numbers used per well in the 96-well microculture plates for the MTT assay

Cell line	Cells/well ($\times 10^4$)		
	24 h	48 h[a]	72 h[a]
Reh-A2	2.5-7.5	1.25	0.75
SB	2.5-5	1.0	0.65
CEM	2.5-5	1.0	0.65
KM3-2B	3.5	1.0	0.5

[a]Cells plated at these concentrations proliferated exponentially during the 48-h and 72-h incubation intervals; cell concentrations used for the 24-h incubations were not shown to provide exponential proliferation in the microcultures.

Table 4

Antibodies used to detect CD antigens

CD antigen	Antibody[a]	Fluorochrome	Predominant Lineage Reactivity
CD34	HPCA 1	PE	Progenitor cells
CD33	Leu M9	FITC	Early myeloid cells
CD13	Leu M7	FITC	Granulocytes
CD10	CALLA	FITC	Lymphoid progenitor
HLA-DR	HLA-DR	FITC	Lymphoid cells
CD19	Leu 12	FITC	B-Lymphoid cells
CD5/7	Leu 1/9	FITC	T-Lymphoid cells
CD3	Leu 4	PE	Pan T-lymphocyte
CD38	Leu 17	FITC	Lymphoid progenitor

[a]Commercial antibodies (BDIS, San Jose, CA, USA) were provided by Dr. J.J. Akabutu, Department of Pediatrics, University of Alberta, Edmonton, AB, Canada.

1 ml of PBS, and centrifuged (5 min, 250 x g) at 10°C and the cell pellet was resuspended in 1 ml of PBS containing 2.5% paraformaldehyde. The stained cells were analyzed for fluorescence by flow cytometry on the day of staining. Stained controls were prepared as described above with an isotypic fluorescent antibody. The isotype control was a mouse IgG antibody specific for keyhole limpet hemocyanin (BDIS, San Jose, CA, USA), an antigen not expressed on human cells or human cell lines. Cell-associated fluorescence was analyzed with a Becton Dickinson FACScan flow cytometer using Lysis II software (BDIS). FITC- and PE-labelled molecules were excited with an argon laser (488 nm) and emitted fluorescence was collected through 530 nm and 560 nm band pass filters for the two dyes, respectively.

2.2.4 Cloning of KM-3 and Reh cell lines

The KM-3 "wild-type" cell line was cloned twice in succession using a serial dilution method (246) to ensure a homogenous cell population. KM-3 cells were serially diluted in RPMI-10% FBS medium containing gentamicin (10 µg/ml), to a final concentration of 1.66 cells/ml. Following dilution, cell suspensions were added to 96-well microculture plates (200 µl/well), so that one cell was present in about every third well. After two weeks of culture, a clone designated KM3-2 was selected for expansion. The KM3-2 clone was expanded and cloned again as described above. The clone so obtained was designated KM3-2B and was used in the present study.

The Reh cell line was cloned using "conditioned" media containing gentamicin (10

μg/ml) and amphotericin B (1%) as two earlier attempts at cloning using conventional RPMI-10% FBS medium were unsuccessful. Conditioned medium is known to contain factors that support cell colony growth, particularly when cells are plated at very low densities (246). Reh-conditioned medium was prepared from cultures of Reh cells in RPMI 1640 medium with 10% FBS which had achieved the "lag phase" of culture growth. Lag phase Reh cell suspensions were centrifuged (5 min, 200 x g) and the medium was sterile-filtered through a 0.22 μm Millipore filter. The conditioned medium supported growth of colonies in the microcultures and allowed the selection of clone Reh-2 which was cloned one further time by the same method. The cell population selected in the second cloning procedure was designated Reh-A2; that clone was used in the present study.

2.3 Nucleoside transport measurements

2.3.1 Procedure for measuring inward fluxes of 2-CdA

In the membrane transport field, the rates at which permeant molecules traverse plasma membranes in one direction or the other are termed "fluxes". Unidirectional inward fluxes are conventionally measured as initial rates of permeant uptake by cells. In this study, all nucleoside flux measurements were conducted in a biological safety cabinet in a 37°C warm room. The NT processes expressed (that is, the "NT phenotype[15]) in the four human ALL cell lines and 5 patient samples were characterized by a modified version

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[15]The "NT phenotype" of a cell describes the NT processes expressed in cells of that type.

of a procedure described previously (216). Briefly, initial rates of nucleoside uptake were determined from time courses of the cellular uptake of [^{3}H]2-CdA. Cells were suspended in sodium-replete medium (DPBS) with or without NT inhibitors, or in sodium-deficient medium (DNMG, Ch^+ medium or Li^+ medium); the concentrations of inhibitors used are given in descriptions of individual experiments in "Results and Discussion". Cell suspensions were exposed to NT inhibitors for 10 min prior to the flux assay. Assays of inward zero-*trans*[16] fluxes, were initiated by mixing 100 µl of [^{3}H]2-CdA with 100-µl cell suspensions (1 x 10^7cells/ml for cultured cells and 2 x 10^7cells/ml for patient leukemic blasts), which had previously been layered over 100 µl of transport oil in a 1.5 ml plastic microcentrifuge tube. Cellular uptake of the ^{3}H-permeant was measured over intervals of 3-60 sec, which were ended by pelleting cells (20 sec, 15000 x g) through transport oil in an Eppendorf Model 5412 microcentrifuge. Cellular uptake of [^{3}H]2-CdA is known to proceed during the pelleting operation and was considered equivalent to that which takes place during a 2-sec interval of [^{3}H]2-CdA exposure (167); accordingly, this 2-sec interval is included in all assay results here reported. Assays were conducted in triplicate.

The "oil-centrifugation" method used in this study to measure inward fluxes of 2-CdA does not allow direct determination of the cellular permeant content at time-zero (T_{zero}). To estimate T_{zero} values for 2-CdA uptake, cells in suspension were incubated in an ice-bath with 10 µM DPM for 10 min prior to rapid addition of [^{3}H]2-CdA which was

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[16]By convention, *zero-trans* flux is defined as a permeant flux from one side (*cis*) of the membrane to the other side (*trans*), where the concentration of nucleoside is initially zero.

followed by immediate centrifugation. Uptake data are reported as the cellular uptake of [^{3}H]2-CdA (pmol of 2-CdA equivalents/10^6 cells). In figures illustrating time courses of 2-CdA uptake, the cellular content of the permeant at T_{zero} is shown and has not been subtracted from the cellular content of 2-CdA measured after various time intervals.

To measure ^{3}H-activity in cell pellets, the supernatant fractions and oil layers were removed by aspiration and the tip portions of the assay tubes that included the cell pellets were placed in scintillation vials. Cells were lysed overnight with 0.5 ml of 5% Triton X-100, and 4 ml of Ecolite scintillation fluid (ICN, Costa Mesa, CA, USA) were added to the lysate, and the ^{3}H-activity of the cell samples were then determined by liquid scintillation counting. Intracellular water volumes in cell pellets were estimated by subtracting the extracellular water space (estimated by measuring cell pellet content of [^{3}H]polyethylene glycol or of [^{14}C]sucrose after a 3-sec interval of exposure) from the total pellet water (determined by measuring [^{3}H]H_2O uptake during 3 sec). The intracellular water volumes were measured to determine (a) the leakiness of the cells and (b) whether cells had swelled or shrunk in the various media under the conditions of the experiment.

Time courses of cellular [^{3}H]2-CdA uptake were analyzed by fitting either a first or second order polynomial equation to uptake data (GraphPad Prism, Version 1.0, GraphPad Software Inc., San Diego, CA, USA):

First order: $Y = A + B \cdot X$

Second order $Y = A + B \cdot X + C \cdot X^2$

In the first order equation, A is the intercept and B is the slope of the line. In the second order equation, A is the intercept and B + 2CX is the slope of the tangent at X. Transport rates were determined from the slope of the line in the case of first order equations, or from the slope of the tangent to the curve at T_{zero} for second order equations.

The F-test was used to determine which equation was most appropriate for the data (GraphPad Prism, Version 1.0).

2.3.2 Concentration-effect plots for inhibition of 2-CdA uptake by NT inhibitors

2.3.2.1 Inhibition of 1 μM [^{3}H]2-CdA influx

The concentration-dependencies of inhibition by NBMPR and DPM of 1 μM 2-CdA uptake were determined for the four lines of cultured cells. For each concentration of inhibitor, 0.4 ml of cell suspension in DPBS (2×10^7 cells/ml) were added to 0.4 ml of graded concentrations (2x) of inhibitor; this step took place 10 min prior to the assay of [^{3}H]2-CdA uptake rates. In control assays, cell suspensions (0.4 ml) were incubated with an equal volume of DPBS. Permeant solutions were prepared by adding 0.4 ml of 4 μM [^{3}H]2-CdA (prepared in DPBS) to 0.4 ml of DPBS containing graded inhibitor concentrations (0.1 nM to 1 μM), or for the control samples, by adding [^{3}H]2-CdA to DPBS lacking inhibitors. Uptake of [^{3}H]2-CdA was initiated by adding 100 μl of ^{3}H-labelled permeant solutions to 100-μl portions of cell suspension (inhibitor-equilibrated cells) layered over oil in 1.5 ml microcentrifuge tubes (in a microcentrifuge), and ended by

spinning cells through the oil layer after 1.0 sec*. The cellular uptake of [^{3}H]2-CdA was measured at T_{zero} and at 3 sec; 2-CdA uptake under these conditions was linear for at least 3 sec (Fig. 3), and transport rates were determined from differences in 2-CdA uptake between T_{zero} and 3 sec. 2-CdA fluxes were expressed as percentages of the control rate of 2-CdA uptake (pmol/10^6cell/sec) measured in cells not exposed to the inhibitor.

IC_{50} values for inhibition of 2-CdA uptake were determined by using a computer program (GraphPad Prism, Version 1.0, GraphPad Software Inc., San Diego, CA, USA) to fit a one-site competition equation to plots of 2-CdA uptake (% control) *versus* the log of the inhibitor concentration:

$$Y = A_2 + \frac{(A_1 - A_2)}{1 + 10^{X - \log IC_{50}}}$$

where Y is 2-CdA uptake, % control, A_1 and A_2 are the upper and lower limits for Y, respectively, X is the inhibitor concentration and IC_{50} is the concentration of the inhibitor that produces a half-maximal response.

2.3.2.2 Inhibition of 100 μM [^{3}H]2-CdA influx

A slightly different procedure was followed to determine the IC_{50} value for inhibition of 100 μM 2-CdA uptake by NBMPR and DPM in cultured lymphoblasts. Briefly, 1.7 ml of cell suspensions in DPBS were incubated with equal volumes of 2x

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*This procedure results in a 3 sec exposure of cells to the ^{3}H-permeant

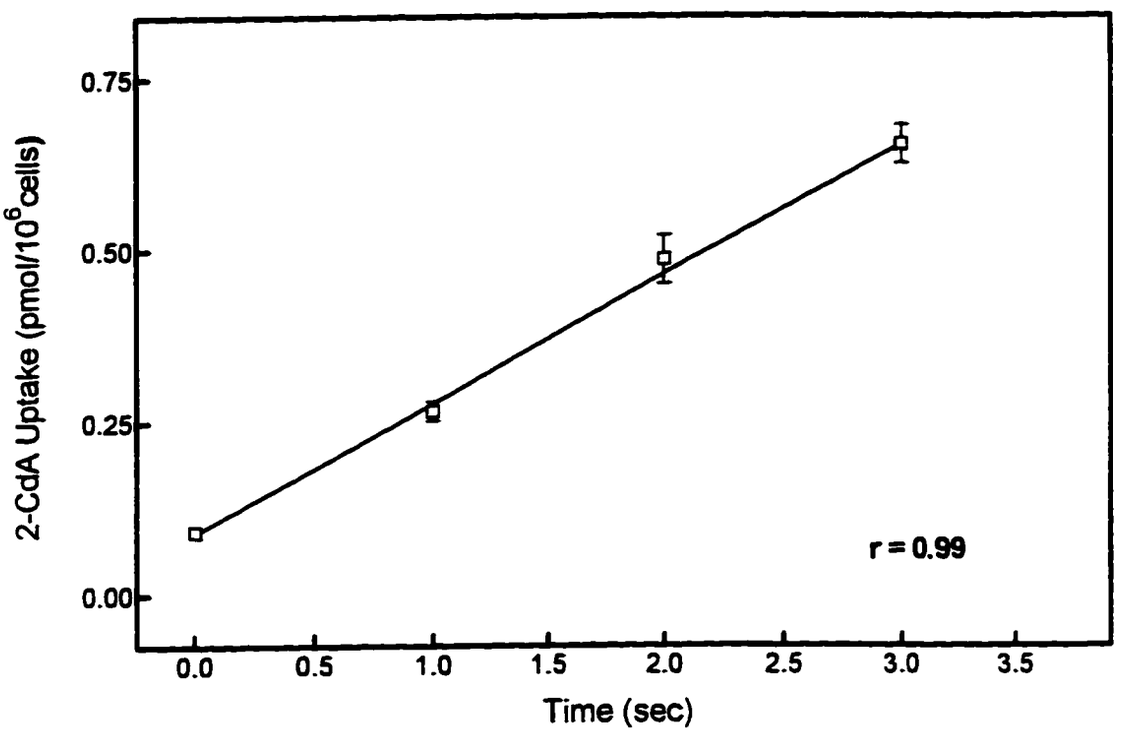

Figure 3. Linear time course of 1 μM 2-CdA uptake in Reh-A2 cells.
Influx of [^{3}H]2-CdA was measured at 37°C in cells suspended in Na^+medium for the indicated time intervals. Time-zero (T_{zero}) values represent 2-CdA uptake in cells exposed to 10 μM DPM at 4°C. 2-CdA transport was terminated by the addition of 35 μM NBMPR with immediate pelleting of the cells through "transport oil". Uptake of 1 μM 2-CdA was linear over a 3 sec time period. Thus, initial rates of uptake may be measured from the slope of the line between T_{zero} and 3 sec. Results reported are means ± SEM of six determinations; where error bars are not shown, the SEM is smaller than the data symbol plotted.

inhibitor solutions (0.1 nM to 3 μM) for 10 min. In control assays, cell suspensions (1.7 ml) were incubated with an equal volume of DPBS (10 min, 37°C) after which 1.7-ml portions of the cell suspension were centrifuged through "transport oil"(30 sec, 15000 x g) in a microcentrifuge. The supernatant was carefully removed and supplemented with [^{3}H]2-CdA to a final concentration of 200 μM. Uptake intervals were initiated by the addition of 100-μl portions of permeant solution to equal volumes of inhibitor-equilibrated cell suspensions layered over oil in 1.5 ml microcentrifuge tubes placed in a microcentrifuge, and ended by switch-on of the latter after graded intervals (3-9 sec); the centrifugation spun cells through the oil layer ending permeant uptake. As permeant uptake by the cells was linear between 3- and 9-sec (Fig. 4), transport rates were determined from the slopes of lines fitted to time courses of 2-CdA uptake during 3-9-sec intervals. The IC_{50} value was determined as described above for inhibition of 1 μM 2-CdA uptake.

2.3.3 Concentration dependence of 2-CdA transport in cultured ALL cells

The concentration dependence of 2-CdA inward fluxes (that is, of 2-CdA inward transport) was determined at 37°C in cultured lymphoblasts of the four human ALL cell lines used in this study. Because the *es* transport process was the only NT system detected in these cells, simple measurement of 2-CdA fluxes would provide kinetic characterization of 2-CdA transport by the *es* NT system in the four lines of cultured lymphoblasts. The cellular uptake of graded concentrations (0.1 nM to 1.5 μM) of [^{3}H]2-

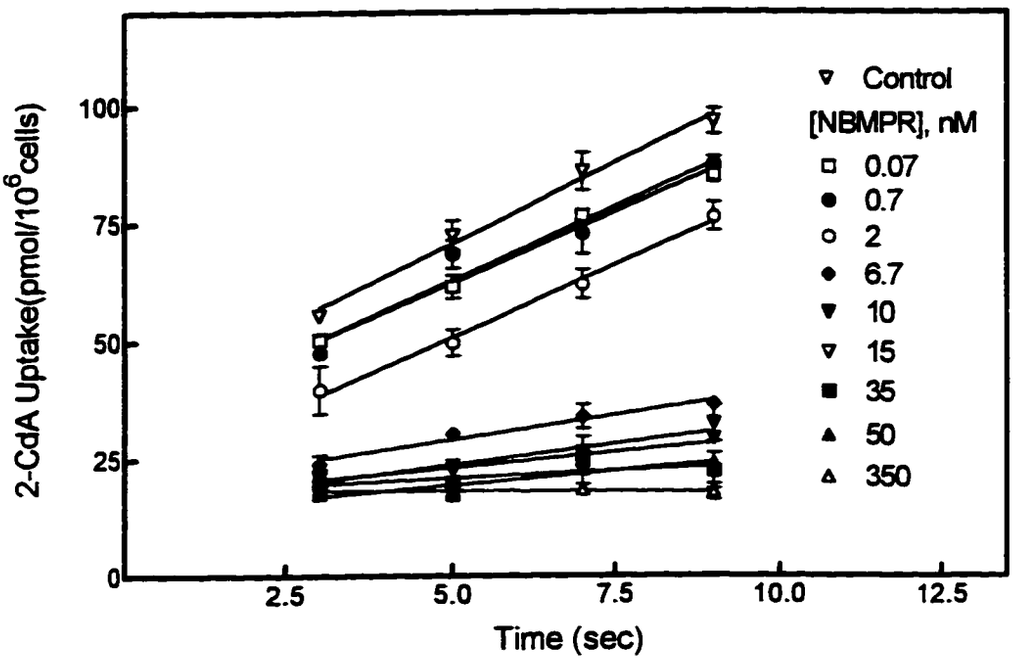

Figure 4. Linear time courses of 100 μM [^{3}H]2-CdA uptake in KM3-2B cells. Replicate assay mixtures in 1.5-ml microcentrifuge tubes contained 1 × 10^6 cells each in 200 μl (final volume)of Na^+ medium (DPBS) with 100 μM [^{3}H]2-CdA and the indicated concentrations of NBMPR. Cells were incubated at 37°C without or with the indicated concentrations of inhibitor for 10 min prior to the transport measurement; uptake intervals were initiated by addition of cells and ended by pelleting cells under "transport oil". Uptake of 100 μM 2-CdA was linear between 3 and 9 sec, hence, initial rates of uptake may be determined from the slope of the line between these time points. Each data point represents means ± SD of triplicate determinations from a single experiment.

CdA was determined at T_{zero} and 3 sec, and the slope of the line was a measure of the inward flux of 2-CdA, that is, the initial rate of permeant uptake. Kinetic constants (K_m and V_{max}) for 2-CdA transport were determined by computer analysis (GraphPad Prism, Version 1.0) of [S]/V *versus* [*S*] plots (Hanes plots) or by nonlinear regression analysis of [S] *versus* V plots using the Michaelis-Menten equation:

$$V = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

where V is the initial rate (that is, the inward flux) of permeant uptake (pmol/10^6cells/sec), [S] is the nucleoside concentration, V_{max} is the maximum initial rate of uptake, and K_m is the Michaelis-Menten constant.

2.3.4 Cellular retention of [^{3}H]2-CdA by NT inhibitors

Cultured ALL cells in DPBS medium (1×10^7 cells/ml) were incubated with graded concentrations of [^{3}H]2-CdA for 1 h to "load" the cells with [^{3}H]2-CdA. Following loading, cell suspensions were diluted 10-fold, with DPBS lacking additives (control), or with DPBS containing NT inhibitors (the concentrations of inhibitors are specified in individual experiments), to initiate efflux of [^{3}H]2-CdA. One milliliter portions containing 10^6 cells were sampled from these suspensions at graded intervals. Cells from those samples were pelleted under oil (20 sec, 15000 x g) to end the efflux processes and the ^{3}H-activity of the cell pellets was determined as described above. Efflux

data are reported as 2-CdA equivalents (pmol/10^6cells), which includes 2-CdA and the mono-, di-, and triphosphate metabolites, and possibly 2-chloroadenine. Time courses of cellular [^{3}H]2-CdA content were analyzed by fitting either one-phase or two-phase exponential decay curves to the efflux data as follows:

One-phase exponential decay $Y = Ae^{-\alpha t}$

Two-phase exponential decay $Y = Ae^{-\alpha t} + Be^{-\beta t}$

where Y is the concentration of 2-CdA and its metabolites in the cell, α and β are the elimination rate constants, A and B are the respective intercepts, and e is the base of natural logarithms. The half-lives of decay ($t_{1/2}$) are $0.693/\alpha$ and $0.693/\beta$.

The F-test was used to determine which equation was most appropriate for the data (GraphPad Prism, Version 1.0). Areas under the 2-CdA equivalents *versus* time curves between 0 and 2 h ($AUC_{0\text{-}2h}$) were calculated using the trapezoidal rule (GraphPad Prism, Version 1.0).

2.4 Enumeration of *es* sites on leukemic cells

2.4.1 Equilibrium binding of[^{3}H]NBMPR

The site-specific binding of [^{3}H]NBMPR at equilibrium was measured at 37°C in lymphoblasts suspended in DPBS medium. Cell suspensions (0.5 ml, 4 x 10^6 cells/ml) were added to 0.5 ml of DPBS containing graded concentrations of [^{3}H]NBMPR (0.125 -

12 nM) either in the presence, or absence of 5 μM (final) nonisotopic NBMPR. The assay mixtures were layered over 100 μl of transport oil in a microcentrifuge tube and incubated for 30 min. A 30-min incubation period was chosen because earlier work by Dagnino that showed that site-specific binding of 20 nM [^{3}H]NBMPR to L1210 cells at 22°C was not progressive beyond 10 min of incubation (247). Following incubation, bound and free ligand were separated by centrifugation of cells through oil, and the [^{3}H]NBMPR content of the supernatant and the cell pellets were determined as described for measurement of [^{3}H]2-CdA in the NT flux assays. Concentrations of free ligand (unbound) were determined from the [^{3}H]NBMPR content of the supernatant. Site-specific binding of NBMPR for each point was determined by subtracting the cell content of [^{3}H]NBMPR measured in the presence of 5 μM nonisotopic NBMPR ("nonspecific binding") from that measured in the absence of NBMPR ("total binding"). The binding constants B_{max} and K_D were calculated from a plot of transformed equilibrium binding data according to the method of Scatchard (248).

2.4.2 Equilibrium binding of 5-(SAENTA-x8)-fluorescein to ALL lymphoblasts measured by flow cytometry

5-(SAENTA-x8)-fluorescein (5-(Sx8)-F) is a fluorescent probe or specifically bound ligand for the *es* nucleoside transporter that has been shown to be useful for the flow cytometric detection and quantitation of *es* transporter sites on cells (176,195).

Freshly isolated leukemic cells from patients were washed twice (5 min, 150 × g)

and resuspended in PBS at 3 x 10^6 cells/ml for determination of the total binding of 5-(Sx8)-F, or resuspended in PBS containing 5 μM NBMPR for measuring nonspecific binding of 5-(Sx8)-F. Prior treatment of cells with NBMPR under these conditions (30 min, 22°C) prevents the site-specific binding of 5-(Sx8)-F to the *es* transporter sites by the preemptive occupancy of those sites by NBMPR. Cell suspensions were protected from light throughout these experiments. To determine total binding of 5-(Sx8)-F, cells suspended in PBS were incubated with graded concentrations (0.1-20 nM) of 5-(Sx8)-F for at least 15 min at 22°C before flow cytometric analysis of cell-associated fluorescence. Nonspecific binding was determined by incubating NBMPR-treated cells with graded concentrations of 5-(Sx8)-F in medium containing 5 μM NBMPR. The autofluorescence of leukemic cells was measured in PBS with and without 5 μM NBMPR.

Cell-associated fluorescence was analyzed with a FACScan flow cytometer (BDIS, San Jose, CA, USA). 5-(Sx8)-F molecules that were cell-associated were excited with 488 nm light from an argon laser and the emitted fluorescence was collected at 530 nm. Fluorescence signals from 10,000 cells for each condition were analyzed using BDIS Lysis II software. The mean relative fluorescence intensity (RFI) obtained from histograms of cell-bound fluorescence was used as a measure of cell-associated 5-(Sx8)-F, and the site-bound content of 5-(Sx8)-F on cells was calculated as the difference between the mean RFI of 5-(Sx8)-F bound in the absence of NBMPR (total binding) and the mean RFI for nonspecific binding, calculated by linear regression analysis of plots of 5-(Sx8)-F fluorescence *versus* concentration of 5-(Sx8)-F (Total, nM) measured in the presence of

5 μM NBMPR.

The concentration of 5-(Sx8)-F giving half-maximal binding (K_D) and the maximum value for *es*-site bound fluorescence of 5-(Sx8)-F (B_{max}) were determined by nonlinear regression analysis (GraphPad Prism, Version 1.0) of equilibrium binding data obtained by flow cytometry using the following equation:

$$Y = \frac{B_{max} \cdot X}{K_D + X}$$

where Y is the specific binding (mean RFI) of 5-(Sx8)-F, X is the total concentration of 5-(Sx8)-F, B_{max} is the maximum number of binding sites/cell and K_D is the equilibrium dissociation constant or concentration of 5-(Sx8)-F giving half-maximal binding.

2.5 MTT assay for cell viability

MTT is a yellow, water-soluble tetrazolium dye that is reduced by live cells to a dark purple formazan product; the MTT dye is not reduced by dead cells. The MTT assay can be used to measure cytotoxicity in proliferating cells, or in cells that are not dividing. The amount of MTT formazan product formed by test cells during a standard interval under cell culture conditions may be determined spectrophotometrically following dissolution of the formazan in a suitable solvent. Previously, reduction of the MTT dye was thought to be a mitochondrial process. It is now known that most cellular reduction of MTT occurs extramitochondrially (249). The MTT assay is widely used to measure cell viability and to screen candidate chemicals for cytotoxic activity in various

applications, for example, in searching for anticancer drug activity.

2.5.1 Drug sensitivity assay

The *in vitro* sensitivity of fresh leukemic lymphoblasts and cultured ALL cells to the nucleoside analogs, 2-CdA, araC and FaraA, was determined using the MTT assay of Mosmann (250), as modified by Alley *et al.* (251). Briefly, in 96-well microculture plates, 100-µl cell suspensions were added to replicate 100-µl portions of RH10F medium containing graded concentrations of the nucleoside analogs; 3-6 replicate microcultures were prepared for each drug concentration. Cell concentrations were adjusted according to the cell type and the interval of culture (Table 3). Fresh leukemic blasts from patients were cultured at the concentration of 1×10^7 cells/ml. Untreated control cultures lacked drugs and were set up with media only. RH10F medium was added to the outer wells to minimize the "edge effect", that is, to reduce evaporation in the outer wells, and to be used as blanks for the microplate reader (see below).

Plates were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for specified intervals of 1 to 4 days. After incubation, 30 µl of MTT solution (1 mg/ml) in RH10F[17] medium was added to each well and the plates were incubated for a further 2 h. During the 2-h interval of MTT reduction, the microculture plates were protected from light. The plates were then centrifuged (7 min, 50 x g) and the supernatant fluid was

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[17]Stock solutions of the MTT reagent (5 mg/ml in PBS) were sterilized by filtration. These stock solutions were stable for up to 4 weeks when protected from light and stored at 4°C. Immediately before use, the MTT stock solutions were diluted with 4 volumes of RH10F.

aspirated from each well. Following this, the crystalline formazan product was dissolved by the addition to each well of 150 µl of dimethylsulfoxide (spectrophotometric grade, Aldrich, Milwaukee, WI, USA) and the absorbance of the well solutions was measured in a microplate reader (Titertek Multiskan Plus, ICN Biomedicals, Mississauga, ON) at 540 nm (A_{540}). The A_{540} of each microwell solution was proportional to cell viability. Cell viabilities in the test wells were expressed as percentages of those in control wells, which lacked the cytotoxic test agent.

The cytotoxic effects of the nucleoside analogs toward cultured lymphoblasts were evaluated by determining the concentration of antileukemic agent producing a half-maximal response (extinguishing MTT dye reduction) in the MTT assay. A computer program (GraphPad Prism, Version 1.0) was used to fit the following four-parameter logistic equation to plots of cell viability (% drug-free control) *versus* the log of the drug concentration:

$$Y = A_2 + \frac{(A_1 - A_2)}{1 + (X / IC_{50})^b}$$

where Y is the response (cell viability, % drug-free control), A_1 and A_2 are the upper and lower limits for Y which were fixed at 100 and 0, respectively (except in the case of Patient J.M.), X is the drug concentration, IC_{50} is the concentration of antileukemic agent producing a half-maximal response, and b is a constant related to the steepness of the plot.

2.5.2 Cytotoxicity of 2-CdA and NBMPR combinations in cultured lymphoblasts

It was hypothesized that inhibition of 2-CdA efflux would tend to sustain cellular pools of 2-CdA and its metabolites, thereby enhancing 2-CdA cytotoxicity. The effect on lymphoblast viability of subjecting ALL lymphoblasts to NBMPR exposure after an episode of 2-CdA exposure was examined in Reh-A2 and CEM cells using the MTT dye reduction assay.

Reh-A2 cell suspensions in RH10F medium (5×10^5 cells/ml) were exposed to graded concentrations of 2-CdA for 1 h to "load" cells with the latter agent. Following loading, cell suspensions were diluted 10-fold (5×10^4 cells/ml), with (a) RH10F medium (control), or (b) RH10F medium with 0.5 μM NBMPR (RH10FN). Cell suspensions were then plated as 200 μl cultures containing 10^4 cells in 96-well microculture plates; the viability of 3-6 replicate microcultures was examined for each 2-CdA concentration in the presence and absence of 0.5 μM NBMPR. Controls for 2-CdA-treated cells contained cells incubated in media alone. Controls for the 2-CdA/NBMPR-treated cells, were composed of cells incubated in medium that contained 0.5 μM NBMPR. Plates were incubated in 5% CO_2 in air at 37°C for 72 h. Following that period of exposure to the test drugs, cells in the microcultures were assayed for drug effects by measuring their ability to reduce the MTT dye under standard conditions. The MTT reagent was added to the microcultures, which were processed as described for the drug sensitivity assays.

As described for the retention experiments, lymphoblasts in suspension were loaded with 2-CdA for 1 hour after which suspensions of the loaded cells were diluted 10-

fold with 2-CdA-free medium either in the presence or absence of 0.5 μM NBMPR to initiate the efflux process. After a 2-h incubation period, cells were washed twice (5 min, 150 x g) with RH10F medium and resuspended at the same concentration (3×10^4 cells/ml and 5×10^4 cells/ml for CEM and Reh-A2 cells, respectively) in RH10F medium and incubated for a further 69 hours prior to an MTT assay that was processed as described above.

2.6 Metabolism of [^{3}H]2-CdA in Reh-A2 and CEM cells

Reh-A2 and CEM cells (100 μl) were suspended in RH10F medium at 10^7 cells/ml and loaded with 1 μM [^{3}H]2-CdA at 37°C for 1 h. After 2-CdA loading, portions of the cell suspension were diluted 10-fold with either RH10F medium without additives (control), or with RH10F medium containing 0.5 μM NBMPR, and incubated at 37°C for a further 2 h. Following incubation, the cell suspensions were chilled in an ice-bath for 10 min and centrifuged (30 sec, 15000 × g) at 4°C, after which the cell pellets were washed with 0.5 ml ice-cold PBS and recovered by centrifugation at 4°C. The wash medium was discarded and the cells were extracted with 250 μl of 70% methanol; the methanolic extracts were stored at -20°C prior to HPLC analysis and the cell pellet residues were retained for further work-up (see below).

Samples for HPLC analysis were centrifuged (30 sec, 15000 x g) at 4°C and the supernatant retained for analysis. Further extraction of 2-CdA and its metabolites from the pellet residues was continued by adding 100 μl of ice-cold 70% methanol to the pellet

that was centrifuged and the methanol extract combined with the previously-retained supernatant. Cellular 2-CdA and its metabolites were determined by HPLC analysis of methanol extracts. The combined extracts were dried in an ice bath under a stream of nitrogen and resuspended in 100 µl of the initial mobile phase buffer (5 mM $NH_4H_2PO_4$). Samples were applied to a Partisil 10-SAX anion exchange column (250 × 4.6 mm, Whatman) and eluted at 2 ml/min (22°C) with the solvent gradient system defined in Table 5. Fractions (0.66 ml) were collected every 20 sec for 32 min. Ecolite (4 ml) solution was added to each vial and the ^{3}H-activity of the samples determined by liquid scintillation counting.

This procedure appeared to resolve 2-CdA and its mono-, di-, and tri-phosphate metabolites. Intracellular 2-CdA and its metabolites were identified by comparison with retention times of several standards, including 2-CdA, dAMP, dADP and dATP (see Table 21). The intracellular concentration of metabolites was calculated as follows:

$$\mu M = \frac{CPM}{SA \cdot IWS \cdot X}$$

where CPM is the total CPM value for each peak, SA (CPM/pmol) is the specific activity of the ^{3}H-permeant, IWS (µl/cell) is the intracellular water space and X is the cell number $\times 10^6$.

Table 5

Elution system for HPLC analysis of 2-CdA and its phosphate metabolites on a Partisil 10-SAX anion exchange column

The composition of the elution buffer used in the linear gradient system for HPLC analysis is expressed as the percentage of each constituent in the final solution as a function of time.

Time (min)	5 mM $NH_4H_2PO_4$ pH 4.0 (%)	700 mM $NH_4H_2PO_4$ pH 4.8 (%)
0	100	0
30	0	100
40	0	100
45	100	0

2.7 Statistical analysis

Unless otherwise stated, the Student's t-test was used to measure statistical significance. The Wilcoxon rank-sum test (252) was used to compare the sensitivity of pediatric ALL lymphoblasts to 2-CdA and araC. The relationship between sensitivity of patient blasts to 2-CdA and araC was assessed by linear regression analysis. The Tukey multiple comparisons test (252) was used to compare intracellular 2-CdA equivalents measured in the presence or absence of NT inhibitors in retention experiments. The Tukey Kramer multiple comparisons test was used to compare nucleoside analog sensitivity in lymphoblasts from the four ALL cell lines. Statistical analysis were performed as two-tailed tests with significance level of at least 0.05. Data are reported as mean ± SD/SEM.

RESULTS and DISCUSSION

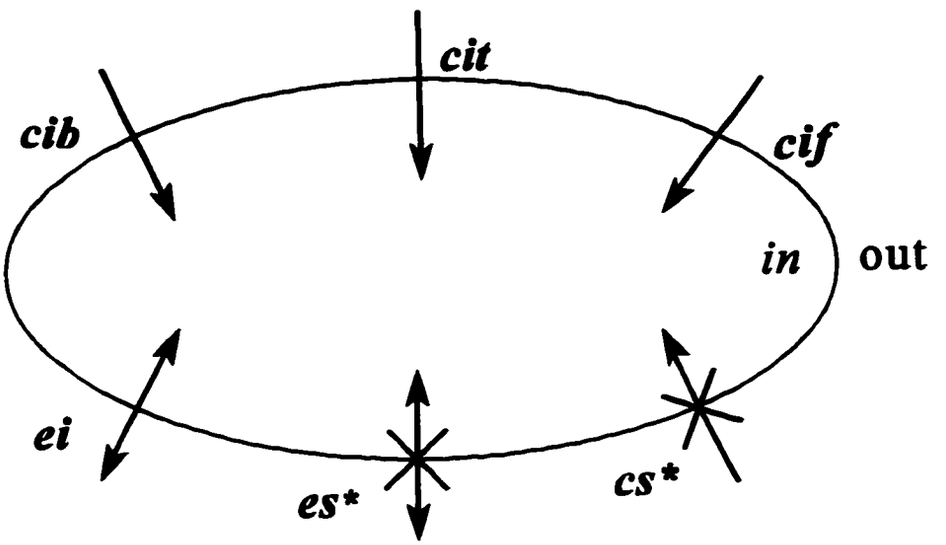
3.1 Nucleoside transport processes found in ALL cells

A goal of this project was to characterize the NT processes by which nucleoside analogs enter bone marrow leukemic lymphoblasts from pediatric ALL patients. 2-CdA was chosen as the permeant for measurement of nucleoside fluxes in this study because (a) this analog is a substrate for both equilibrative (facilitated diffusion) and Na^+-nucleoside co-transport permeation systems (158,210), and (b) this project was concerned with the role of nucleoside transport processes in the cytotoxicity of 2-CdA in lymphoblasts from pediatric ALL patients. Because the low patient accrual rates reflect the population size of this community and because marrow samples are small and of a "once-only" nature, cultured lymphoblast lines of human ALL origin were used as model systems to develop methods and to enable kinetic and mechanistic studies of NT processes in ALL cells. It was recognized from the start that cultured cell lines may not be adequate models for fresh leukemia cells from patients.

Analysis of 60-sec time courses that were measured by a rapid sampling technology at 37°C, yielded initial rates for cellular uptake of 2-CdA under various conditions. Initial rates of permeant uptake by cells measure unidirectional inward fluxes of that permeant, and are by definition, transport rates (170). NT processes were identified by measuring the effects on 2-CdA transport rates of (a) the replacement of Na^+ ions in the medium, and (b) the addition of the classical NT inhibitors, NBMPR and DPM, to the medium. The *es* process was recognized as a

Na^+-independent component of 2-CdA uptake that was inhibited by nanomolar concentrations of NBMPR. ***Ei*** transport activity was measured as the difference between the initial rates of Na^+-independent 2-CdA uptake in the presence of NBMPR and DPM, that is, as the difference between 2-CdA fluxes under these two conditions. The difference between 2-CdA uptake in Na^+-replete and Na^+-deficient medium was used to identify the presence of Na^+-dependent NT processes. A kinetic approach was also used to characterize the membrane permeation of 2-CdA in four human ALL cell lines, that is, inward fluxes of 2-CdA into the cultured lymphoblasts were assayed at graded concentrations of 2-CdA in order to define the kinetic constants for the influx processes .

In other studies, the enhancement of nucleoside uptake in the presence of NBMPR has been used to identify Na^+-dependent processes. Belt *et al.* (218) reported that FB was concentrated in leukemic blasts from AML patients in the presence of DPM. The concentrative uptake of nucleosides by cells in medium containing NBMPR is indicative of the involvement of a Na^+-dependent process that is insensitive to the classical NT inhibitors, NBMPR and DPM (Fig. 5). In the presence of these inhibitors, efflux by the ***es*** system is blocked, but the Na^+-dependent system is not, allowing the permeant to be concentrated intracellularly. To observe this effect, longer time courses (min) of permeant uptake are necessary. However, in the present study, Na^+-substitution experiments were used to identify Na^+-dependent processes. The cations used in this study (NMG^+, Ch^+ and Li^+) to replace Na^+ ions are not believed to participate in Na^+/nucleoside co-transport processes.



* The *es* and *cs* nucleoside transport processes are blocked in the presence of 1 μM NBMPR and DPM

Figure 5. Concentrative uptake of nucleoside analogs in the presence of nucleoside transport inhibitors.

In the presence of the classical NT inhibitors, NBMPR and DPM the *es* and *cs* nucleoside transport systems are inhibited, thereby, blocking permeant influx and efflux by these transporters. However, influx by Na^+-dependent systems (*cif*, *cit* and *cib*) is permitted in the presence of these inhibitors, thus, allowing the permeant to concentrate intracellularly. The expression of the *ei* transporter may compromise the ability of NBMPR and DPM to concentrate the permeant intracellulary by allowing permeant efflux in the presence of these inhibitors.

3.1.1 Effect of Na^+ ion replacement on the uptake of 2-CdA in KM-3 and Reh lymphoblasts

The Figs. 6A and 6B show time courses of the uptake of 1 μM [^{3}H]2-CdA in KM-3* and Reh* cells suspended in Na^+-replete medium (DPBS) and in Na^+-deficient media containing other cations (NMG^+, Ch^+ and Li^+) in place of Na^+. No significant difference was observed between the uptake of 2-CdA in the four media, suggesting that no Na^+-dependent process was involved in the influx of 2-CdA in these cells. Because the time courses of 2-CdA uptake were similar in cells suspended in the three sodium-substituted media and because NMG^+ of high purity was less costly than choline, NMG^+ was selected as the Na^+ ion replacement for the remainder of the study.

3.1.2 Cloning of KM-3 and Reh cell lines

Following these demonstrations of nucleoside flux measurements, the KM-3 and Reh cell lines were cloned twice to insure that the NT characteristics identified in these studies were those of a single genotype. Examination of the KM-3 immunophenotype showed that cells of this line expressed surface antigens associated with both B- and T-lineages (Table 6), suggesting the possible presence of two or more genotypes in the KM-3 cell line. To obtain a clonal population of cells, the KM-3 cell line was cloned twice using a limiting dilution method (246), yielding finally the KM3-2B clone line, which expressed CD5 and CD7 antigens, but not the CD19 or CD3

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* These designations refer to uncloned cell lines, that is, to "wild-type" cell lines.

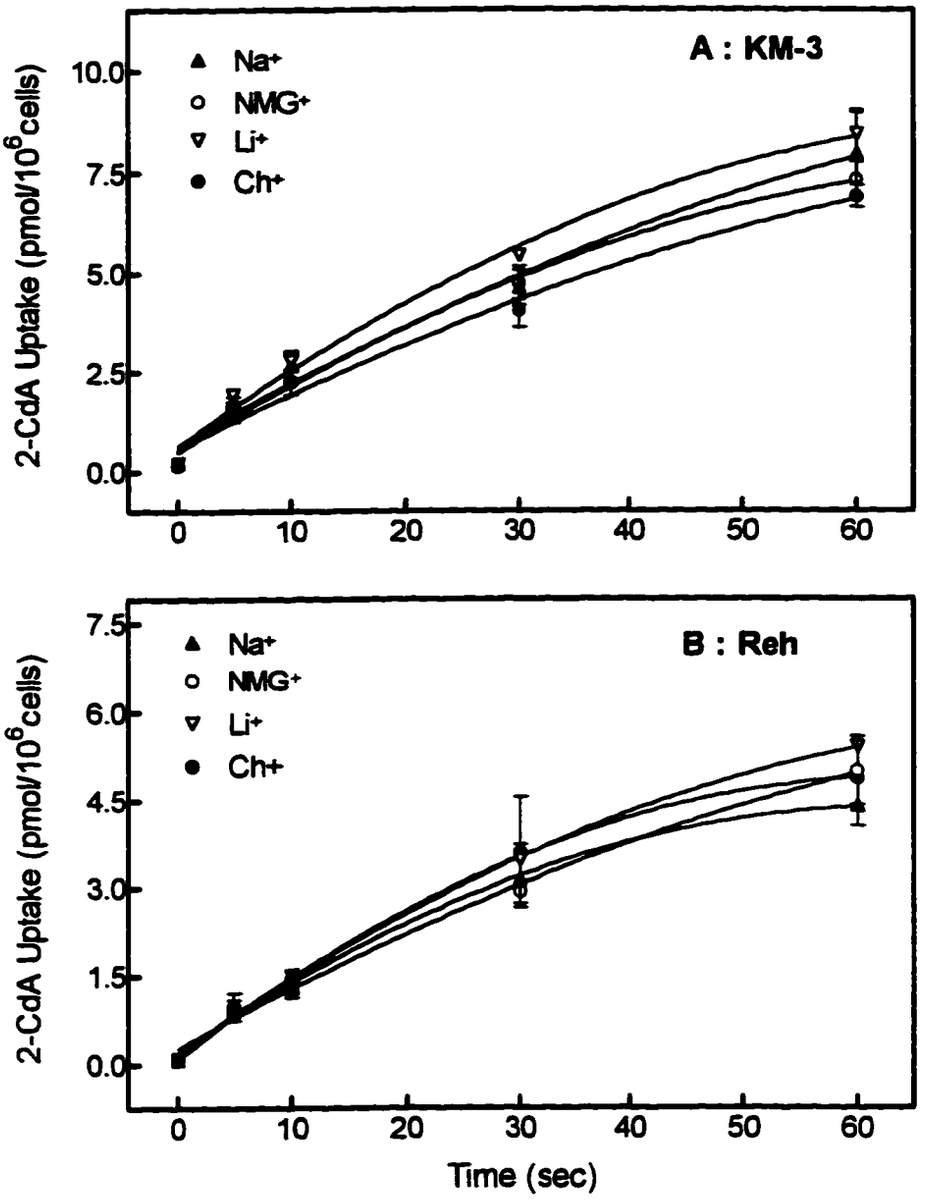

Figure 6. [^{3}H]2-CdA uptake in KM3 and Reh lymphoblasts: effects of Na^+ substitutes.

Assay mixtures contained 1 x 10^6 cells in Na^+ medium (DPBS) (▲), NMG^+ medium (DNMG) (○), Li^+ medium (▽), or Ch^+ medium (●), and also contained 1 μM 2-CdA. Data in Panel A and B were obtained with KM-3 cells and Reh cells, respectively. Assay mixtures were contained in 1.5-ml microcentrifuge tubes and were layered over 100 μl of "transport oil". After incubations at 37°C for the indicated time intervals, cells were centrifugally pelleted under the oil layer and assayed for ^{3}H-content. Data represent means ± SEM from three or more separate experiments, each performed in triplicate; where absent, error bar (SEM) values are covered by the data symbol.

Table 6

Antigenic profile of KM-3 "wild type" cells and clonal KM3-2B cells

Cells were stained with commercially available, fluor-conjugated antibodies directed at the indicated antigens (Table 4) and the percentage of cells expressing each antigen was determined by flow cytometry[18].

CD antigen	Predominant lineage reactivity	KM-3	KM3-2B
CD34	Progenitor	-	nd
CD33	Early myeloid cells	-	nd
CD13	Granulocyte	-	-
CD10	Lymphoid progenitor	+	-
HLA-DR	Lymphoid cells	-	-
CD19	B-lymphoid cells	±	-
CD5/7	T-lymphoid cells	++	++
CD3	pan T-lymphocyte	±	-
CD38	Lymphoid progenitor	_	nd

nd, not determined; -, absent; ±, < 10% positive; +, 10-50% positive;++, > 50% positive.

[18]Immunophenotyping of the KM-3 and Reh (Table 7) cell lines was performed by John Chan (Department of Pediatrics, Cross Cancer Institute, Edmonton, AB, Canada)

Table 7

Antigenic profile of Reh "wild-type" cells and clonal Reh-A2 cells

Cells were stained with commercially available, fluor-conjugated antibodies directed at the indicated antigens (Table 4) and the percentage of cells expressing each antibody was determined by flow cytometry.

Antibody	Predominant lineage reactivity	Reh	Reh-A2
CD34	Progenitor	±	-
CD33	Early myeloid cells	-	nd
CD13	Granulocyte	-	nd
CD10	Lymphoid progenitor	++	++
HLA-DR	Lymphoid cells	±	±
CD19	B-lymphoid cells	±	±
CD5/7	T-lymphoid cells	-	-
CD3	pan T-lymphocyte	-	-
CD38	Lymphoid progenitor	+	+

nd, not determined; -, absent; ±, < 10% positive; +, 10-50% positive; ++, > 50% positive.

antigens (Table 6). The immunophenotype of the KM3-2B clone was consistent with that of T-lineage ALL. Similarly, a clone of Reh cells, designated Reh-A2, was established by cloning a population of Reh "wild-type" cells. The cloning was then repeated so that the selected population, designated Reh-A2, was ultimately cloned twice. Table 7 compares the expression on the Reh lines of cell surface antigens reactive with the same panel of antibodies listed in Table 4 and it is seen that Reh cells and clonal Reh-A2 cells are of the same immunophenotype. For the remainder of this study, the KM3-2B and Reh-A2 clonal lines were used in place of the KM-3 and Reh cell lines.

3.1.3 Is *es* transport the only NT process expressed in the four ALL cell lines used in this study ?

The uptake of 1 μM 2-CdA in lymphoblasts of the four lines of cultured ALL cells is shown in Figs. 7A-10A, which summarize experiments that measured fluxes in cells suspended in Na^+-replete medium (DPBS), in Na^+-deficient medium (DNMG), in DPBS medium containing 50 nM NBMPR, or either 100 nM DPM or 700 nM DPM. The [^{3}H]2-CdA concentration was 1 μM in these initial experiments, a concentration chosen to increase the possibility of identifying Na^+-dependent transport processes, since the equilibrative nucleoside transporters appear to have a lower affinity for their substrates than the concentrative transporters (168,253). The concentration of NT inhibitors used in these experiments was based on earlier studies in leukemic cells from CLL patients (210). Although it was realized that these concentrations of inhibitors

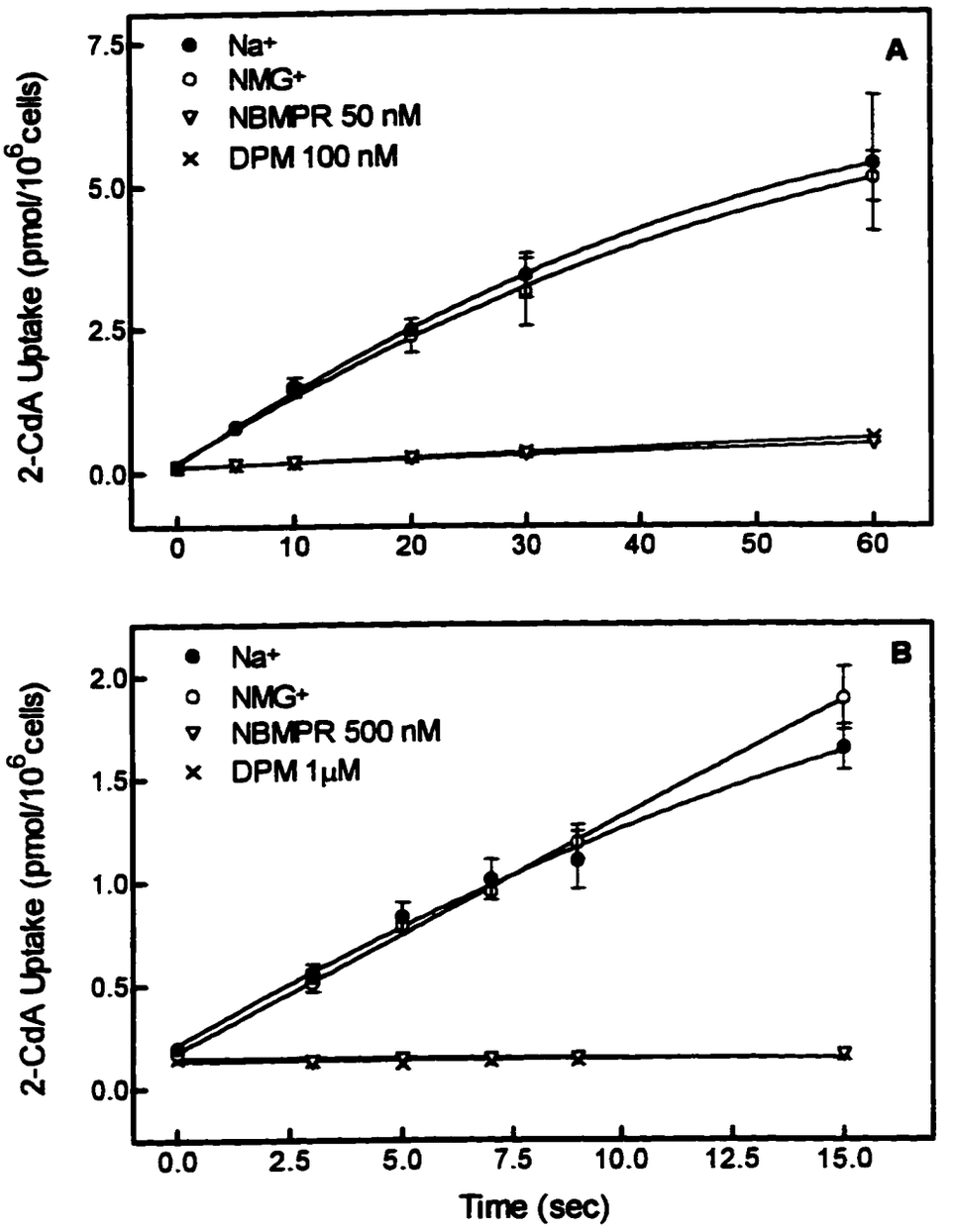

Figure 7. Time courses of [^{3}H]2-CdA uptake in Reh-A2 cells: effects of Na^+ substitution and NT inhibitors.

Assay mixtures contained 1×10^6 cells in Na^+ medium (DPBS) without inhibitors (●), or with NBMPR (▽) (50 nM and 500 nM in Panels A and B, respectively), or with DPM (×) (100 nM and 1 μM in Panels A and B, respectively), or in NMG^+ medium (DNMG)(○). Uptake of 1 μM 2-CdA was measured at 37°C at the indicated time intervals and cells were exposed to inhibitors for 10 min prior to assays of 2-CdA uptake. Data are means ± SD of triplicate determinations from a single experiment; where absent, error bars (SD) are covered by the data symbol.

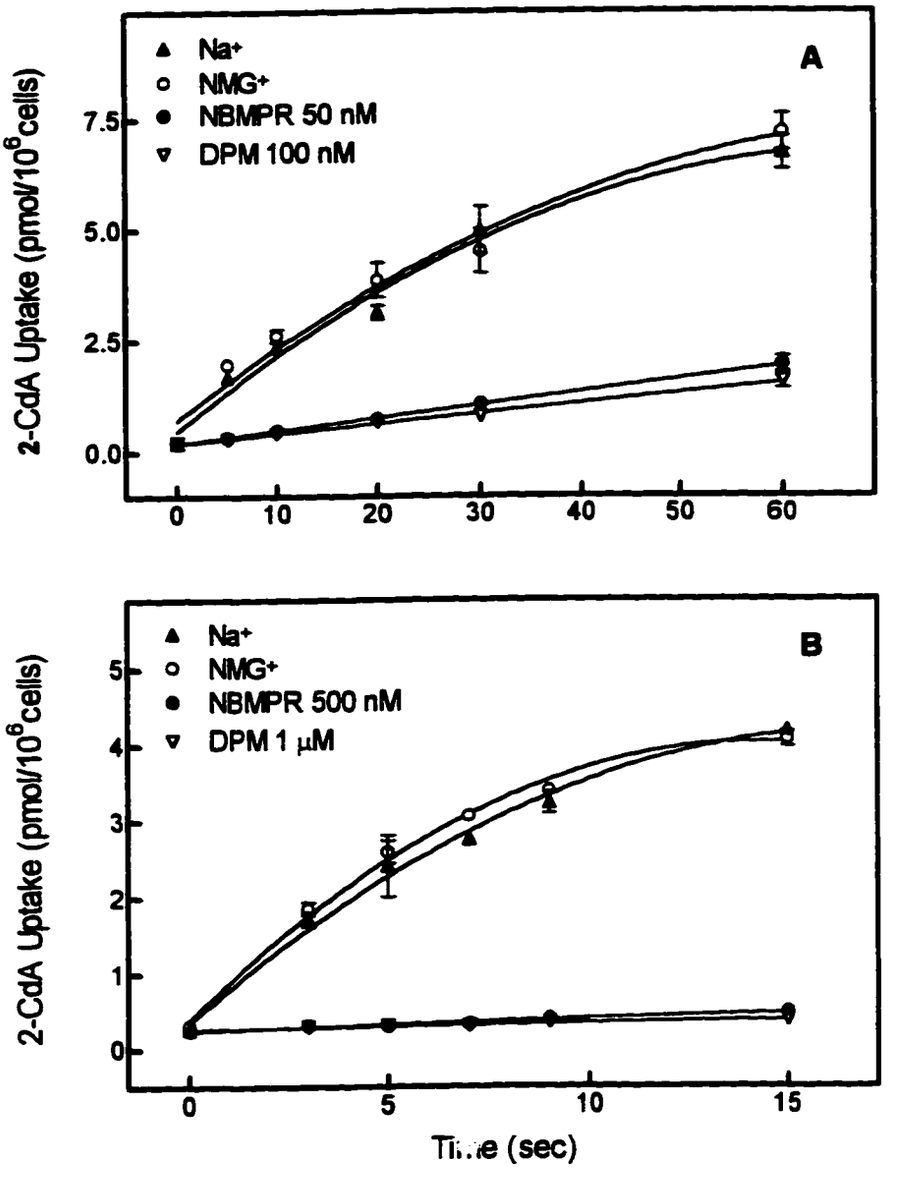

Figure 8. Time courses of [^{3}H]2-CdA uptake in CEM cells: effects of Na^+ substitution and NT inhibitors.

Assay mixtures contained 1 × 10^6 cells in Na^+medium (DPBS) without inhibitors (▲), or with NBMPR (●) (50 nM and 500 nM in Panels A and B, respectively). or with DPM (▽) (100 nM and 1 μM in Panels A and B, respectively), or in NMG^+ medium (DNMG) (○). Uptake of 1 μM [^{3}H]2-CdA was measured at 37°C at the indicated time intervals and cells were exposed to inhibitors for 10 min prior to assays of 2-CdA uptake. In Panel A data are means ± SEM of data from three separate experiments (each experiment was performed in triplicate), and in Panel B results reported are means ± SD of triplicate determinations from a single experiment; where absent, error bars are covered by the data symbol.

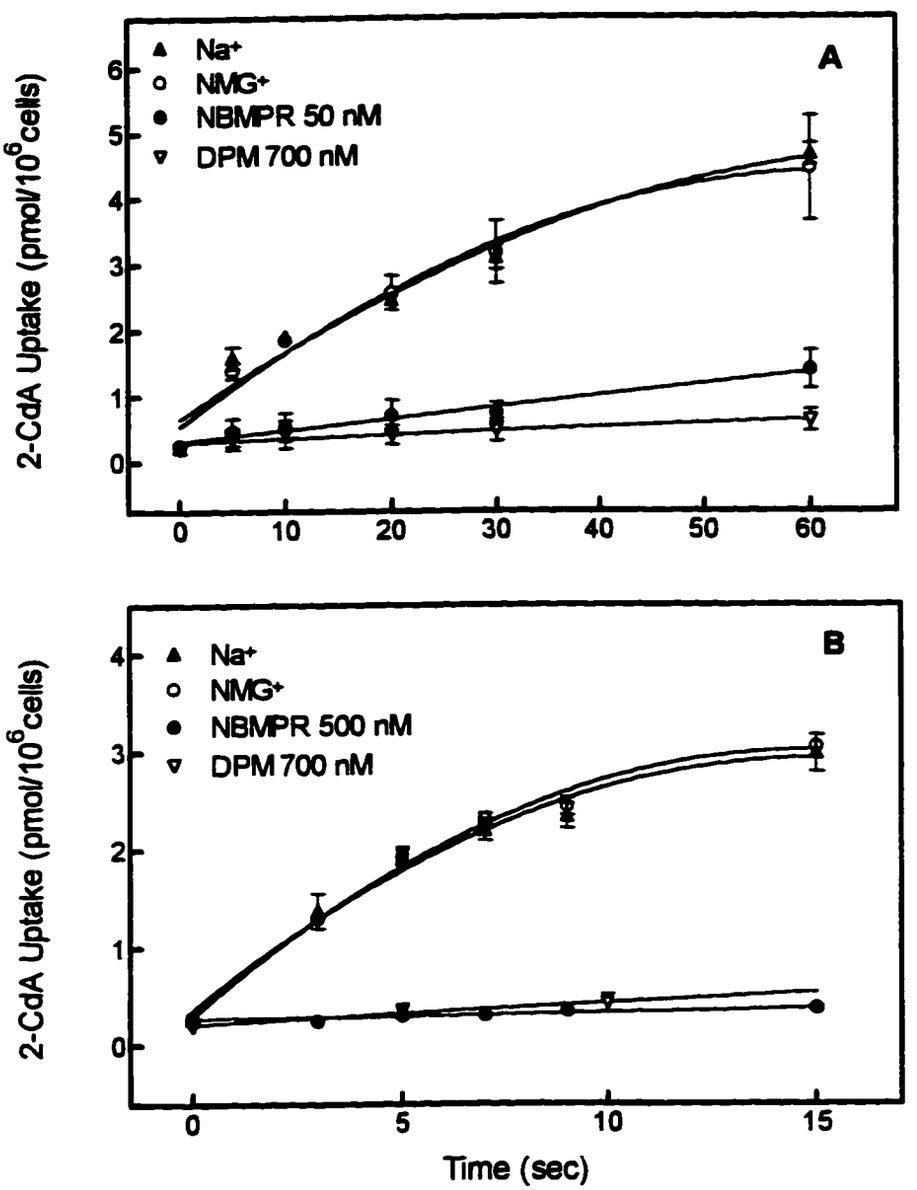

Figure 9. Time courses of [^{3}H]2-CdA uptake in SB cells: effects of Na^+ substitution and NT inhibitors.

Assay mixtures contained 1×10^6 cells in Na^+medium (DPBS) without inhibitors (▲), or with NBMPR (●) (50 nM and 500 nM in Panels A and B, respectively), or with DPM (∇) (700 nM in Panels A and B, respectively), or in NMG^+medium (DNMG) (○). Uptake of 1 μM [^{3}H]2-CdA was measured at 37°C at the indicated time intervals and cells were exposed to inhibitors for 10 min prior to assays of 2-CdA uptake. In Panel A data are means ± range of data from two separate experiments (each experiment was performed in triplicate), and in Panel B results reported are means ± SD of triplicate determinations from a single experiment; where absent, error bars are covered by the data symbol.

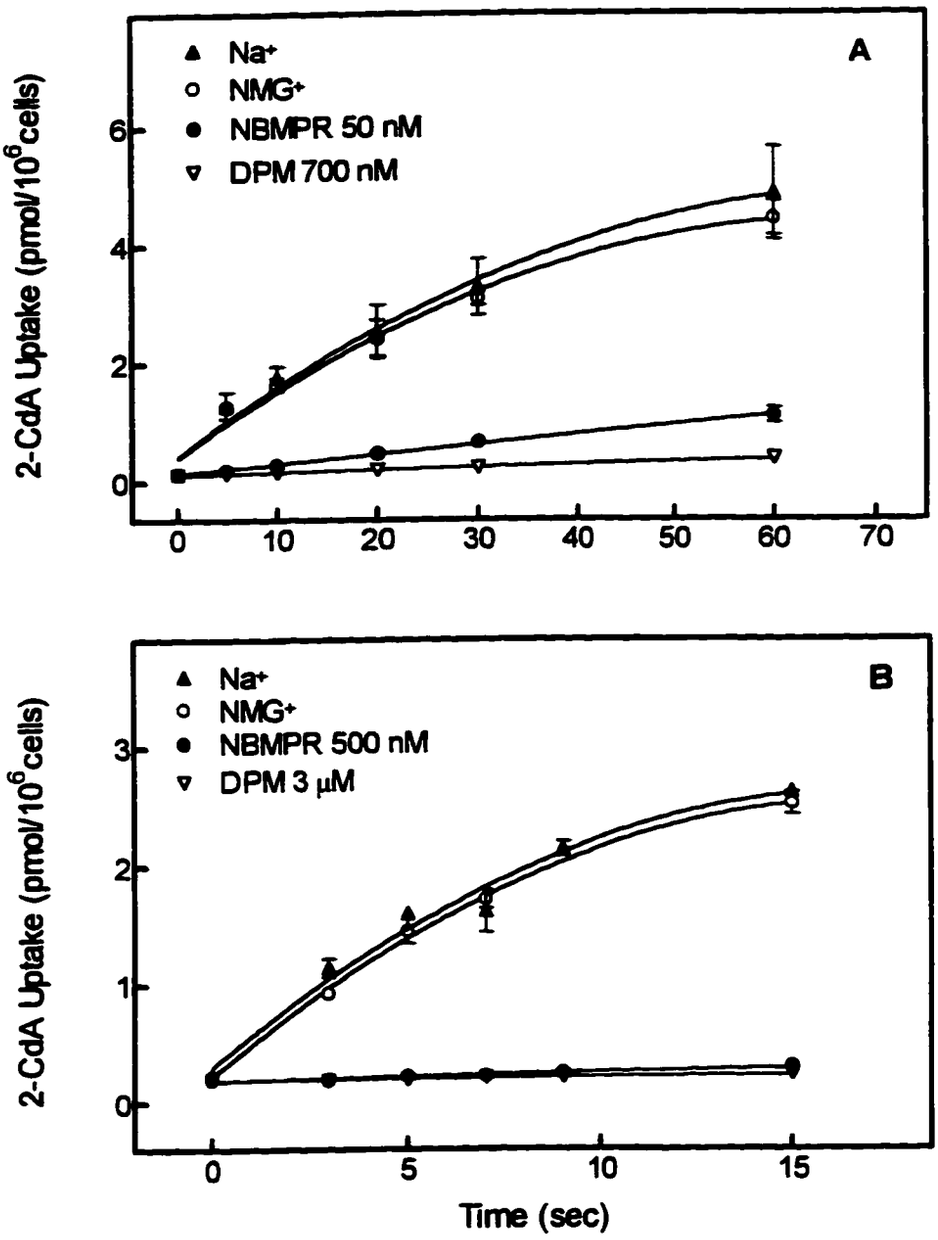

Figure 10. Time courses of [^{3}H]2-CdA uptake in KM3-2B cells: effects of Na^+substitution and NT inhibitors.

Assay mixtures contained 1 × 10^6 cells in Na^+medium (DPBS) without inhibitors (▲), or with NBMPR (●) (50 nM and 500 nM in Panels A and B, respectively), or with DPM (▽) (700 nM and 3 μM in Panels A and B, respectively), or in NMG^+ medium (DNMG) (○). Uptake of 1 μM [^{3}H]2-CdA was measured at 37°C at the indicated time intervals and cells were exposed to inhibitors for 10 min prior to assays of 2-CdA uptake. In Panel A data are means ± SEM of data from four separate experiments (each experiment was performed in triplicate), and in Panel B results reported are means ± SD of triplicate determinations from a single experiment; where absent, error bars are covered by the data symbol.

may not be appropriate for the characterization of NT processes in ALL cells, they were a starting point for the measurement of such processes in ALL cells.

No significant difference was apparent in the rates of uptake of 2-CdA by lymphoblasts in either the Na^+-replete or Na^+-deficient media in the four cell lines studied. These findings argue for the absence of Na^+-dependent transport processes that contribute to 2-CdA uptake in Reh-A2, SB, CEM and KM3-2B cell lines. In Reh-A2 cells (Fig. 7A), the uptake of 1 μM 2-CdA was blocked, virtually completely, by either 50 nM NBMPR or 100 nM DPM; this result suggests that Reh-A2 cells lack *ei* activity. Similarly, in CEM cells (Figs. 8A), the *es* process was the only transporter activity detected. However, in CEM cells, approximately 12% of 2-CdA uptake appeared to be inhibitor-insensitive and, therefore, possibly not transporter-mediated. It is possible that in this experiment, 2-CdA uptake in the presence of inhibitors was a consequence of 2-CdA diffusion in "leaky" cells. However, in these experiments the intracellular water volume was approximately 14% of the total pellet water space indicating that the cells were of low permeability to [^{3}H]polyethylene glycol, and therefore, not "leaky". In SB (Fig. 9A) and KM3-2B lymphoblasts (Fig. 10A), the *es* process contributed significantly to 2-CdA influx, yet *ei* activity (NBMPR-insensitive) accounted for approximately 10% and 9% of the influx, respectively. Activity of the *ei* process was shown by the greater inhibition of 2-CdA influx in the presence of 700 nM DPM relative to that in the presence of 50 nM NBMPR. In this cell line, transporter-independent uptake of 2-CdA (that is, inhibitor-insensitive permeation of 2-CdA) was less than 4% in the presence of 700 nM DPM. Interestingly, when 100 nM DPM was used in place of 700 nM DPM, transporter-independent uptake of 2-CdA was about

15% in both KM3-2B and SB cell lines (data not shown). These results highlights the importance of determining the appropriate conditions that are necessary to detect NT processes in cells.

To confirm the expression of *ei* transport activity in SB and KM3-2B cells, further experiments were conducted to define NT phenotypes. Belt *et al.* (205) defined NBMPR-insensitive nucleoside transport activity as that which took place in the presence of 1 μM NBMPR. Concentrations of NBMPR and DPM that abolished 1 μM 2-CdA uptake by the *es* transporter (determined in inhibitor concentration-effect studies (3.1.4)) were used in place of 50 nM NBMPR and 700 nM DPM. The experiment of Fig. 10B showed that in the presence of high inhibitor concentrations, *ei* transport activity was no longer detected in KM3-2B cells. This was evident in the virtually complete blockade of 2-CdA uptake in the presence of either 500 nM NBMPR or 3 μM DPM. Similarly, Fig. 9B shows that in SB cells, the level of *ei* NT activity was reduced to approximately 3% in the presence of 500 nM NBMPR. Thus, *es* transport activity was the only NT process detectable in SB cells, as contributions to uptake of less than 5% are difficult to measure accurately under these conditions.

The inhibitor concentrations used were also important in determining the contribution of inhibitor-insensitive permeation to 2-CdA uptake in CEM cells. As stated earlier, approximately 12% of 2-CdA influx could not be inhibited in these cell lines by either 50 nM NBMPR or 100 nM DPM (Fig. 8A). However, in CEM cells more than 98% of 2-CdA uptake in the lymphoblasts was inhibited by 500 nM NBMPR or by 1 μM DPM (Fig. 8B), showing that inhibitor-insensitive entry of 2-CdA was at most, a minor component of 2-CdA uptake. This result agrees with previous

nucleoside permeation studies in CEM cells (summarized in ref 209) which showed that the *es* transporter to be the only NT process present in these cells.

Table 8 summarizes the NT processes found in the four ALL cell lines; the quantitation of those processes was based on the fractional contribution to total 2-CdA uptake of fluxes measured in the presence of inhibitors or in Na^+-deficient medium. Inward fluxes of 1 μM 2-CdA in this study ranged from 0.12 pmol/10^6cell/sec in Reh-A2 cells to 0.42 pmol/10^6cells/sec in CEM cells. In general, the findings of this work show that the *es* transporter was the only NT process detected in the four cell lines, Reh-A2, SB, CEM and KM3-2B. The present study has shown that to characterize NT processes in leukemic cells, it is important to first determine the conditions that will allow detection of each NT process present in a cell population. For example, inhibitor concentrations that are too low may not completely block *es* transport activity, a circumstance that may lead to a false conclusion about *ei* activity.

The nucleoside transport processes contributing to the uptake of 100 μM 2-CdA in Reh-A2 and KM3-2B cell lines were also explored in experiments that were aimed at defining relationships between 2-CdA fluxes and concentrations of that permeant. As well, the use of inhibitors to identify the NT processes required definition of inhibitor concentration-effect relationships for NBMPR and DPM inhibition of 100 μM 2-CdA influx in the two cell lines (3.1.4). Early time courses of 2-CdA (100 μM) uptake were measured in sodium-replete medium (DPBS), with or without NBMPR or DPM (using the concentrations of inhibitors that completely blocked 100 μM 2-CdA influx by the *es* transporter determined in inhibitor concentration-effect experiments), or in sodium-deficient medium (DNMG). Shown in Figs. 11A and 11B are 15-sec time

Table 8

Immunophenotype and NT phenotype characteristics of ALL cell lines

Cell line	Immunophenotype	NT phenotype[a]		
		% *es*	% *ei*	% Na^+-dependent uptake
Reh-A2	Pre-B ALL	99	-	-
SB	B-ALL	99	-	-
CEM	T-ALL	94	3	-
KM3-2B	T-ALL	97	2	-

[a]Inward flux of 1 μM 2-CdA (37°C) in cells of a particular type measured under conditions that permit only *es* transporter activity (or only *ei*, or Na^+-linked activity), with expression of the measured rate as a percentage of 2-CdA flux in the absence of inhibitors , that is, as a percentage of the total inward flux of 2-CdA.

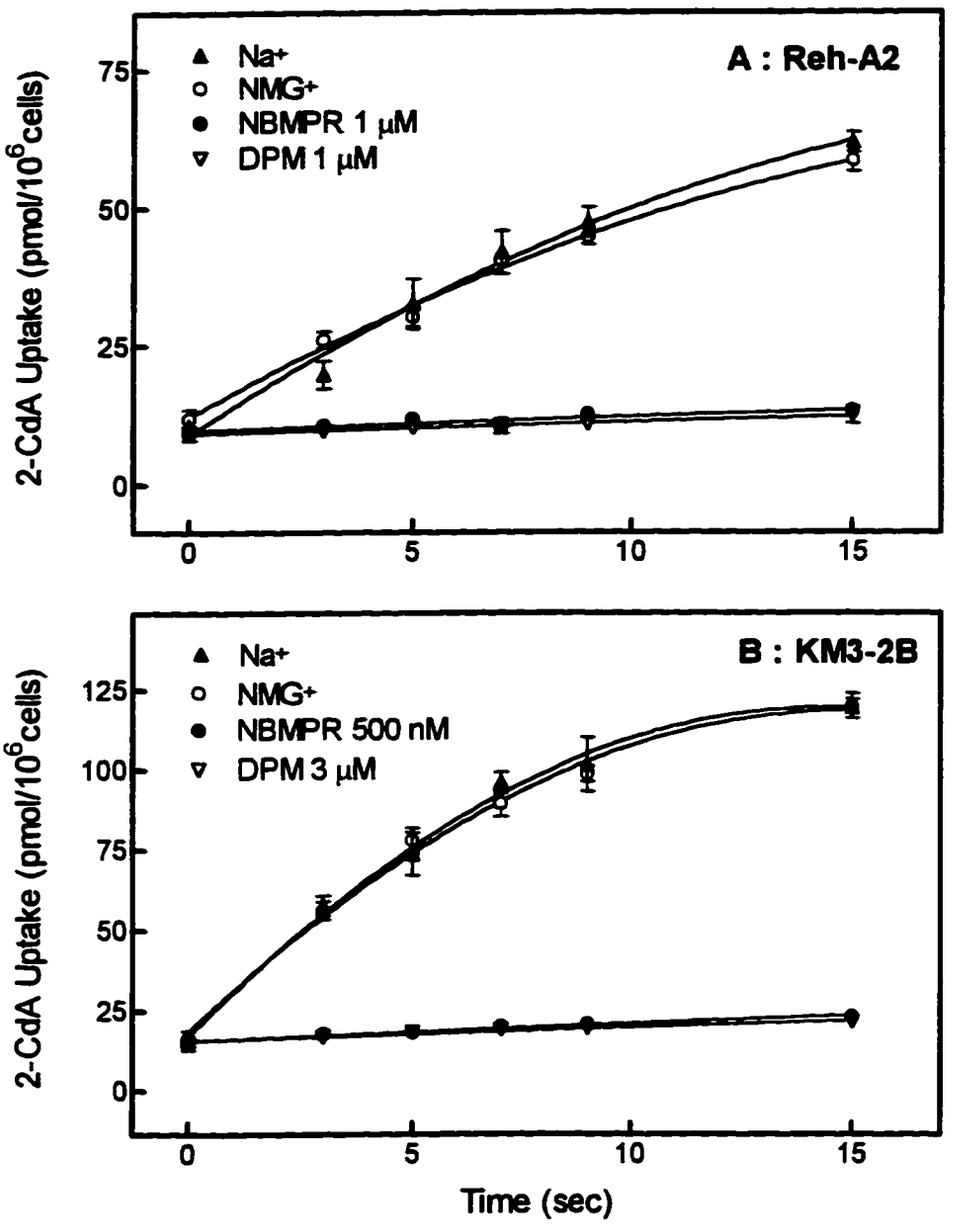

Figure 11. Time courses of 100 μM 2-CdA uptake in Reh-A2 (Panel A) and KM3-2B (Panel B) cells: effects of Na^+ substitution and NT inhibitors.
Assay mixtures contained 1 x 10^6 cells in 200 μl (final volume) of Na^+medium (DPBS) without inhibitors (▲), or with NBMPR (●) (1 μM and 500 nM in Panels A and B, respectively), or with DPM (▽) (1 μM and 3 μM in Panels A and B, respectively), or in NMG^+ medium (DNMG) (○). Uptake of 100 μM [^{3}H]2-CdA was measured at 37°C at the indicated time intervals; cells were exposed to inhibitors for 10 min prior to assays of 2-CdA uptake. In Panel A (Reh-A2 cells) points represent means ± SD of triplicate determinations from a single experiment, and in Panel B (KM3-2B cells) results reported are means ± SEM of data from three separate experiments (each experiment was performed in triplicate); where absent, error bars are covered by the data symbol.

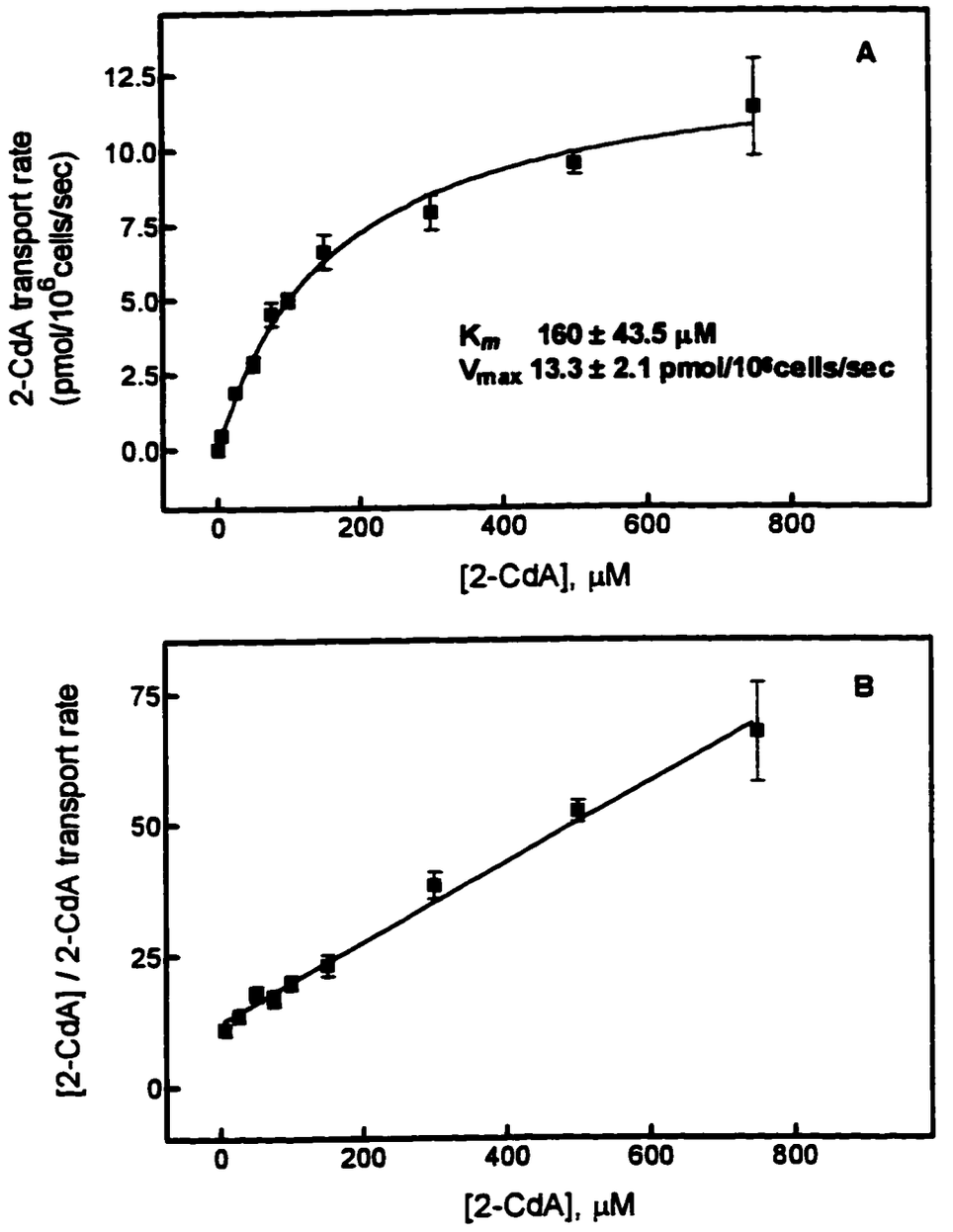

Figure 12. Kinetics of 2-CdA influx in Reh-A2 cells.
Panel A: Rates of [^{3}H]2-CdA inward transport (unidirectional inward fluxes) were measured as the initial rates of 2-CdA uptake (measured between T_{zero} and 3 sec) and were plotted as a function of 2-CdA concentration. The curve represents the best fit to the data of the Michaelis-Menten equation, as described in the Materials and Methods. The kinetic constants for the data presented are V_{max} and K_m values of 13.3 ± 2.1 pmol/10^6cells/sec and 160 ± 43.5 μM, respectively.
Panel B: Hanes plot (S/V *versus* S) analysis of the data in Panel A. The linear form of the plot is consistent with the activity of a single nucleoside transport system in Reh-A2 cells. Data are means ± SEM of three separate experiments; where error bars are absent, the SEM is smaller than the symbol.

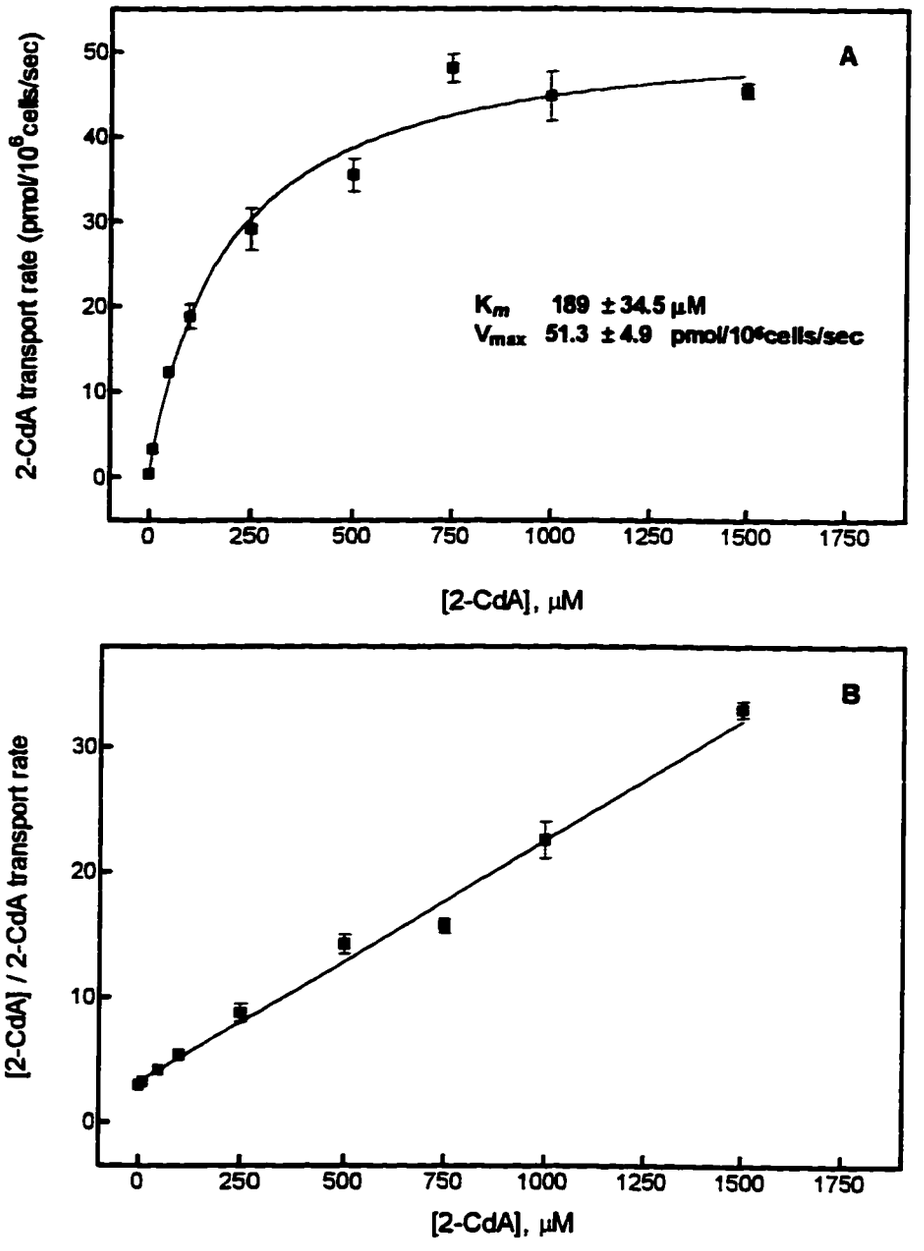

Figure 13. Kinetics of 2-CdA influx in KM3-2B cells.
Panel A: Rates of [^{3}H]2-CdA inward transport (unidirectional inward fluxes) were measured as the initial rates of 2-CdA uptake (measured between T_{zero} and 3 sec) and were plotted as a function of 2-CdA concentration. The curve represents the best fit to the data of the Michaelis-Menten equation, as described in the Materials and Methods. The kinetic constants for the data presented are V_{max} and K_m values of 51.3 ± 4.9 pmol/10^6cells/sec and 189 ±34.5 μM, respectively.
Panel B: Hanes plot (S/V *versus* S) analysis of the data in Panel A. The linear form of the plot is consistent with the activity of a single nucleoside transport system in KM3-2B cells. Data are means ± SEM of three separate experiments; where error bars are absent, the SEM is smaller than the symbol.

courses of 100 μM 2-CdA uptake in Reh-A2 and KM3-2B cell lines. Both NBMPR and DPM effected major inhibition of the uptake of 2-CdA in both cell lines, and the virtual absence of NBMPR/DPM-insensitive 2-CdA fluxes confirmed the lack of *ei* transporter activity in these cells. Uptake of 2-CdA was less than 1% in the presence of either NT inhibitor. Furthermore, little difference was observed between the uptake of 2-CdA in the presence or absence of Na^+ ions. These findings support earlier experiments, which showed the *es* transporter to be the only significant NT process contributing to 2-CdA uptake in Reh-A2 and KM3-2B cell lines.

3.1.4 Concentration dependence of 2-CdA transport rates in ALL cell lines

Kinetic experiments involved measurement of inward fluxes of 2-CdA as a function of 2-CdA concentration. Inward fluxes were defined as the initial rates of [^{3}H]2-CdA uptake in the lymphoblasts measured at 37°C. Initial rates of 2-CdA uptake by the cultured cells were measured as the difference in 2-CdA uptake by 10^6 cells after (a) 3 sec of permeant exposure, and (b) zero sec of exposure. The data of Figs. 12A and 13A showed that uptake of 2-CdA in both Reh-A2 and KM3-2B lymphoblasts was saturable. Furthermore, plots of the data according to the Hanes equation (S/V *versus* S plots) were linear, indicating that the inward fluxes obeyed Michaelis Menten kinetics, and that a single transport process was responsible for 2-CdA uptake in cells of the Reh-A2 and KM3-2B clones (Figs. 12B and 13B). In CEM and SB cells, the rate-concentration relationships of 2-CdA transport were also saturable and similar to those shown in Figs. 12A and 13A.

Table 9 summarizes the kinetic constants found in the present study for the

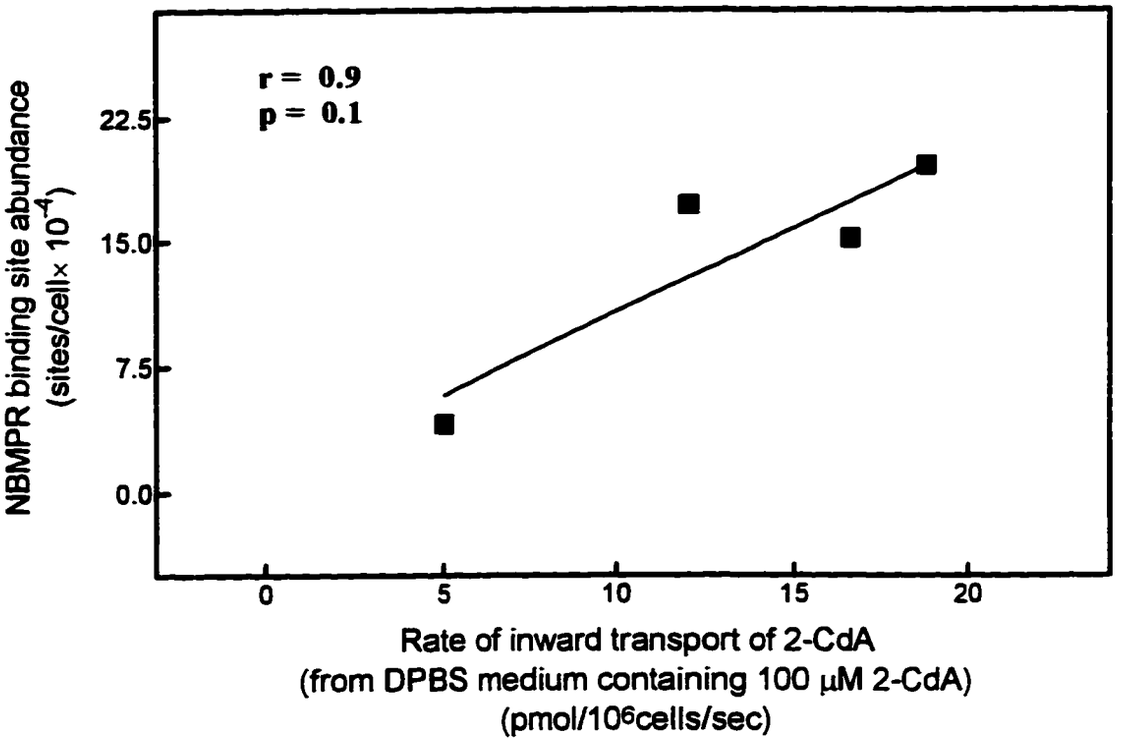

Figure 14. Comparison of 2-CdA uptake with nucleoside transport capacity in cultured ALL lymphoblasts.
The initial rates of 2-CdA uptake at 37°C were determined from short time courses of 2-CdA uptake measured in cells suspended in DPBS containing 100 μM [^{3}H]2-CdA and nucleoside transport capacity was measured by determining the maximum number of [^{3}H]NBMPR binding sites.

Table 9

Kinetics of 2-CdA transport in cultured ALL cell lines

Inward fluxes of 2-CdA were measured as initial rates of cellular 2-CdA uptake during the first 3 sec of cell exposure to [^{3}H]2-CdA at 37°C. V_{max} values (pmol/10^6cells/sec) were established in rate-concentration studies with kinetic analysis based on the Michaelis Menten equation. B_{max} values (*es* sites/cell) were determined from mass law analysis of the equilibrium binding of [^{3}H]NBMPR in the ALL cell lines at 37°C (refer to Table 11).

	Kinetic parameters[a]			
Cell line	K_m (μM)	V_{max} (pmol/10^6cells/sec)	V_{max}/K_m	V_{max}/B_{max} (molecules/*es* site/sec)
Reh-A2	160 ± 43.5	13.3 ± 2.1	0.08	195
SB	71.8 ± 17.3	18.9 ± 3.4	0.26	66
CEM	203 ± 18.6	54.9 ± 3.7	0.27	217
KM3-2B	189 ± 34.5	51.3 ± 4.9	0.27	158

[a]The kinetic parameters presented are: K_m, permeant concentration at half-maximal flux; V_{max} , maximum inward flux of 2-CdA; V_{max}/K_m, 2-CdA transport efficiency; V_{max}/B_{max}, 2-CdA translocation capacity.

2-CdA transport processes in the four ALL cell lines. This table presumes that each of the four cell lines expresses a single nucleoside transporter, the *es* transporter. Evidence for this has been provided earlier in NT phenotype and later in inhibitor concentration-effect experiments. The kinetic constants listed are, K_m (permeant concentration at half-maximal flux) and V_{max} (maximal inward flux of 2-CdA). Maximal 2-CdA influx varied four-fold among the cell lines tested, ranging from 13.3 to 54.9 pmol/10^6cells/sec. The K_m values ranged from 71.8 ± 17.3 μM in SB cells to 201.3 ± 34.5 μM in KM3-2B cells. These K_m values are similar to values reported for araC influx in fresh myeloblasts and lymphoblasts (254). The "efficiency" of 2-CdA transport (V_{max}/K_m) was approximately 0.27 for SB, CEM and KM3-2B cells, but was notably lower for Reh-A2 cells at 0.08. In conclusion, there was little difference between the transportability of 2-CdA in SB, CEM and KM3-2B cells, however, Reh-A2 cells appeared to have a reduced ability relative to the other lines studied here to transport this agent.

Jarvis *et al.* (255) have shown that V_{max} values for uridine transport were roughly proportional to the number of NBMPR sensitive sites in a variety of cell types. In the present study, a relationship ($r = 0.9$, $p = 0.1$), although not significant, was apparent between the inward flux of 100 μM 2-CdA uptake and the abundance of *es* transporter sites in the four ALL cell lines (Fig. 14). If more cell lines were included in the study, it is conceivable that a significant correlation would be established between the maximum velocity of 2-CdA uptake and the *es* site content in ALL cells.

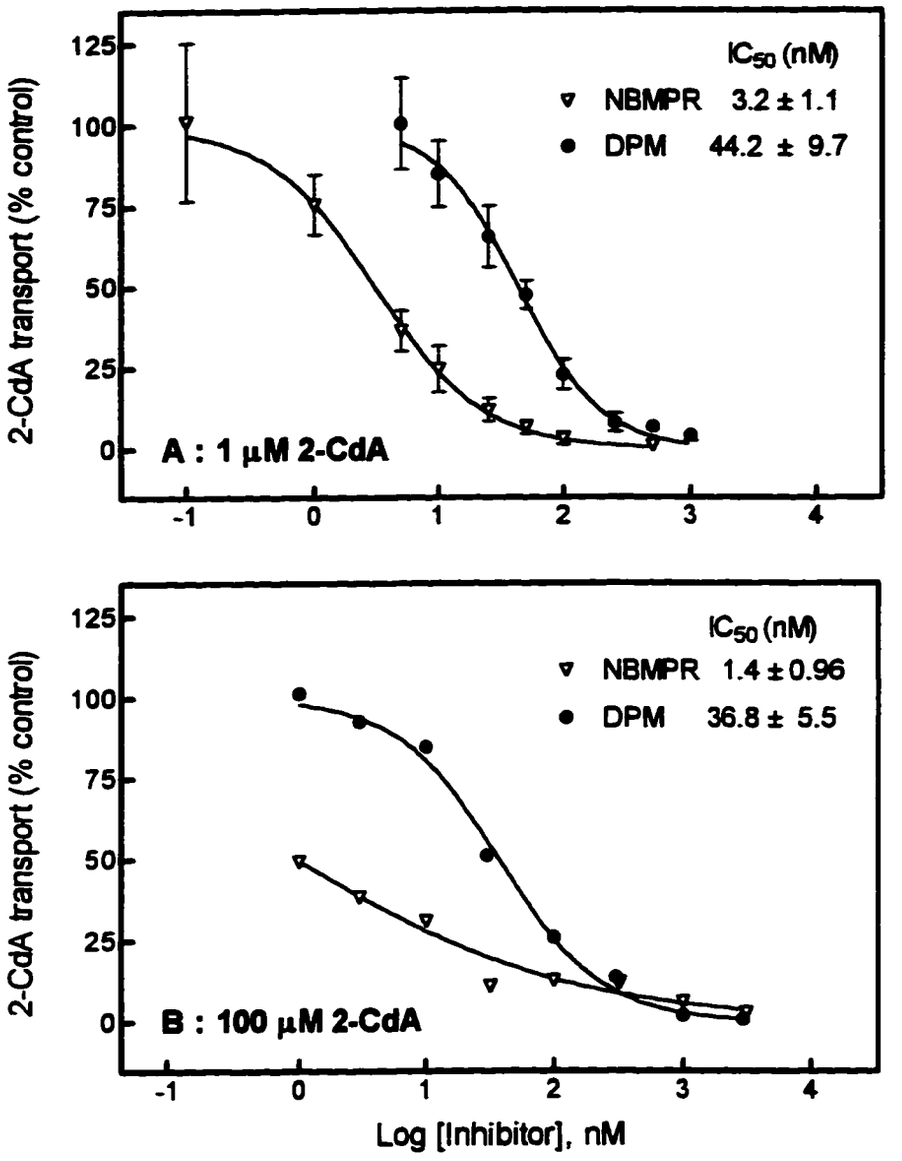

Figure 15. Concentration-effect plots of inhibition by NBMPR (∇) and DPM (●) of 2-CdA transport (1 μM and 100 μM in Panels A and B, respectively) in Reh-A2 cells.

The data in Panel A are means of rates of inward transport of [^{3}H]2-CdA measured as unidirectional inward fluxes of 2-CdA as described in Fig. 3. Panel B shows rates of 2-CdA inward transport, measured as described in Fig. 4. The control values (100 %) represents transport rates measured in cells suspended in medium without inhibitors. The initial rates of 1 μM and 100 μM influx in control (untreated) cells were 0.18 ± 0.02 pmol/10^6cells/sec and 3.59 ± 0.25 pmol/10^6cells/sec, respectively. Data in Panel A are means ± SEM obtained from 3-4 experiments; data in Panel B are means of two separate experiments. Each experiment was performed in triplicate.

2-CdA translocation capacity calculated as the V_{max}/B_{max} ratio (that is; maximum rate of 2-CdA influx/ maximum number of NBMPR binding sites per cell) ranged from 66 molecules/carrier/sec to 217 molecules/carrier/sec in the cell lines examined in this study (Table 9). These data are similar to results presented by Jarvis *et al.* (255), supporting the idea that the kinetic properties of transport processes in the cell reflect the abundance of transporter sites on the membrane.

3.1.5 Sensitivity of 2-CdA uptake to NBMPR and DPM in ALL cell lines

The relationship between concentrations of NBMPR and DPM, and inhibition of inward transport of 2-CdA was explored (a) to confirm previous observations suggesting activity of an *ei* nucleoside transporter in SB and KM3-2B cells (3.1.3), and (b) to determine whether significant inhibitor-insensitive uptake of 2-CdA occurred in CEM cells. The experiments of Figs. 15A-17A measured the effects of graded concentrations of DPM and NBMPR on the uptake of 1 μM 2-CdA in Reh-A2, SB and KM3-2B cell lines, respectively. The previously described (2.3.1), rapid-sampling assay was used to measure uptake of [^{3}H]2-CdA at T_{zero} and 3 sec of exposure of cells to the ^{3}H-substrate. Since uptake of 2-CdA was linear over a 3-sec interval (Fig. 3), the uptake of [^{3}H]2-CdA by cells during the initial 3 sec of 2-CdA exposure is equivalent to the rate of inward transport of 2-CdA in these cells.

NBMPR and DPM concentrations of approximately 500 nM and 1 μM, respectively, were required to block 1 μM 2-CdA uptake in the four cell lines (Figs. 15A-17A). The concentration-effect plots for inhibition of cellular uptake of 2-CdA

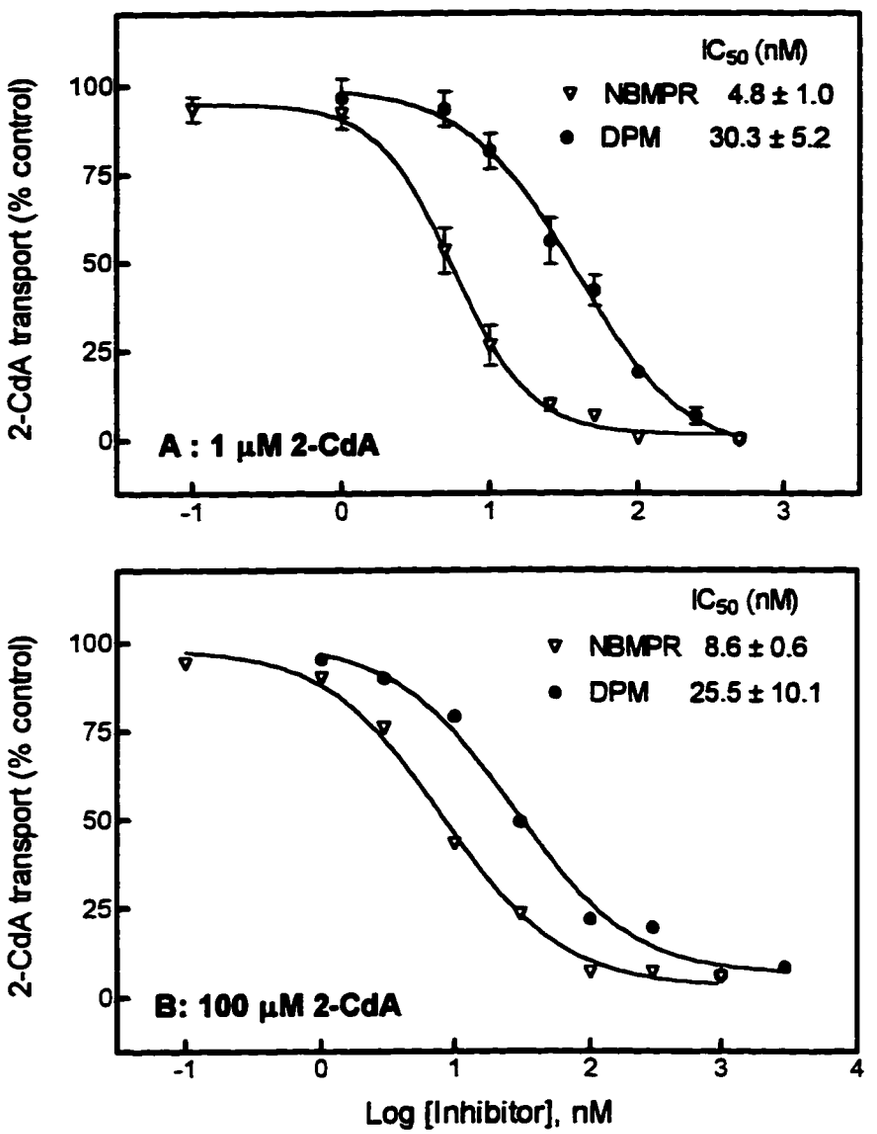

Figure 16. Concentration-effect plots of inhibition by NBMPR (∇) and DPM (●) of 2-CdA transport (1 μM and 100 μM in Panels A and B, respectively) in SB cells.

The data in Panel A are means of rates of inward transport of [^{3}H]2-CdA measured as unidirectional inward fluxes of 2-CdA as described in Fig. 3. Panel B shows rates of 2-CdA inward transport measured as described in Fig. 4. The control values (100 %) represent transport rates measured in cells suspended in medium without inhibitors. The initial rates of 1 μM and 100 μM influx in control (untreated) cells were 0.36 ± 0.01 pmol/10^6cells/sec and 7.87 ± 0.19 pmol/10^6cells/sec, respectively. Data in Panel A are means ± SEM obtained from four separate experiments; data in Panel B represent the means of two separate experiments. Each experiment was performed in triplicate.

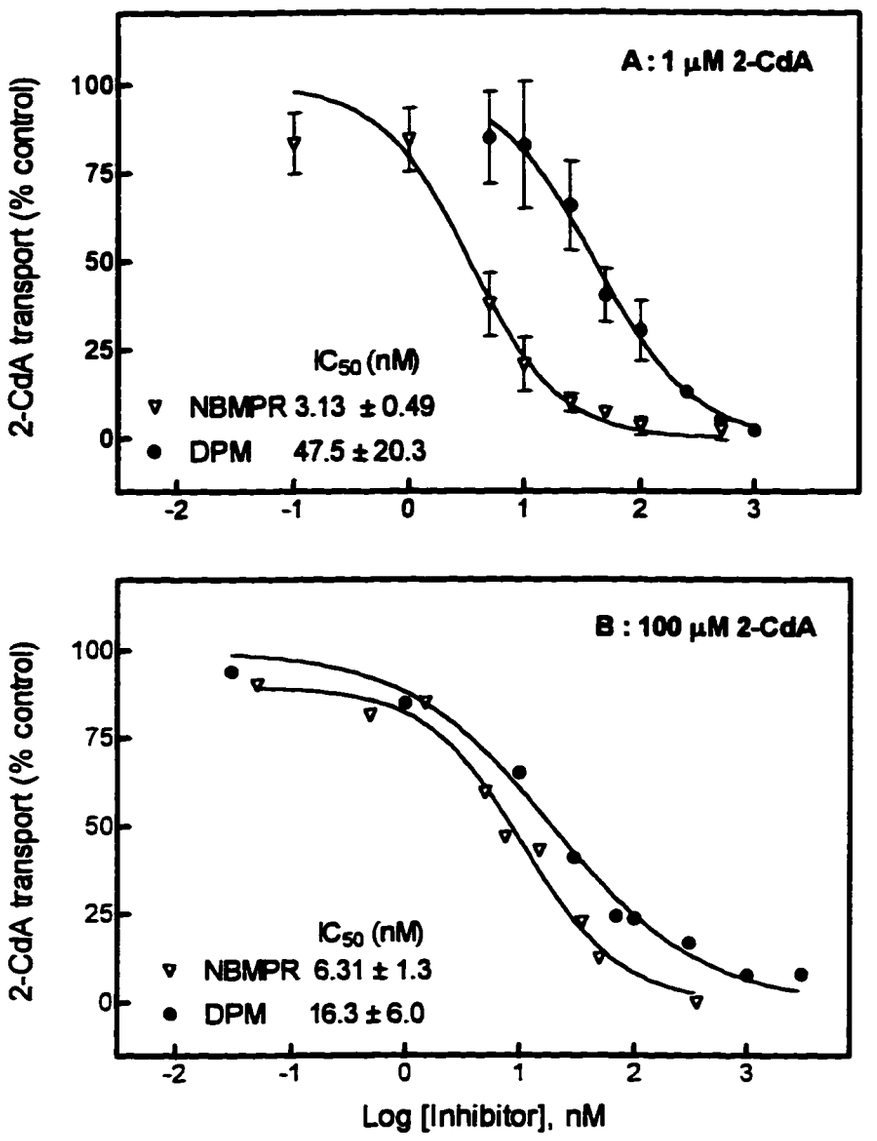

Figure 17. Concentration-effect plots of inhibition by NBMPR (∇) and DPM (●) of 2-CdA transport (1 μM and 100 μM in Panels A and B, respectively) in KM3-2B cells.

The data in Panel A are means of rates of inward transport of [^{3}H]2-CdA measured as unidirectional inward fluxes of 2-CdA as described in Fig. 3. Panel B shows rates of 2-CdA inward transport measured as described in Fig. 4. The control values (100 %) represent transport rates measured in cells suspended in medium without inhibitors. The initial rates of 1 μM and 100 μM influx in control (untreated) cells were 0.38 ± 0.02 pmol/10^6cells/sec and 7.34 ± 0.32 pmol/10^6cells/sec, respectively. Data in Panel A are means ± SEM obtained from 3-4 experiments; points in Panel B report results from one (∇) or two (●) experiments. Each experiment was performed in triplicate.

were monophasic (see Figs. 15A-17A), suggesting that the *es* transporter was the sole NT process contributing to 2-CdA influx in these cell lines. A biphasic inhibition of permeant fluxes by NBMPR has previously been used to illustrate the presence of both the *es* and the *ei* transport processes in lymphoid and myeloid cell lines (209,247). Table 10 summarizes the inhibition of 2-CdA uptake by NBMPR and DPM in cells of the four ALL lines tested. NBMPR was a potent inhibitor of 1 μM 2-CdA influx, with an IC_{50} value of approximately 4 nM for inhibition of 2-CdA uptake in the four ALL cell lines. This result was consistent with reported IC_{50} values for inhibition of uptake of several nucleoside permeants by NBMPR (256,257). As expected (168), NBMPR was a more potent inhibitor of 2-CdA influx than DPM ($p < 0.01$). IC_{50} values for the DPM inhibition of 1 μM 2-CdA uptake ranged from 30 to 50 nM (Table 10).

The experiments of Figs. 15B-17B measured the effects of graded concentrations of DPM and NBMPR on the uptake of 100 μM 2-CdA in Reh-A2, SB and KM3-2B cell lines, respectively. In these experiments, the transport rates for 100 μM 2-CdA uptake were determined differently (2.3.2.2) from the earlier inhibition studies with 1 μM 2-CdA uptake (2.3.2.1). Fig. 4 shows an example of early time courses of 100 μM 2-CdA uptake in the absence or presence of graded concentrations of NBMPR in KM3-2B cells. The uptake of 100 μM 2-CdA in either the absence or presence of NBMPR was linear between 3 and 9 sec and, therefore, rates of 2-CdA transport determined by linear regression analysis of 2-CdA influx over this interval were evidently initial rates.

Table 10

Inhibition of inward fluxes of 2-CdA by the NT inhibitors, NBMPR and DPM

Initial rates of 1 μM [^{3}H]2-CdA uptake (that is, inward fluxes of 1 μM [^{3}H]2-CdA) by cells of the cultured ALL lines were estimated from differences in the lymphoblast content of [^{3}H]2-CdA after exposure of the cells to the ^{3}H-permeant for intervals of zero sec and 3 sec. Transport rates were expressed as percentages of 2-CdA transport rates measured in the absence of inhibitors (2.3.2.1). Initial rates of 100 μM 2-CdA uptake were determined from linear time courses (3-9 sec) of 2-CdA uptake and were expressed as a percentage of 100 μM 2-CdA transport rates in the absence of inhibitors (2.3.2.2). IC_{50} values were determined from plots of 2-CdA uptake (% control) *versus* concentration of inhibitor.

	IC_{50} values (nM) for the inhibition of inward fluxes of 2-CdA, measured under these conditions:			
	1 μM 2-CdA[a]		100 μM 2-CdA[b]	
Cell Line	NBMPR	DPM	NBMPR	DPM
Reh-A2	3.2 ± 1.1	44.2 ± 9.7	1.38 ± 1.0	36.8 ± 5.5
SB	4.8 ± 1.0	30.0 ± 5.2	8.6 ± 0.6	25.5 ± 10.1
CEM	4.3 ± 0.6	37.5 ± 8.9	nd	nd
KM3-2B	3.2 ± 0.5	47.5 ± 20.3	6.3 ± 1.3	16.3 ± 6.0

nd, not determined

[a]IC_{50} values represent means ± SEM of data obtained from three to four experiments (each experiment was performed in triplicate).

[b]IC_{50} values represent the mean ± range of data obtained from two experiments (each was performed in triplicate).

The concentration-effect relationships for inhibition of 2-CdA (100 μM) transport by NBMPR and DPM in Reh-A2, SB and KM3-2B cells were monophasic (Figs. 15B-17B), and similar to inhibition of 1 μM 2-CdA transport by these agents (Figs. 15A-17A). The monophasic NBMPR-inhibition plots, which were found with both 2-CdA concentrations, supported conclusions from NT phenotype recognition experiments to the effect that *ei* transporter activity does not contribute significantly to 2-CdA uptake in these cell lines. The experiment of Fig. 15B shows that 1 μM NBMPR and 1 μM DPM were required to inhibit 100 μM 2-CdA influx in Reh-A2 cells. Similarly, 500 nM NBMPR and 3 μM DPM were required to inhibit 100 μM 2-CdA transport in KM3-2B cells (Fig. 16B).

In summary, NT phenotype studies and NBMPR inhibition studies shown in Figs. 15-17 support the conclusion that the *es* transporter is primarily responsible for the uptake of 2-CdA in Reh-A2, SB, CEM and KM3-2B cells. However, it is possible that, with different permeants, nucleoside transport processes other than the *es* process may be detected in these cell lines.

3.1.6 NT processes in lymphoblasts from five pediatric ALL patients.

An aim of this study was to identify and characterize in lymphoblasts from pediatric ALL patients, the processes responsible for 2-CdA uptake. However, measurement of nucleoside fluxes typically requires large numbers of cells (10^6 per assay sample tube) and as cell numbers harvested from patient samples were low, these experiments were conducted with cells from only five patients. Earlier studies in this laboratory identified *es*, *ei* and *cs* NT processes in leukemic cells from CLL and AML

patients, and it was apparent that expression of these processes and their relative activities varied remarkably from patient to patient (210).

The experiments of Figs. 18A and 18B showed that in lymphoblasts from a Pre-B ALL patient (H.D.) and from a T-ALL patient (R.R.), rates of inward transport of 2-CdA (2 μM) were essentially the same in sodium medium (DPBS) and in medium without sodium ions (DNMG). These experiments also showed that the inward flux of 2-CdA was blocked by 50 nM NBMPR or by 100 nM DPM. Thus, in the lymphoblasts from these two patients, properties of the observed 2-CdA fluxes indicate that the *es* transporter alone was responsible for the cellular uptake of 2-CdA.

Shown in Figs. 19A and 19B are short time courses of [^{3}H]2-CdA (2 μM) uptake in leukemic cells from Pre-B ALL patient (J.H.) and from T-ALL patient (E.P.), respectively. The data of Fig. 19A show that 2-CdA uptake was higher in Na^+ medium (DPBS) than in Na^+-deficient medium (DNMG), suggesting that a Na^+-dependent process was contributing to 2-CdA influx. Because NBMPR inhibited 2-CdA uptake, this component of 2-CdA uptake was attributed to the *cs* NT process (210). However, approximately 20% of 2-CdA uptake was not inhibited by 50 nM NBMPR (Fig. 19A), a result that could mean that the concentration of NBMPR was too low to block *es* activity fully. As well, Fig. 19A data show that 2-CdA uptake was greater in the presence of 100 nM DPM than in the presence of 50 nM NBMPR. It was concluded that lymphoblasts from Patient J.H. expressed at least two NT processes, the *es* and the *cs* transport systems. The uptake of 2-CdA was linear over the 30-sec incubation period (Fig. 19A), suggesting perhaps that internalized 2-CdA was metabolized and did not participate in transport equilibria and that 2-CdA

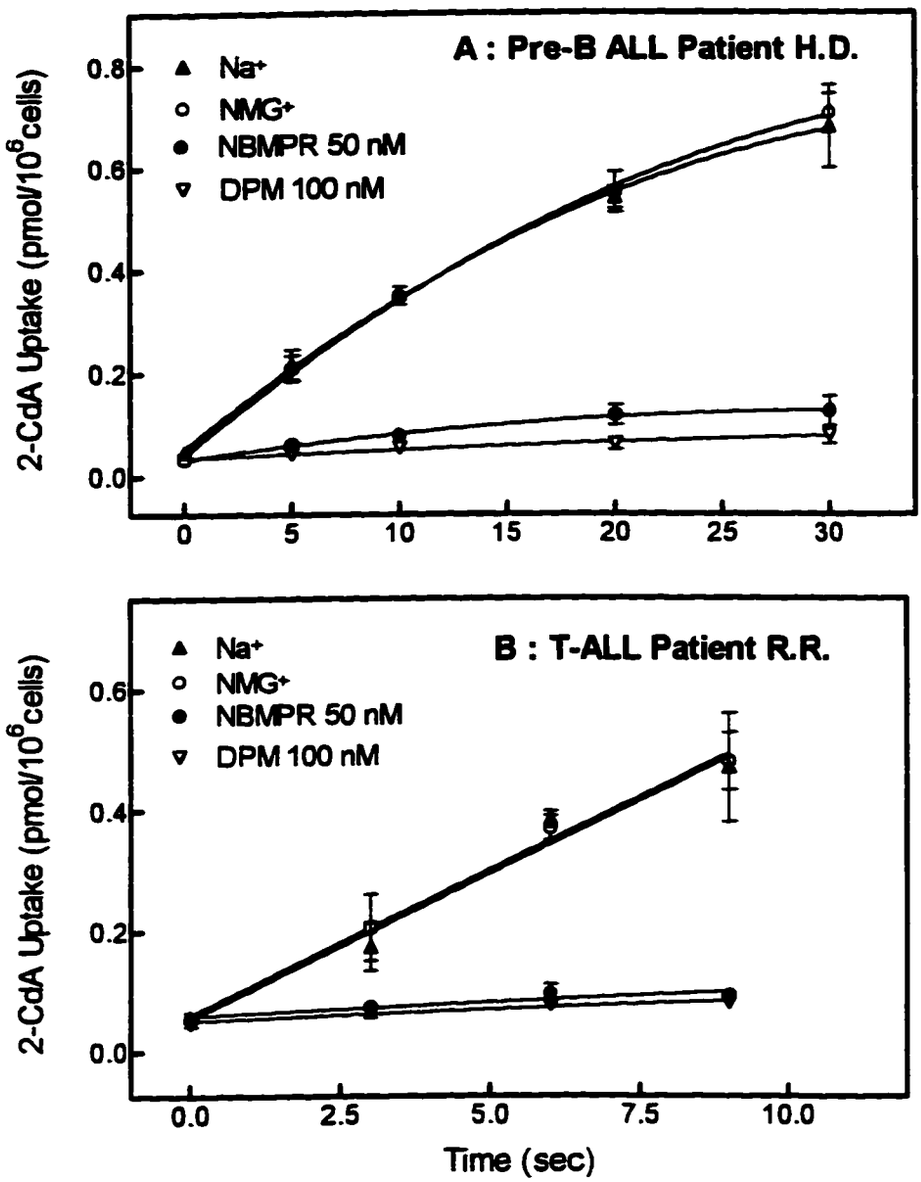

Figure 18. Time courses of 2-CdA uptake in lymphobalsts from Pre-B ALL Patient H.D. (Panel A) and from T-ALL Patient R.R. (Panel B): Effects of Na+ substitution and NT inhibitors.

Assay mixtures contained 2 x 10^6 cells in 200 µl (final volume) of Na^+ medium (DPBS) without inhibitors (▲), or with 50 nM NBMPR (●), or with 100 nM DPM (▽), or in NMG^+ medium (DNMG) (○). Uptake of 2 µM [^{3}H]2-CdA was measured at 37°C at the indicated time intervals; cells were exposed to inhibitors for 10 min prior to assays of 2-CdA uptake. The data are means ± SD of triplicate determinations from a single experiment; where absent, error bars (SD) are smaller than the data symbol.

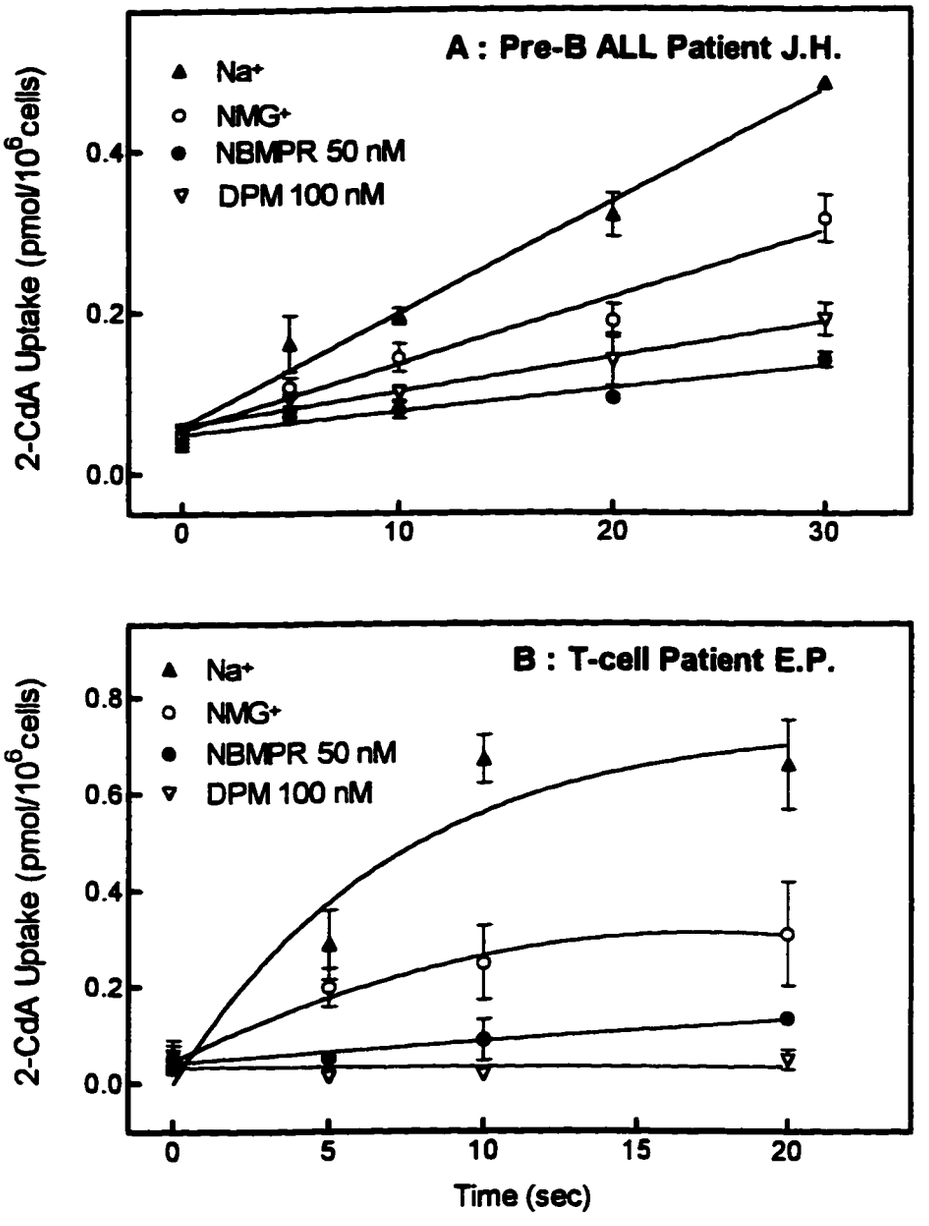

Figure 19. Na^+-linked uptake 2-CdA in lymphoblasts from Pre-B ALL Patient J.H. (Panel A) and from T-ALL Patient E.P. (Panel B).
Assay mixtures contained 2 x 10^6 cells in 200 µl (final volume) of Na^+-replete medium (DPBS) without inhibitors (▲), or with 50 nM NBMPR (●), or with 100 nM DPM (▽) , or in Na^+-deficient medium (DNMG) (○). Uptake of 2 µM [^{3}H]2-CdA was measured at 37°C at the indicated time intervals; cells were exposed to inhibitors for 10 min prior to assays of 2-CdA uptake. Data points are the means ± SD of triplicate determinations from a single experiment.

metabolites were retained by cells during this period.

The experiment of Fig. 19B describes 2-CdA uptake in lymphoblasts from T-ALL patient, E.P. Second order polynomial equations were fitted to time courses of 2-CdA uptake in cells from this patient. After a 30-sec interval, the uptake curve plateaued, suggesting that transport equilibria and/or efflux processes for 2-CdA or its metabolites were influencing the 2-CdA content of the leukemia cells. The initial rate of 2-CdA influx was measured as the slope of the tangent to the time course curve at T_{zero}. The difference between 2-CdA uptake in cells in sodium-replete and in sodium-deficient medium may be attributed to expression of the *cs* system, since 2-CdA uptake in Na^+-replete medium was blocked significantly in the presence of NBMPR. Complete inhibition of 2-CdA influx by 100 nM DPM, shows that in lymphoblasts from this patient (E.P.), 2-CdA uptake was mediated entirely by NT processes. The influx of 2-CdA was greater in the presence of NBMPR than in DPM-containing medium. This component of uptake may be attributable to expression of the *ei* transporter in cells from this patient. However, it is also possible, that the 50 nM NBMPR concentration was too low to fully inhibit *es* activity. Therefore, the conditions of this experiment allow no conclusion about the expression or absence of *ei* NT activity in this patient's lymphoblasts.

Fig. 20 demonstrates the transport of 2-CdA in leukemic cells from B-ALL patient, D.G. A difference between the uptake of 2-CdA in sodium-replete and sodium-deficient medium was observed after a 20-sec uptake interval. The difference between the uptake of 2-CdA in the presence of NBMPR and DPM may be due to the *ei* process. However, as discussed for Patient E.P., because the concentration of

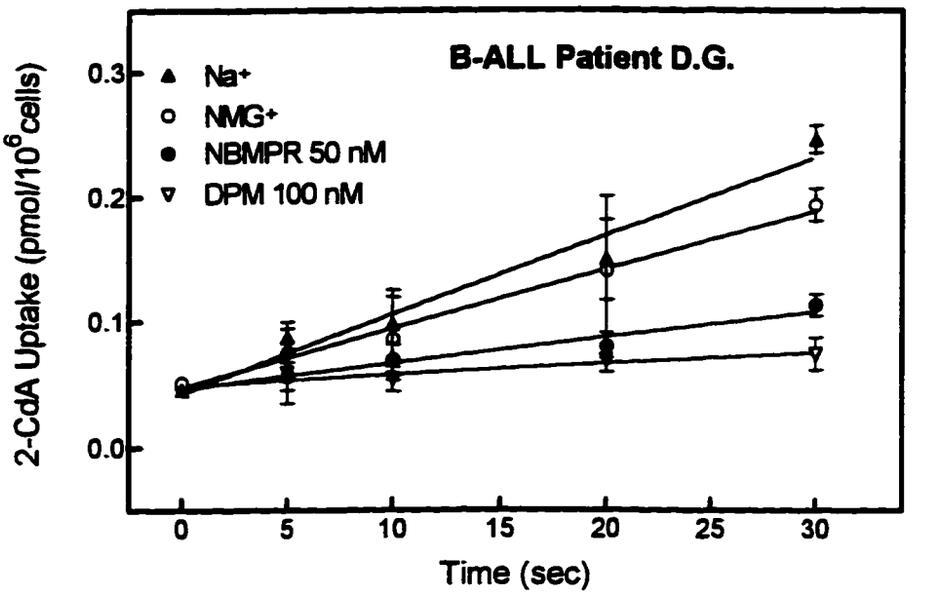

Figure 20. Uptake 2-CdA in lymphoblasts from Pre-B ALL Patient D.G: Effects of Na^+ substitution and NT inhibitors.
Assay mixtures contained 2 x 10^6 cells in 200 µl (final volume) of Na^+-replete medium (DPBS) without inhibitors (▲), or with 50 nM NBMPR (●), or 100 nM DPM (∇), or in Na^+-deficient medium (DNMG) (○). Uptake of 2 µM [^{3}H]2-CdA was measured at 37°C at the indicated time intervals; cells were exposed to inhibitors for 10 min prior to assays of 2-CdA uptake. Data points are means ± SD of triplicate determinations from a single experiment.

NBMPR required to completely inhibit uptake of 2-CdA via the *es* process was not determined for cells from this patient, conclusions regarding *ei* activity cannot be reached.

In summary, the *es* NT system was expressed in cells from all five patients. The relative contribution to uptake by the *es* NT process could not be determined quantitatively, as conditions for the demonstration of the *ei* process in the presence of *es* NT activity were not established. Therefore, detection of 2-CdA uptake by other NT processes may have been compromised. In two of the five patients tested the *cs* transporter was expressed, confirming previous observations that several NT processes may be expressed in fresh leukemic cells (210).

Initial rates of 2-CdA uptake in sodium-replete medium differed by approximately 7-fold between patient samples, ranging from 0.007 pmol/10^6cells/sec to 0.05 pmol/10^6cells/sec. White *et al.* (151) have reported that at concentrations below 1 μM the rate of araC accumulation in freshly isolated human acute leukemia cells was determined by the transport rate. Earlier studies by Kufe *et al.* (152,240) have also shown a correlation between araCTP accumulation, its incorporation into DNA and cytotoxicity of araC in human leukemia cells. It is possible that differences in the sensitivity to 2-CdA of lymphoblasts from pediatric ALL patients may derive in part from differences in their abilities to transport 2-CdA.

NT phenotype recognition experiments conducted in patient samples in this study highlight the difficulties in characterization of the multiple NT processes that may be expressed in lymphoblast samples. First, it is important to determine the conditions that will demonstrate each process expressed. Second, current methodology requires

large cell samples to compensate for low levels of ^{3}H-permeant acquired in cell samples during the short intervals of permeant uptake that are an intrinsic part of the initial rate strategy in measuring nucleoside fluxes. With the recent cloning of several NT proteins, it has been suggested that hybridization techniques may be useful for screening patient cells to characterize NT protein expression, as such techniques would typically require very few cells. However, hybridization technology would not determine whether a NT protein was functionally expressed in the plasma membrane, or whether it participated in the uptake of a particular nucleoside drug. Therefore, current functional assays for recognizing NT phenotypes must be improved to permit the use of smaller cell numbers per assay, because alternative assays for the characterization of NT processes in cells from patients are currently not available.

3.1.7 Site-specific binding of NBMPR to cell lines of ALL origin

NBMPR binds with high affinity to the *es* transporter (K_D < 1 nM), but the site of binding has not yet been identified. Site-specific binding of [^{3}H]NBMPR has been used for enumeration of these transport proteins in cells (173,193,194). The experiment of Fig. 21 measured the site-specific equilibrium binding of NBMPR to Reh-A2 cells. This experiment showed that the binding of NBMPR to Reh-A2 cells was saturable, and that NBMPR is bound to a single class of sites, as is evident from a Scatchard plot of the binding data (Fig. 21B). Mass law analysis of the data yielded the following constants: K_D, 0.4 ± 0.03 nM, and B_{max}, 4.1 ± 0.6 × 10^{4}sites/cell.

Similar experiments with SB, CEM and KM3-2B cell lines are summarized in Fig. 22 and Table 11. The NBMPR binding constants for these three cell lines were

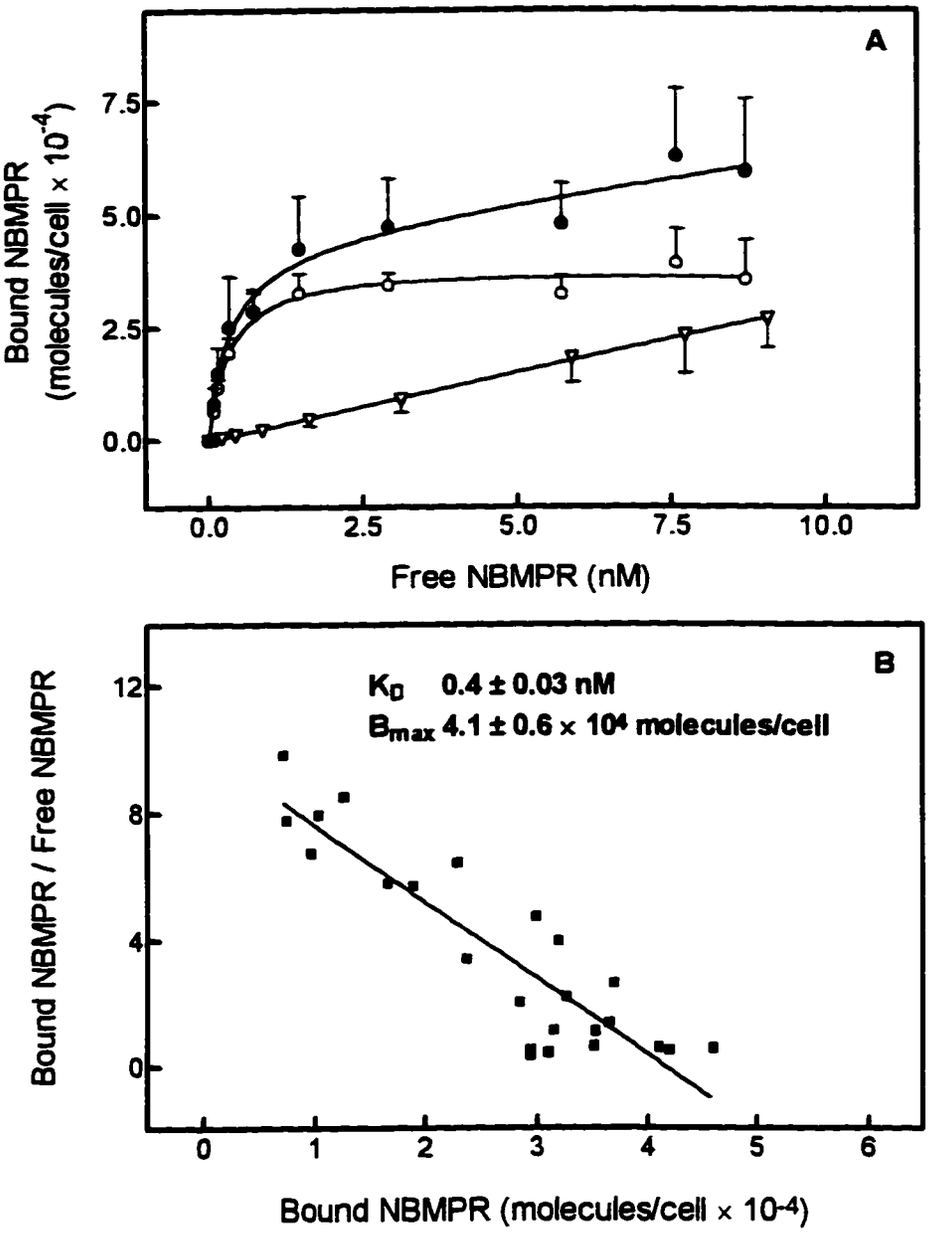

Figure 21. Binding of [^{3}H]NBMPR to Reh-A2 cells.
Panel A: Equilibrium binding of [^{3}H]NBMPR to Reh-A2 cells. Cells were incubated at 37°C with graded concentrations of [^{3}H]NBMPR in DPBS for 30 min. Cell content of [^{3}H]NBMPR was measured in the absence (●) or presence (∇) of 5 μM non-isotopic NBMPR. The specific binding data (○) represent the difference between the total (●) and nonspecific (∇) binding for each point. Data are means ± SD from three separate experiments.
Panel B: Scatchard analysis of the NBMPR binding data from three separate experiments (data pooled). Mass law analysis of specific binding data from these experiments yielded B_{max} and K_D values of $4.1 \pm 0.6 \times 10^4$ sites/cell and 0.4 ± 0.03 nM, respectively.

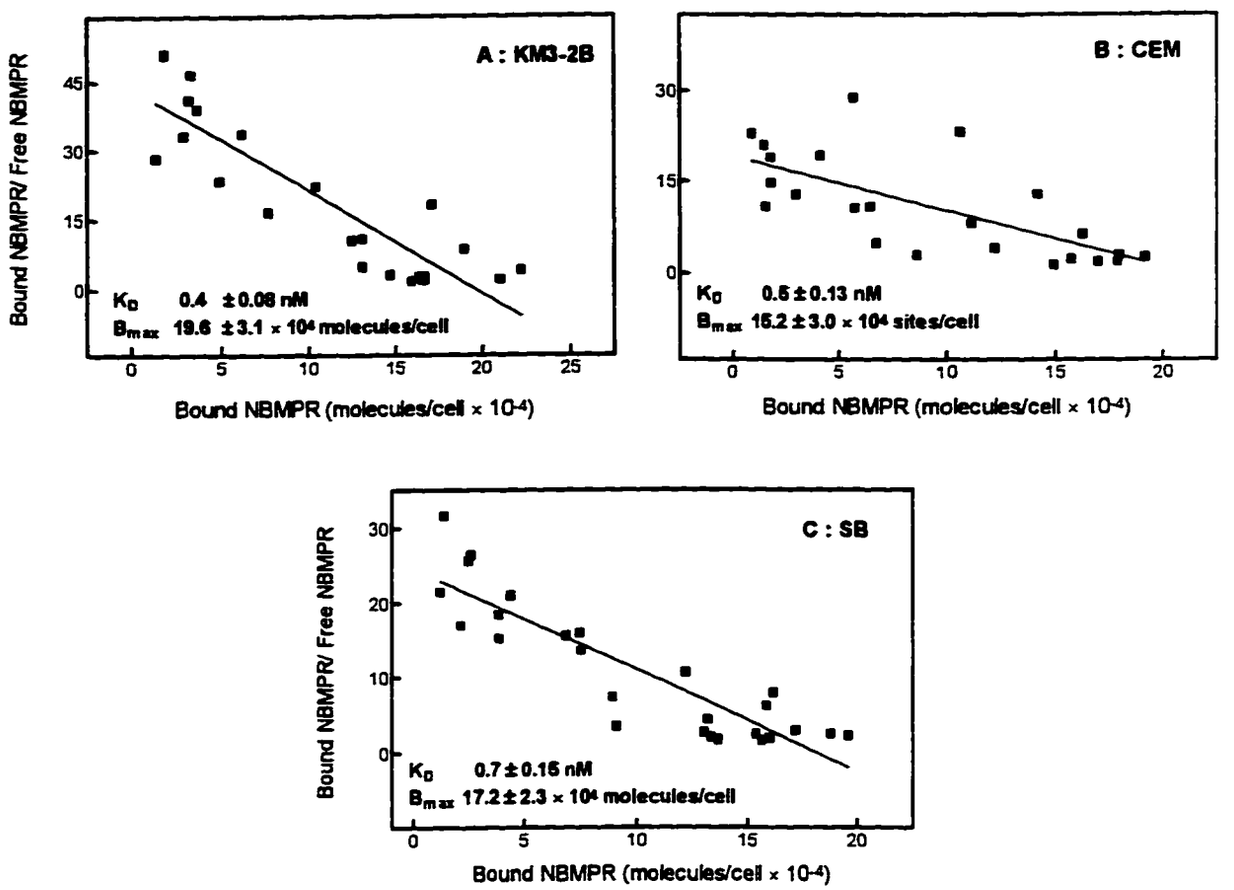

Figure 22. Binding of [^{3}H]NBMPR to cultured ALL cells.
NBMPR binding site content was determined in KM3-2B cells (Panel A), CEM cells (Panel B) and SB cells (Panel C). Cells were incubated at 37°C with graded concentrations of [^{3}H]NBMPR in DPBS for 30 min. Cell content of [^{3}H]NBMPR was measured in the absence or presence of 5 μM non-isotopic NBMPR to determine total and nonspecific binding of NBMPR, respectively. Site-specific binding was determined as the difference between the total and nonspecific binding of NBMPR. The data were subjected to mass law analysis by the method of Scatchard to yield the B_{max} and K_D values. The data presented in each panel are the pooled results of 3 separate experiments.

Table 11

Binding of [^{3}H]NBMPR to cultured human ALL lymphoblasts

Cell line	B_{max} (sites/cell)	K_D (nM)
Reh-A2	$4.1 \pm 0.6 \times 10^4$	0.4 ± 0.02
SB	$17.2 \pm 2.3 \times 10^4$	0.7 ± 0.15
CEM	$15.2 \pm 3.0 \times 10^4$	0.5 ± 0.13
KM3-2B	$19.6 \pm 3.1 \times 10^4$	0.4 ± 0.08

The binding constants B_{max} and K_D were calculated from plots of equilibrium binding data transformed according to the method of Scatchard. Values represent means ± SEM of data obtained from three experiments.

similar, with B_{max} values ranging from 15.2 - 19.6 × 10^4 sites/cell. The B_{max} value obtained for CEM cells closely agreed with a value previously reported for this cell line (151). However, Young *et al.* (235) have reported that CEM cells express 3-fold fewer sites than reported in the present study. The K_D value for NBMPR binding in all cell lines was approximately 1 nM, a value typically observed in cells expressing the *es* transporter (157). This result showed the high affinity of NBMPR for the *es* transporter in the four cell lines examined in this study. However, Reh-A2 cells expressed about one fourth as many *es* sites as SB, CEM and KM3-2B cells. A possible explanation for this may include the smaller size of Reh-A2 cells compared to the other three cell types (Table 12).

3.1.8 Flow cytometric enumeration of NBMPR-binding sites in lymphoblasts from pediatric ALL patients

The number of NBMPR-binding sites in lymphoblasts from adult ALL patients has been reported to be low relative to NBMPR site content of cultured leukemia cells of human origin. A study by White *et al.* (151) reported approximately 200-8000 NBMPR-binding sites/cells in lymphoblasts from adult ALL patients at initial diagnosis. However, the number of *es* NT sites on lymphoblasts from pediatric ALL patients has not been previously reported. The present study utilized a flow cytometric (FCM) technique, employing 5-(SAENTA-x8)-fluorescein (5-(Sx8)-F), a fluorescent probe for the NBMPR binding site on the *es* transporter, to determine the abundance of the *es* NT sites on lymphoblasts from pediatric ALL patients. A previous study has shown a high correlation between the NBMPR site content of lymphoblasts measured by the

Table 12

Intracellular water volumes of four ALL cell lines

Intracellular water volumes in cell pellets were estimated by subtracting the extracellular space (estimated by measuring either [^{3}H]polyethylene glycol or [^{14}C]sucrose uptake during a 3-sec interval) from the total pellet water (determined by measuring [^{3}H]H_2O uptake during 3 sec).

Cell line	Intracellular water volume ($\times 10^{-6}$ µl/cell)
Reh-A2	0.68 ± 0.03[1]
SB	1.12 ± 0.12
CEM	1.55 ± 0.1
KM3-2B	1.1 ± 0.1

[1]mean ± SEM of 7-12 determinations

[^{3}H]NBMPR binding assay and the FCM assay using 5-(Sx8)-F (195). A major advantage of the 5-(Sx8)-F / FCM evaluation of *es* site content over the radioligand binding assay is that FCM requires far fewer cells.

Results from this project and previous work by Belt *et al.* (209) have shown the *es* transporter to be the major NT process expressed in human leukemia cell lines. Because of these observations, it was expected that the measurement of the *es* site content of lymphoblasts from ALL patients would be a measure of the transport capabilities of these cells.

Shown in Fig. 23A (lymphoblasts from Pre-B ALL Patient S.C.) and in Fig. 24A (cultured Reh-A2 lymphoblasts) are fluorescence intensity *versus* frequency histograms representing the flow cytometric analysis of ALL cells with or without staining by the fluorescent, *es*-specific probe 5-(Sx8)-F. In the presence of 20 nM 5-(Sx8)-F, mean relative fluorescence intensity (mean RFI) histograms have shifted to the right, demonstrating the binding of the SAENTA-fluorescein conjugate to both cell populations (Figs. 23,24). Cell-associated fluorescence acquired at equilibrium with 20 nM 5-(Sx8)-F was blocked by prior treatment of the cells with 5 μM NBMPR, as in the instances of cells from Patient S.C. (Fig. 23B) and in Reh-A2 cells (Fig. 24B). As a consequence of the blockade of site-bound 5-(Sx8)-F (RFI histogram 2 in Figs. 23B and 24B) by prior treatment of cells with NBMPR, the 5-(Sx8)-F fluorescence histogram shifted to the left (RFI histogram 1 in Figs. 23B and 24B), resembling that of unstained cells (RFI histogram 1 in Figs. 23A and 24A).

The data of Fig. 25 illustrate the equilibrium binding of graded concentrations of 5-(Sx8)-F to cells from Pre-B ALL Patient R.L. as determined by flow cytometry.

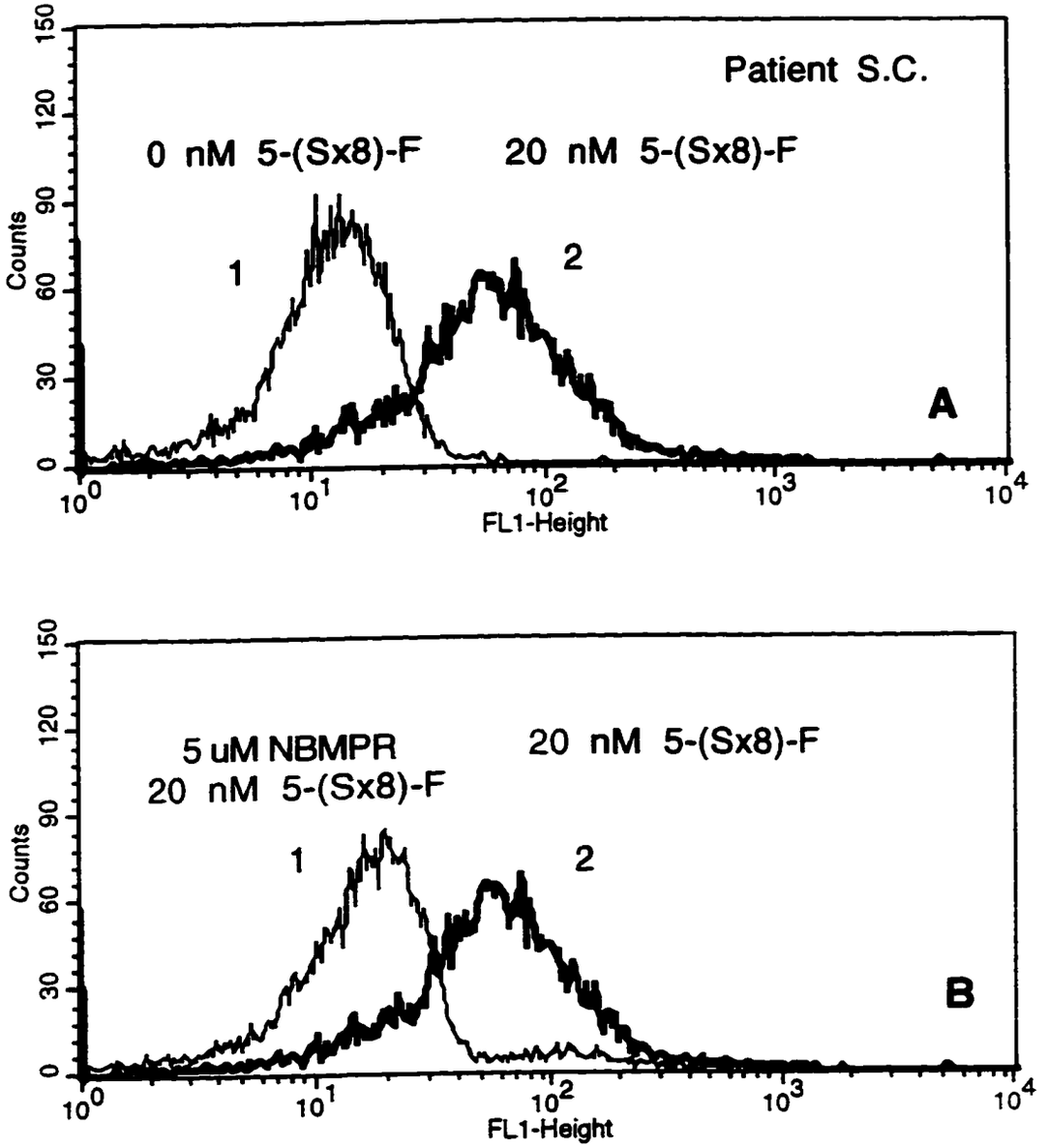

Figure 23. Flow cytometric analysis of lymphoblasts from Pre-B ALL Patient S.C. after staining with the *es*-specific fluorescent probe 5-(SAENTA-x8)-fluorescein.

Patient lymphoblasts were incubated with 20 nM 5-(Sx8)-F at 22°C for 30 min in the absence (Histogram 2 in Panels A and B), or in the presence (Histogram 1 in Panel B) of 5 μM NBMPR. Prior treatment with NBMPR blocked binding of 5-(Sx8)-F to lymphoblasts from Patient S.C. Histograms of cell-associated fluorescence were obtained by flow cytometric analysis of the stained cells with excitation at 488 nm and emission collected at 530 nm. Each fluorescence histogram represents analysis of 10,000 cells. The autofluorescence histogram (Histogram 1 in Panel A) was obtained with untreated (unstained) cells.

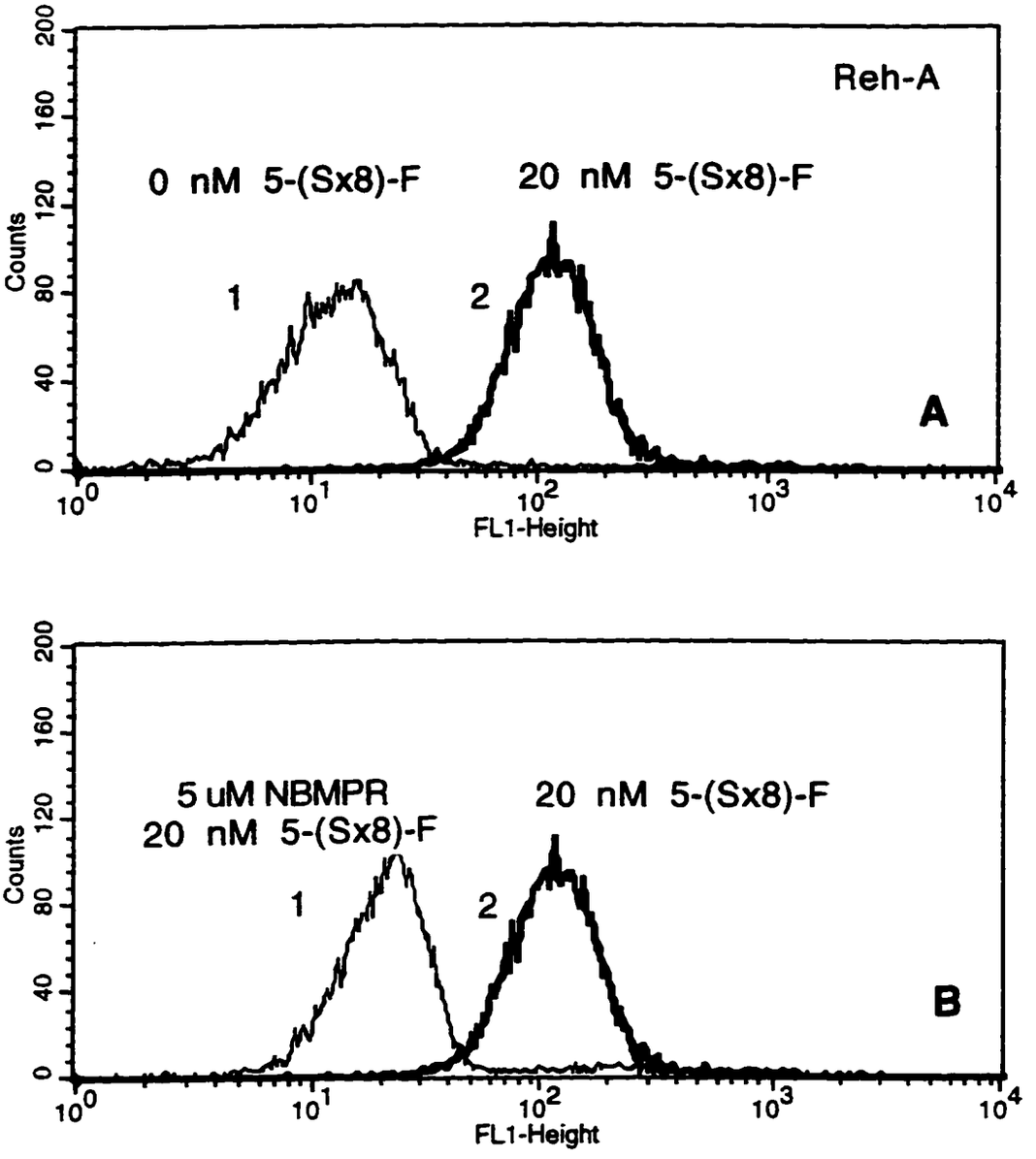

Figure 24. Flow cytometric analysis of Reh-A2 lymphoblasts after staining with the *es*-specific fluorescent probe 5-(SAENTA-x8)-fluorescein.
Cultured lymphoblasts were incubated with 20 nM 5-(Sx8)-F at 22°C for 30 min in the absence (Histogram 2 in Panels A and B), or in the presence (Histogram 1 in Panel B) of 5 μM NBMPR. Prior treatment (30 min) with NBMPR blocked binding of 5-(Sx8)-F to Reh-A2 cells. Histograms of cell-associated fluorescence were obtained by flow cytometric analysis of the stained cells with excitation at 488 nm and emission collected at 530 nm. Each fluorescence histogram represents analysis of 10,000 cells. The autofluorescence histogram (Histogram 1 in Panel A) was obtained with untreated (unstained) cells.

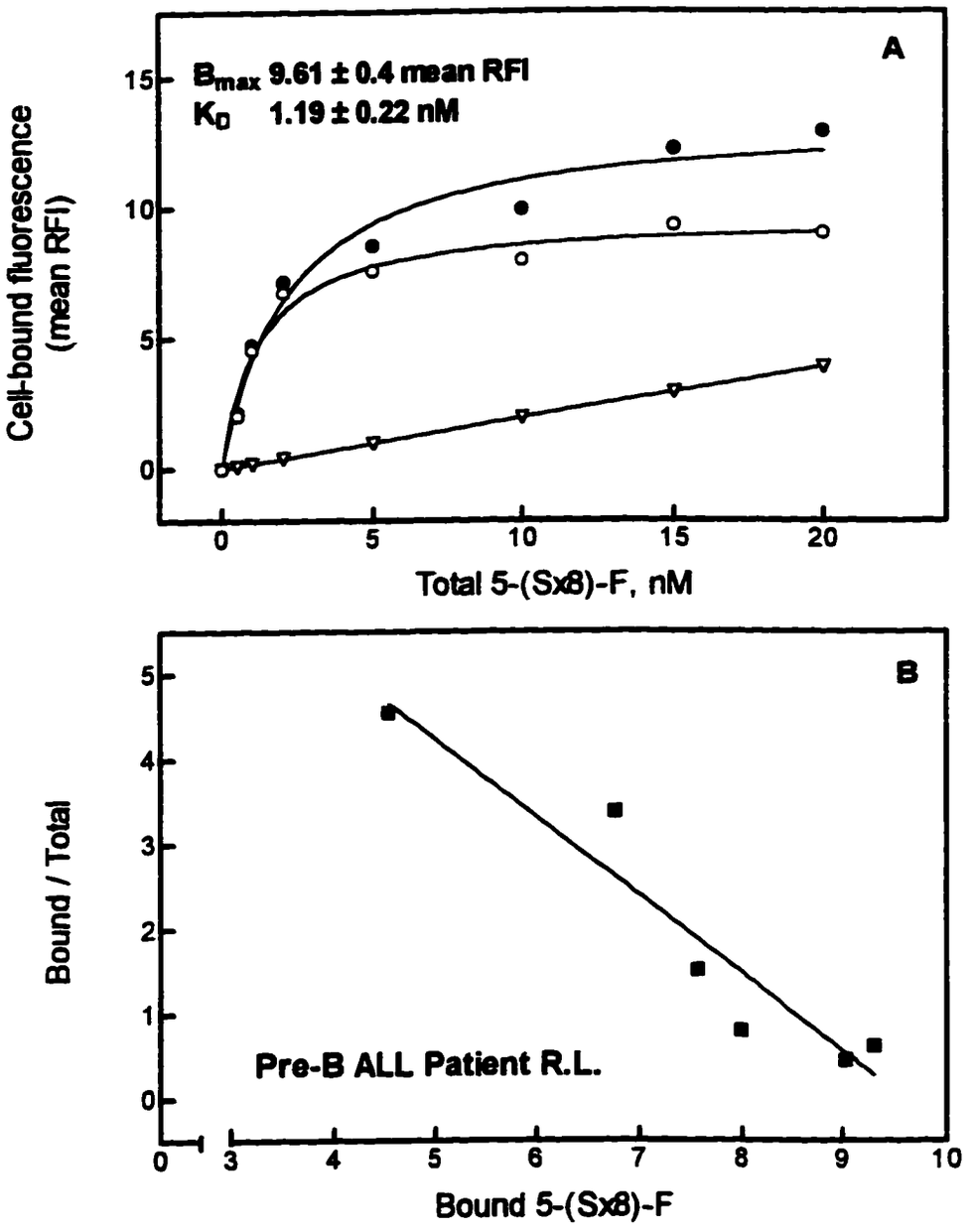

Figure 25. Binding of 5-(SAENTA-x8)-fluorescein to lymphoblasts from Pre-B ALL Patient R.L.

Panel A: Equilibrium binding of 5-(Sx8)-F to lymphoblasts from Pre-B ALL Patient R.L. Leukemic cells were incubated with graded concentrations of 5-(Sx8)-F for 30 min at 22°C in the absence (●) or presence (∇) of 5 μM NBMPR. Cell-associated fluorescence was determined by measuring the mean fluorescence intensity of 10,000 cells for each data point. The specific binding data (○) represents the difference between the total (●) and nonspecific (○) fluorescence. The curve fitted to specific 5-(Sx8)-F binding data yielded B_{max} and K_D values of 9.61 ± 0.4 mean RFI and 1.19 ± 0.22 nM, respectively.

Panel B: Scatchard analysis of the 5-(Sx8)-F binding data shown in Panel A.

The mean RFI value obtained from each flow histogram (RFI *versus* frequency of events), was a quantitative measure of bound 5-(Sx8)-F. Each mean RFI value was determined by measuring the fluoresence of 10,000 cells. This experiment showed that the binding of the SAENTA fluor was saturable and *es*-selective, in that bound 5-(Sx8)-F was reduced with prior treatment of cells with NBMPR. Nonspecific binding of 20 nM 5(Sx8)-F was 30% of the total signal in cells from this patient, after correction for autofluorescence. Mass law analysis of these binding data yielded the constants, K_D 1.19 ± 0.4 nM, and B_{max}, 9.61 ± 0.4 mean RFI units/cell.

Quantitation of *es* sites by flow cytometry requires a means to relate the cell associated 5-Sx8-F fluorescence signal to the cellular NBMPR binding site content, which is conventionally measured by the site-specific binding of [^{3}H]NBMPR. Others have used a method in which fluorescence signals were converted to "molecules of equivalent soluble fluorescence" (MESF) using a standard curve generated from observation in the flow cytometer of use, of the fluorescence of commercial polystyrene microspheres containing known amounts of fluorescein (195). Correlating "maximum cell associated fluorescence" (MESF) with maximum values obtained for the content of [^{3}H]NBMPR sites in human myeloid leukemia cells yielded a constant of 0.41 MESF per transporter site. In the present study, a factor of 200 was used to convert B_{max} mean RFI values to *es* nucleoside transporter sites (236). This factor was obtained with cultured leukemic cells, by comparing B_{max} values measured at 22°C by the FCM technique with B_{max} values determined by equilibrium binding of [^{3}H]NBMPR at 22°C in the same cells (236). Converting mean RFI values to NBMPR sites in cells from Patient R.L. yielded an approximate B_{max} value of 1920 ± 80 sites/cell.

Fig. 26A shows data for the site-specific binding of 5-(Sx8)-F to fresh leukemia cells from Pre-B ALL Patient S.C.; in these cells, the nonspecific binding of 20 nM 5-(Sx8)-F was 11% of the total signal after correction for autofluorescence. The B_{max} value obtained in cells from Patient S.C. was 40.6 ± 0.7 mean RFI units/cell (8120 NBMPR sites/cell); 5-(Sx8)-F was bound with high affinity to nucleoside transporter sites (K_D 1.74 ± 0.14 nM). Fig. 26B shows a mass law analysis (Scatchard plot) of the binding data of Fig. 26A; the linear plot indicates specific binding of 5-(Sx8)-F to a single population of high affinity sites on the leukemic blasts from this patient. Similar 5-(Sx8)-F binding data were obtained with lymphoblasts from a T-ALL patient (Fig. 27). Three of the 13 individuals listed in Table 13 were T-ALL patients; while this is a small sample, obvious differences in the ability of T-ALL patients or Pre-B ALL patients to bind 5-(Sx8)-F were not apparent (K_D values ranged from 0.82 ± 0.1 nM to 12.1 ± 2.5 nM).

Marked patient to patient differences were apparent in the maximum binding of 5-(Sx8)-F to lymphoblasts from pediatric ALL patients, as measured by flow cytometry (Table 13). The maximum cell-bound fluorescence (B_{max}) ranged from 2.83 to 40.6 mean RFI units/cell (566 to 8120 *es* sites/cell). This 20-fold difference in 5-(Sx8)-F binding capacity reflects a wide variation in *es* site abundance among fresh pediatric ALL lymphoblasts. A wide variation in *es* site expression was also observed in lymphoblasts from adult ALL patients and in myeloblasts from AML patients (151,236,258,259). In these studies, *es* expression was measured by either the conventional method which determined site-specific binding of [^{3}H]NBMPR (151,258,259), or by a flow cytometric method that used the *es*-specific stain 5-(Sx8)-F

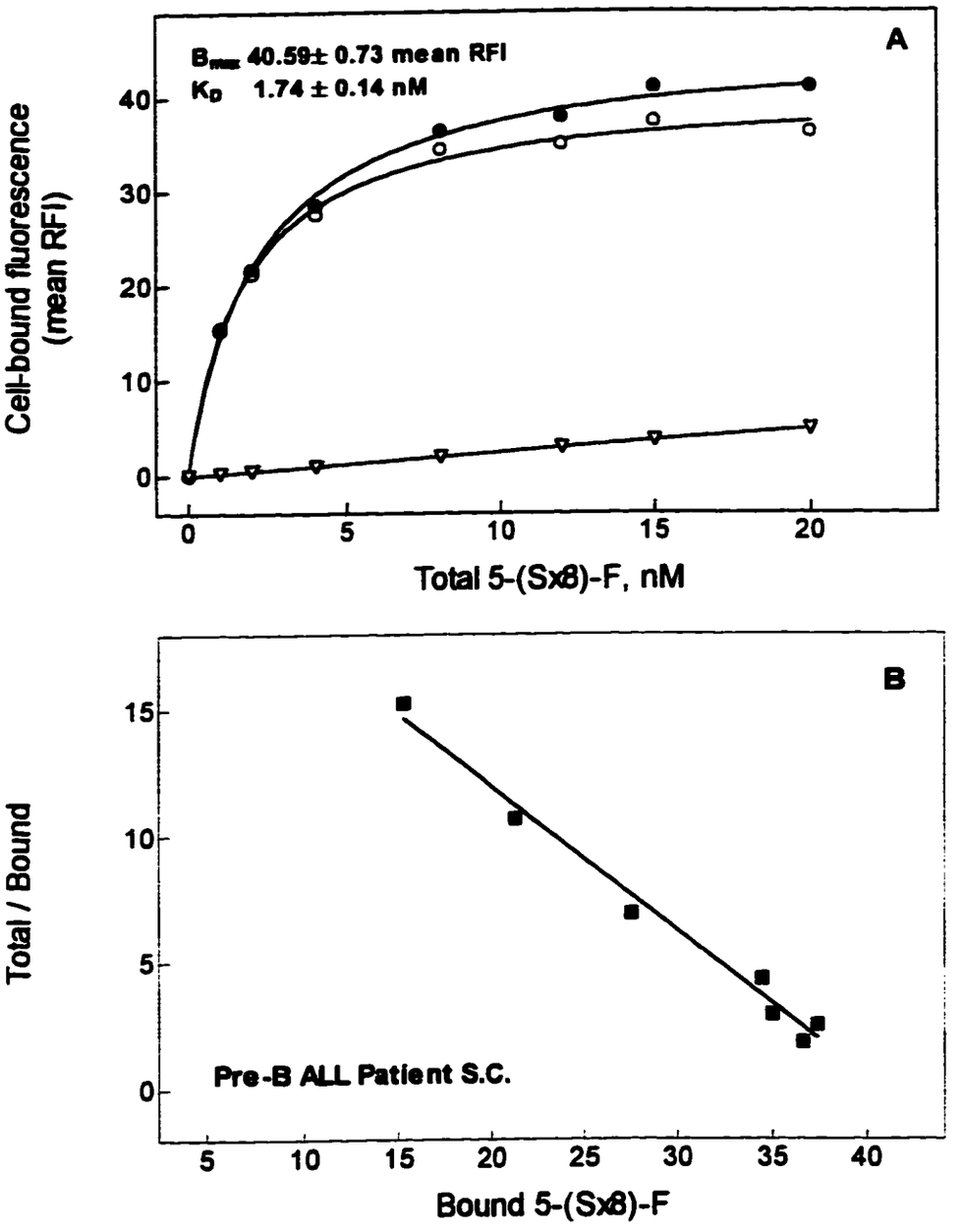

Figure 26. Binding of 5-(SAENTA-x8)-fluorescein to lymphoblasts from Pre-B ALL Patient S.C.

Panel A: Equilibrium binding of 5-(Sx8)-F to lymphoblasts from Pre-B ALL Patient S.C. Leukemic cells were incubated with graded concentrations of 5-(Sx8)-F for 30 min at 22°C in the absence (●) or presence (∇) of 5 μM NBMPR. Cell-associated fluorescence was determined by measuring the mean fluorescent intensity of 10,000 cells for each data point. The specific binding data (○) represents the difference between the total (●) and nonspecific (○) fluorescence. The curve fitted to specific 5-(Sx8)-F binding data yielded B_{max} and K_D values of 40.59 ± 0.73 mean RFI and 1.74 ± 0.14 nM, respectively.

Panel B: Scatchard analysis of the 5-(Sx8)-F binding data shown in Panel A

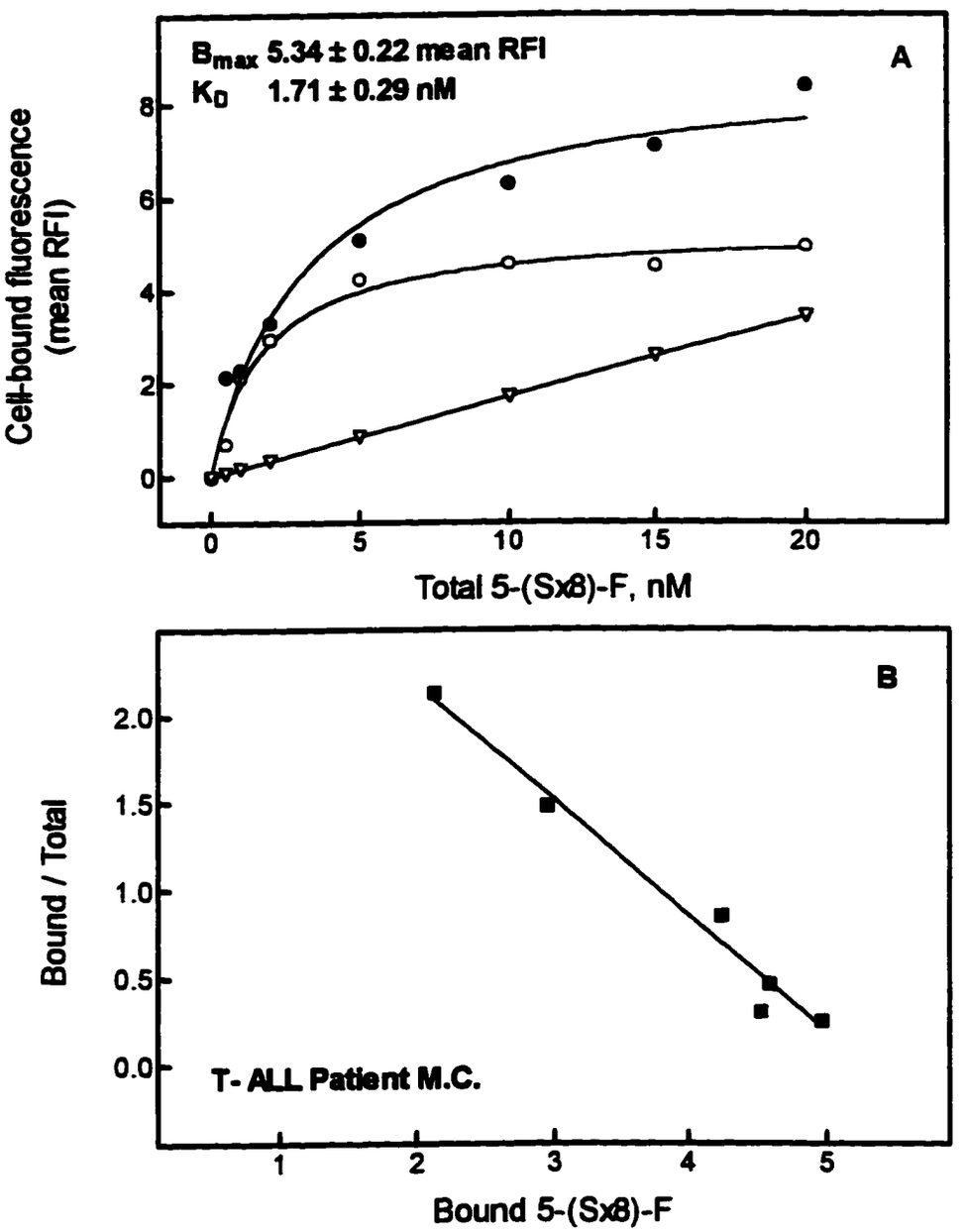

Figure 27. Binding of 5-(SAENTA-x8)-fluorescein to lymphoblasts from T-ALL Patient M.C.

Panel A: Equilibrium binding of 5-(Sx8)-F to lymphoblasts from T-ALL Patient M.C. Leukemic cells were incubated with graded concentrations of 5-(Sx8)-F for 30 min at 22°C in the absence (●) or presence (▽) of 5 μM NBMPR. Cell-associated fluorescence was determined by measuring the mean fluorescent intensity of 10,000 cells for each data point. The specific binding data (○) represents the difference between the total (●) and nonspecific (○) fluorescence. The curve fitted to specific 5-(Sx8)-F binding data yielded B_{max} and K_D values of 5.34 ± 0.22 mean RFI and 1.71 ± 0.29 nM, respectively.

Panel B: Scatchard analysis of the 5-(Sx8)-F binding data shown in Panel A.

Table 13

Characteristics for binding of 5-(SAENTA-x8)-fluorescein to leukemic lymphoblasts from children with ALL

Es nucleoside transporter expression is shown as the B_{max} value (mean RFI) for 5-(Sx8)-F binding determined from flow cytometric measurements and has also been converted to an *es* site content (*es* sites/cell) by means of a conversion factor of 200 (236).

	Equilibrium binding of 5-(Sx8)-F to lymphoblasts from ALL patients		
Patient	B_{max} (mean RFI)	B_{max} (*es* sites/cell)[c]	K_D (nM)
CC	2.83 ± 0.3[b]	566 ± 60	3.16 ± 0.8
JD	2.97 ± 0.2	594 ± 40	2.36 ± 0.5
MH	3.76 ± 1.9	752 ± 380	4.09 ± 4.6
JM	4.91 ± 0.6	982 ± 120	1.07 ± 0.8
MC[a]	5.34 ± 0.2	1070 ± 40	1.71 ± 0.3
NR	9.34 ± 0.8	1870 ± 160	5.08 ± 1.3
RL	9.61 ± 0.4	1920 ± 80	1.19 ± 0.2
GA	10.3 ± 0.6	2070 ± 120	1.51 ± 0.3
RA	17.0 ± 0.9	3410 ± 180	4.08 ± 0.6
ND	24.1 ± 1.9	4840 ± 380	7.98 ± 1.5
MB	30.8 ± 0.5	6160 ± 100	0.82 ± 0.1
RR[a]	32.0 ± 3.5	6400 ± 700	3.23 ± 1.1
BB[a]	33.3 ± 3.6	6670 ± 720	12.1 ± 2.5
SC	40.6 ± 0.8	8120 ± 160	1.74 ± 0.2

[a]T-ALL patients
[b]mean ± SE
[c]Calculated with a conversion factor of 200 (236)

(236). The K_D value for 5-(Sx8)-F binding to the *es* transporter in lymphoblasts in the present study ranged from 0.82 to 12.1 nM, showing that 5-(Sx8)-F binds with high affinity to the *es* transporter site in lymphoblasts from pediatric ALL patients. These values are higher than K_D values (0.1-1 nM) obtained for NBMPR binding to *es* transporter proteins in several cell types (Table 11) (157), suggesting NBMPR binds with greater affinity to the *es* nucleoside transporter than 5-(Sx8)-F.

3.2 Cytotoxicity of 2-CdA and araC in ALL cells

An aim of the present study was to compare the cytotoxicity of 2-CdA and araC toward leukemic lymphoblasts obtained at diagnosis from pediatric ALL patients. The sensitivity of the cells to 2-CdA and araC was measured by the *in vitro* tetrazolium dye (MTT) reduction assay. AraC was employed in this context of this study because; (a) like 2-CdA, it is a nucleoside and (b) this agent has current use in remission induction and consolidation protocols in ALL therapy (1,2,156). AraC has also been widely used as a prophylactic agent to reduce the possibility of CNS involvement in childhood ALL (1,12). The MTT assay was used in this study to investigate the *in vitro* drug sensitivity of fresh leukemic cells, since it had previously been shown that *in vitro* sensitivity data obtained with the MTT assay may be a good predictor of clinical outcome in pediatric ALL and acute non-lymphoblastic leukemia (260,261). Furthermore, a number of retrospective studies of chemotherapy in adult AML also found the MTT assay to be a good predictor of clinical outcome (262).

3.2.1 MTT assay conditions

In lymphoblasts from pediatric ALL patients a linear relationship was demonstrated between formation of the reduced dye product (formazan), measured colorimetrically as absorbance at 540 nm (A_{540}), and cell concentration. That linearity was found over a range of cell concentrations (10^4-10^6 cells per well) in four patient samples (Fig. 28). The ability of patient cells to survive in the microcultures in the absence of test agents was demonstrated for three samples from patients. The small size of most patient samples has meant that this analysis cannot be conducted with every patient sample. Leukemic cell survival in the microcultures, a determinant of assay sensitivity (263), was assessed colorimetrically by determining the concentration (A_{540}) of the formazan product formed before and after three days in culture. After three days, cell survival was 60, 93 and 224% of the time-zero value for leukemic lymphoblasts from patients R.L., M.C. and J.D., respectively. This difference in viability highlights the difficulties of culturing fresh ALL cells, since leukemic cells from some patients appear to survive poorly *in vitro*.

With cultured ALL cells the absorbance (A_{540}) of the reduced formazan product was linearly related to cell number in a range from 1×10^3 to 3×10^5 cells/well in all four of the ALL lymphoblasts lines (data not shown). These findings are similar to those observed for other lymphoblastic cell lines (264). Exponentially proliferating cultures were used in all 48 h and 72 h cytotoxicity experiments.

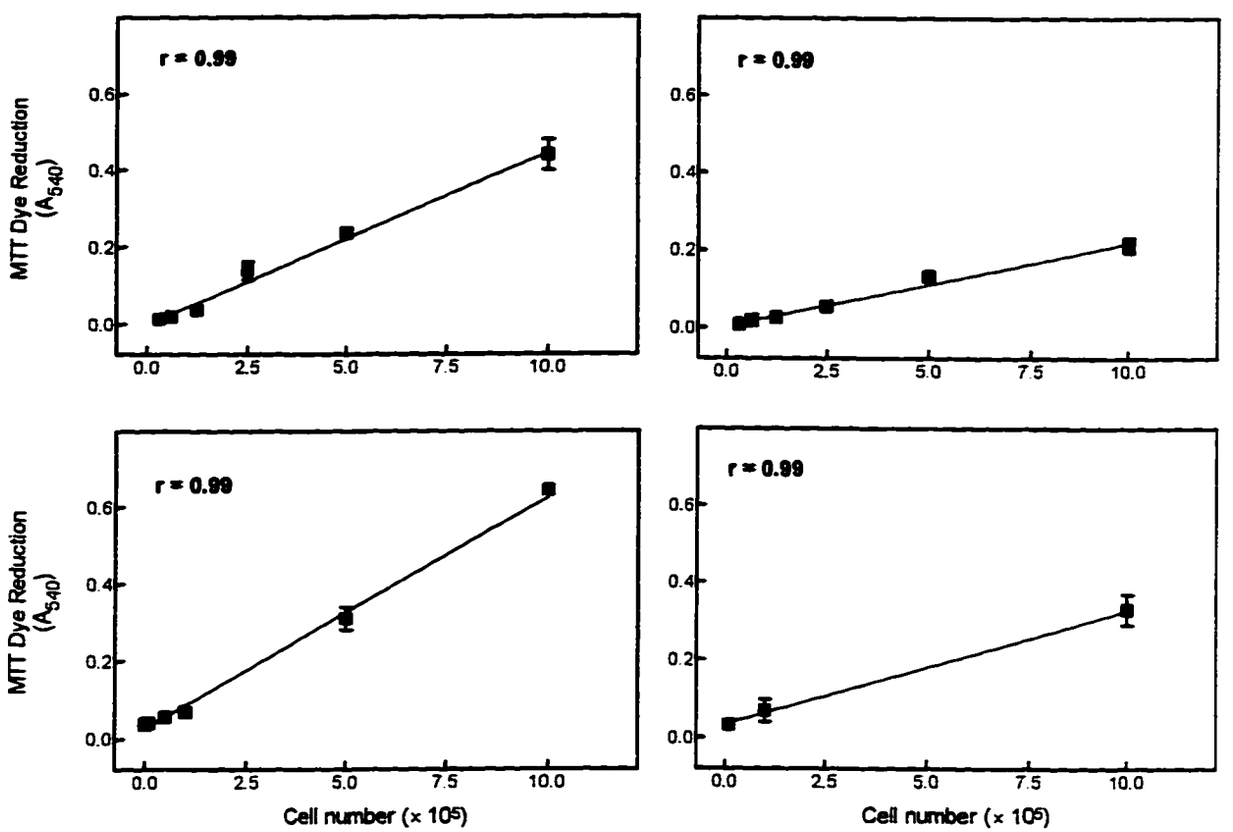

Figure 28. Lymphoblast cell concentration *versus* absorbance at 540 nm (MTT dye reduction).
Cell concentration was linearly related to absorbance (540 nm) over a range of 10^4-10^6 cells for four patient samples tested.

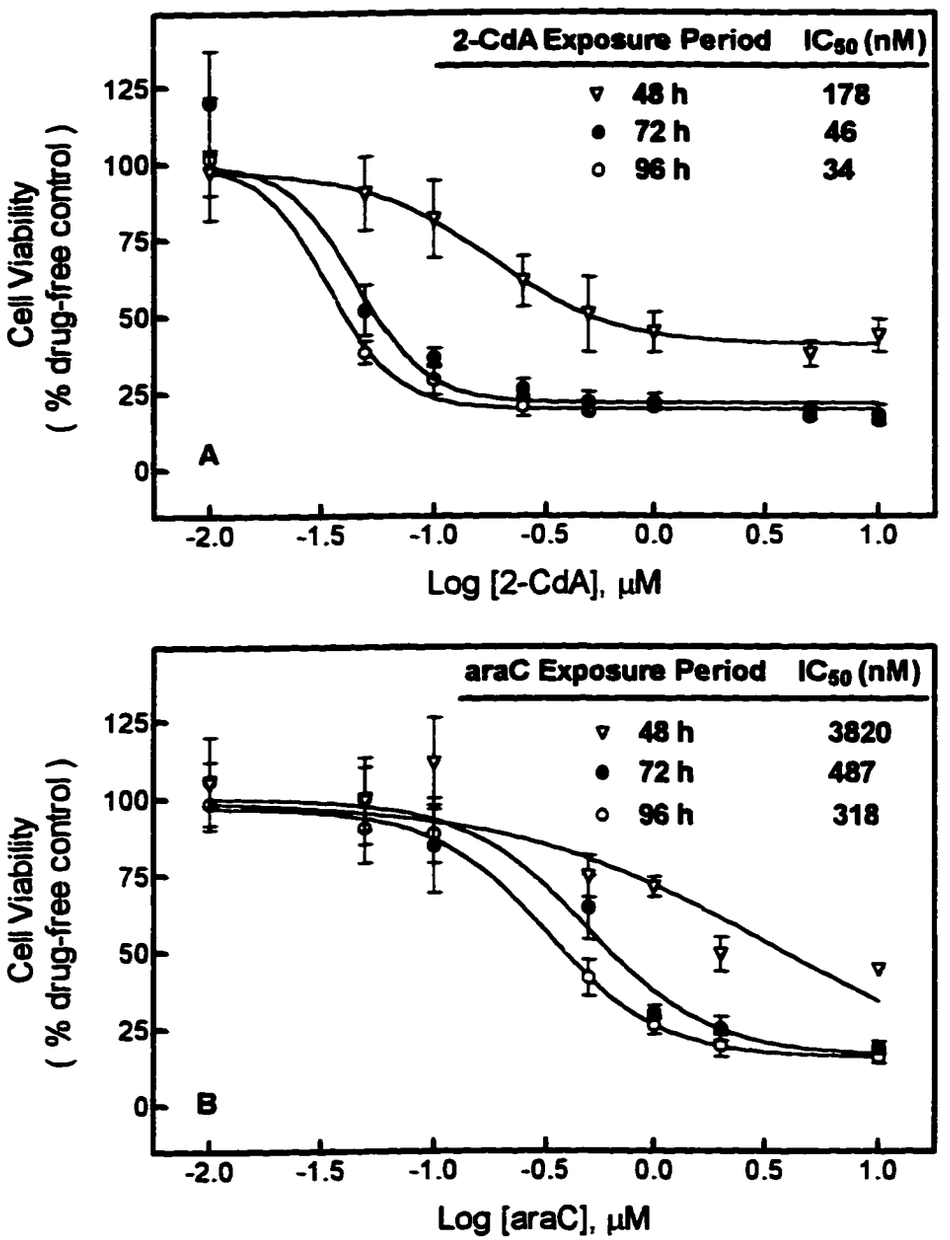

Figure 29. Concentration-and time-dependent effects of 2-CdA and araC toxicity in lymphoblasts from Pre-B ALL Patient N.D.

Leukemic cells from Pre-B ALL patient N.D. were incubated in multiwell culture plates in media containing graded concentrations of 2-CdA (Panel A) or AraC (Panel B), for 48 h (▽), 72 h (●) or 96 h (○); MTT dye reduction, measured after these drug exposure intervals, was expressed as a percentage of that in control cultures (drug-free) and provided a direct measure of cell viability in each culture. Each datum represents the mean ± SD of six replicate determinations; where absent, the SD is smaller than the symbol.

3.2.2 The cytotoxic effects of 2-CdA and araC are concentration- and time-dependent in lymphoblasts from pediatric ALL patients

The experiments of Figs. 29A and 29B show that the cytotoxic effects of 2-CdA and araC were concentration-and time-dependent in lymphoblasts from Pre-B ALL Patient N.D. A marked difference was observed in the sensitivity of leukemic cells to both 2-CdA and araC when drug exposure periods were changed from 48 h to 72 h. However, little difference was found in the sensitivity to both nucleoside analogs during exposure periods of 72 and 96 h. Table 14 summarizes the time-dependent effects of 2-CdA and araC in lymphoblasts from three patients. In cells from patients N.D. and S.C. there was little difference in sensitivity to both nucleoside analogs between 72-h and 96-h exposure periods, and, at least 72 h was required before significant cytotoxicity was apparent in lymphoblasts from patients J.M. and N.D. Based on these results and on data from cell lines (Figs. 34 and 35), a 72-h drug exposure period was chosen to determine drug sensitivity in fresh leukemic cells.

3.2.3 Variable sensitivity of ALL lymphoblasts from pediatric patients to 2-CdA and araC

Figs. 30 and 31 illustrate the variability in response to both agents among patient samples. The IC_{50} value of 46 nM (Fig. 30A) for cytotoxicity of 2-CdA to the leukemic cells from Pre-B ALL Patient N.D. is similar to the maximum 2-CdA plasma concentration (23-85 nM) found after *i.v.* administration of 2-CdA (141). The IC_{50} value for araC cytotoxicity toward ALL lymphoblasts from Patient N.D. was 487 nM (Fig. 30A), and although 10-fold greater than that for 2-CdA in the same experiment,

Table 14

Time-dependence of the toxicity from 2-CdA and araC to leukemic blasts from three patient samples

IC_{50} values were determined from concentration-effect plots following exposure of pediatric ALL lymphoblasts to the nucleoside analogs for the indicated time intervals. The MTT dye reduction assay was used to measure cell viability in the drug-treated cultures.

	2-CdA cytotoxicity IC_{50} (nM)			araC cytotoxicity IC_{50} (nM)		
Patient	48 h	72 h	96 h	48 h	72 h	96 h
JM	>5000	1660	nd	>5000	2650	nd
ND	178	46	34	3820	487	318
SC	nd	84	86	nd	374	363

nd, not determined.

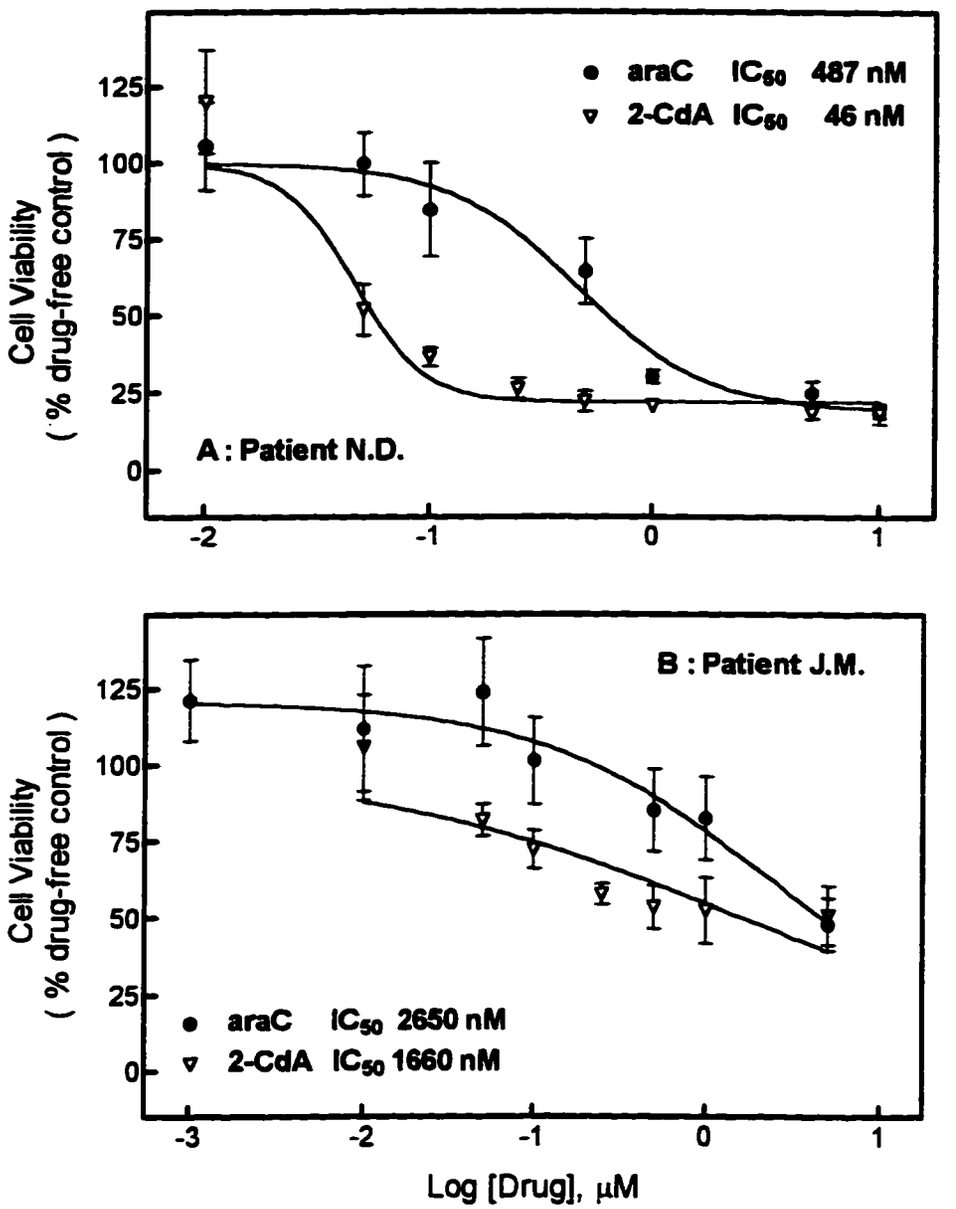

Figure 30. Sensitivity of leukemic cells from Pre-B ALL patients N.D. (Panel A) and J.M. (Panel B) to 2-CdA (▽) and araC (●).
Cells from both patients were exposed to graded concentrations of each drug for 72 h, following which the cell viability was measured by the MTT dye reduction assay. The curves fitted represent the best fit to the sigmoidal dose-response (variable slope) relationship as descried in Materials and Methods. Each datum represents the mean ± SD of six replicate determinations from a single experiment.

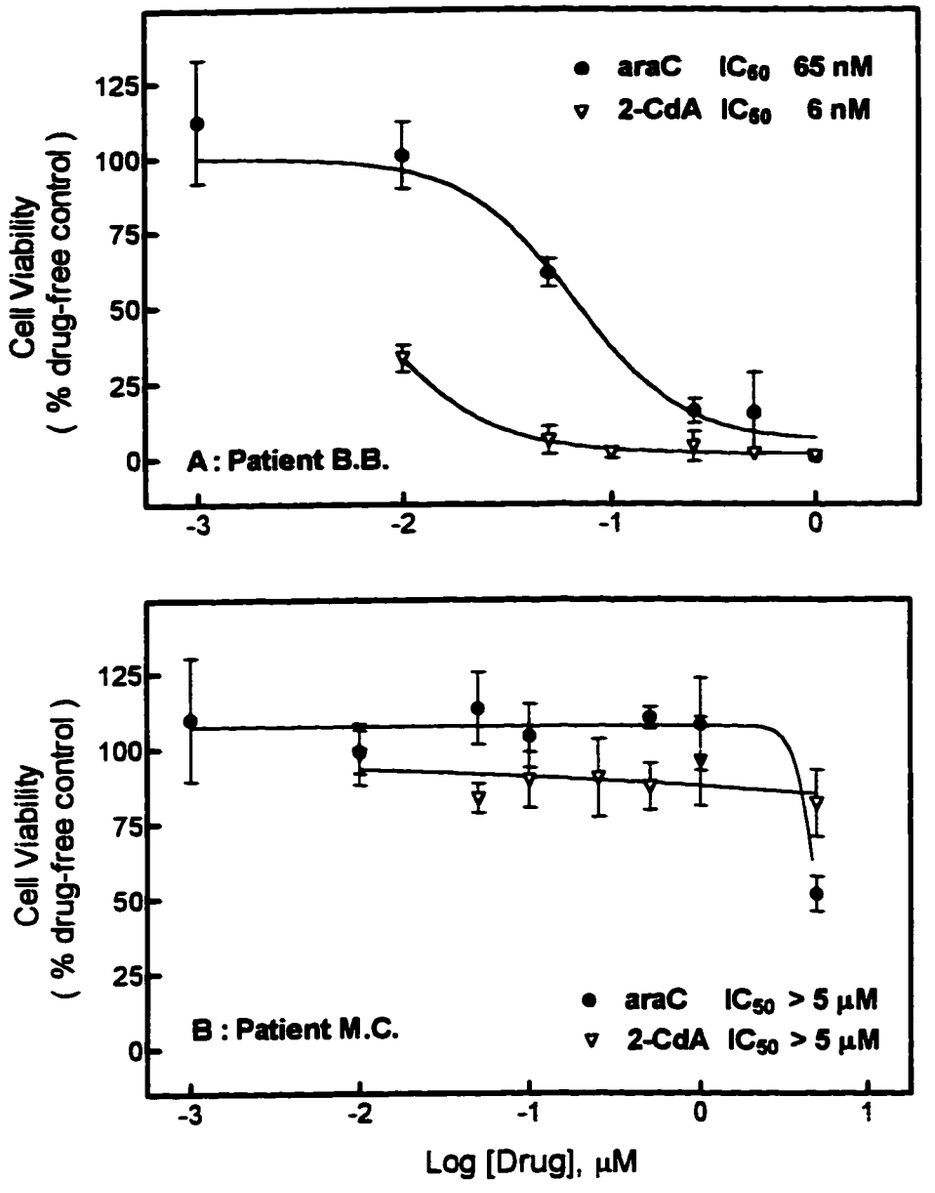

Figure 31. Sensitivity of leukemic cells from T-ALL patients B.B. (Panel A) and M.C. (Panel B) to 2-CdA (▽) and araC (●).
Cells from both patients were exposed to graded concentrations of each drug for 72 h. Following drug exposure, cell viability was measured by the MTT dye reduction assay; Each datum represents the mean ± SD of six replicate determinations from a single experiment.

this value is also in a concentration range (60-800 nM) that is achievable in plasma with conventional araC chemotherapy (156). However, cells from Pre-B ALL Patient J.M. were less sensitive to both 2-CdA and araC, as IC_{50} values of 1660 nM and 2650 nM, respectively, demonstrate (Fig. 30B). Fig. 31B reports resistance to 2-CdA and araC in a T-ALL sample, with less than 10% cell kill even after 3 days exposure to 2-CdA. However, leukemic cells from another T-ALL patient (B.B.) showed remarkable sensitivity to 2-CdA and araC, with IC_{50} values of 6 nM and 65 nM, respectively (Fig. 31A). These results illustrate the wide range of nucleoside analog sensitivity of leukemic lymphoblasts from pediatric ALL patients.

Table 15 shows that interpatient variation in sensitivity to the nucleoside analogs was considerable, with IC_{50} values for 2-CdA ranging from 6 nM to > 5 μM, and for araC, from 65 nM to > 5 μM. Similarly, Pieters and colleagues have reported a wide variation in sensitivity to a number of antitumor agents in leukemic blasts from ALL patients (261). That group also observed that cells from relapsed patients were significantly more resistant *in vitro* to 6-thioguanine, prednisolone, araC, mafosfamide (a derivative of cyclophosphamide) and mustine hydrochloride than cells from newly diagnosed patients. However, no difference in resistance patterns was seen for daunorubicin, vincristine (VCR) or L-asparaginase. Campana *et al.* (263) also observed considerable interpatient variation in ALL blast sensitivity for VCR, 6-thioguanine, araC and teniposide. In that study, a novel stroma-supported immunocytometric assay (SIA) was used to evaluate drug sensitivity in lymphoblasts from patients.

In the present study, half of the patient samples had IC_{50} values for 2-CdA of less than 100 nM; it is noted that concentrations of 2-CdA achievable in the plasma are

Table 15

Interpatient differences in sensitivity of leukemic cells from children with ALL to 2-CdA and araC

IC_{50} values were determined from concentration-effect plots following 72-h exposure of ALL lymphoblasts to the nucleoside analogs. The MTT dye reduction assay was used to measure cell viability in the drug-treated cultures. Patient samples are listed in the order of entry into this study.

Patient	Immunopheno-type	BM blasts (%)	IC_{50} 2-CdA (nM)	IC_{50} araC (nM)
HD	Pre-B ALL	nd	≤ 10	nd
MH	Pre-B ALL	95	43	nd
RB	Pre-B ALL	93	799	2290
RR*	T-ALL	na	408	nd
CC	Pre-B ALL	95	79	372
BB	T-ALL	77	6	65
JM	Pre-B ALL	95	1660	2650
ND	Pre-B ALL	65	46	487
SC	Pre-B ALL	99	84	374
GA	Pre-B ALL	44	148	469
DC	Pre-B ALL/AML	nd	1010	>5000
RL	Pre-B ALL	69	33	946
MC	T-ALL	97	> 5000	> 5000
JD	Pre-B ALL	77	171	1540

BM, bone marrow; nd, not determined; na, not applicable; *, peripheral blood sample.

in the 23-85 nM range (141). The leukemic cell samples that were most resistant to 2-CdA and araC were from a T-ALL leukemia and from a mixed lineage leukemia, immunophenotypes often associated with an unfavorable outcome (49); however it is also seen that the samples that were most sensitive to 2-CdA and araC in the present study included a T-ALL sample, illustrating again the diversity of the ALL lymphoblast response to the nucleoside analogs. It is noted that leukemic cells showed significantly ($p < 0.005$) greater sensitivity to 2-CdA ($n = 13$) than to araC ($n = 10$); this significance was evident with Wilcoxon's signed rank test (252). Therefore, it is evident, that while 2-CdA may be an effective agent for treatment of some pediatric patients, for others it may be of little benefit. As the number of ALL samples in this study was small, a greater number of patient samples is required to determine if this agent has potential utility in pediatric ALL.

In contrast to results reported here, *in vitro* studies by Kumangi *et al.* (145) showed little interindividual variation in cytotoxicity of 100 nM 2-CdA to the 20 samples of pediatric ALL lymphoblasts examined. The Kumangi results also showed that 2-CdA was effective in cells from patients who had relapsed after initial treatment with multiagent chemotherapy and in cells from patients who had an unfavorable karyotype. A possible explanation for the difference may be found in the design of the two studies. Kumangi and colleagues (145) used the SIA assay to assess cell viability, and exposed cells to 2-CdA for a period of 96-120 h.

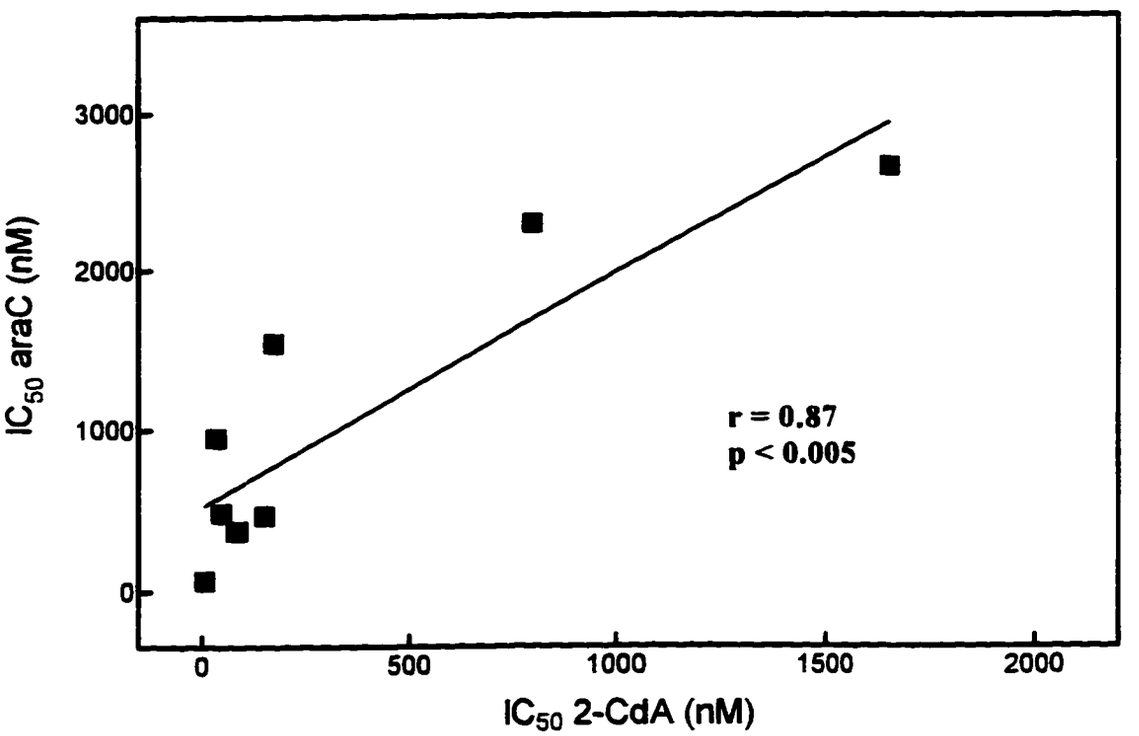

Figure 32. Correlation of 2-CdA and araC sensitivity in leukemic cells from pediatric ALL patients.
The coordinates of each point are the IC_{50} values for 2-CdA and araC in individual patient samples. A statistically significant ($p < 0.005$) linear correlation of sensitivity to 2-CdA and araC was observed in the ALL samples.

3.2.4 Correlation between 2-CdA and araC cytotoxicity in lymphoblasts from pediatric ALL patients

Fig. 32 represents an attempt to test for a correlation between 2-CdA and araC cytotoxicity in leukemic cells from ALL patients. A statistically significant ($p < 0.005$, $r = 0.87$) linear relationship between the sensitivity of the leukemic cells to 2-CdA and to araC was observed. It may be noted that two samples could not be plotted in this analysis because their IC_{50} values for 2-CdA and araC cytotoxicity were in excess of 5 μM. This linear relationship may indicate a common mechanism of cytotoxicity or resistance for both nucleoside analogs. A study by Pieters and colleagues (261) found decreased sensitivity to araC in lymphoblasts from relapsed patients relative to the araC sensitivity of lymphoblasts from newly diagnosed patients. Therefore, to assess the potential usefulness of 2-CdA in patients resistant to araC, it would be meaningful to compare the cytotoxicity of 2-CdA and araC in cells from newly diagnosed pediatric ALL patients relative to that in patients who relapsed after remission induction treatment with araC.

3.2.5 Comparison of sensitivity to 2-CdA and araC to *es* NT expression in leukemic lymphoblasts from children with ALL

It has been stated that the "intrinsic sensitivity of the malignant cell to a drug is probably more important in treating hematological malignancies than systemic exposure in producing therapeutic responses" (141). Previous studies have indicated that the *es* site content of the blast cell surface was a determinant of araC influx and araCTP formation (194,265). This laboratory has recently provided supporting evidence for the

role of membrane transport as a determinant of araC efficacy in acute leukemia, and that a deficiency in transporter expression may be the basis of cellular resistance to araC under certain conditions (236).

The present study asked if the level of *es* nucleoside transporter expression on blast cells from pediatric ALL patients was a determinant of 2-CdA or araC cytotoxicity. A 14-fold difference in *es* site content was observed among patient samples (Table 13). This wide variation in *es* site content among blasts from leukemic patients, which was also observed in previous studies (151,236,258,259), suggests that membrane transport of nucleoside analogs may be a determinant in the intrinsic sensitivity of leukemic blasts to these agents. Table 16 presents IC_{50} values for the sensitivity of leukemic cells to 2-CdA and araC and compares these values to *es* site abundance in leukemic blasts. A correlation between *es* abundance and sensitivity to 2-CdA was not apparent (Fig. 33A), suggesting that *es* site content may not be the major determinant in the response to 2-CdA. Nevertheless, cells from patient samples that showed the least sensitivity to 2-CdA had low *es* site expression. This is consistent with the evidence that NT processes are required for entry of nucleoside analogs into cells. However, a relationship, although not statistically significant, was apparent between araC sensitivity and *es* site expression (Fig. 33B). A study with greater sample numbers is needed to characterize the relationship between *es* site content and sensitivity to nucleoside analogs in lymphoblasts from pediatric ALL patients.

Confounding factors in this study may include the presence of other NT processes that are not identified by this flow cytometric technique. For example, although cells may have a low *es* content, it is conceivable that they may express

Table 16

Comparison of sensitivity to 2-CdA and araC with *es* nucleoside transporter expression in leukemic lymphoblasts from children with ALL

IC_{50} values were determined from concentration-effect plots following 72-h exposure to the nucleoside analogs.

	Sensitivity (IC_{50} (nM) in MTT assay) of patient lymphoblasts to nucleoside analogs		*es* nucleoside transporter content of lymphoblasts[a]
Patient	2-CdA (nM)	araC (nM)	B_{max} (mean RFI)
CC	79	372	2.83 ±0.3
JD	171	1540	2.97 ± 0.2
MH	43	nd	3.76 ± 1.9
JM	1660	2650	4.91 ± 0.6
MC	> 5000	> 5000	5.34 ± 0.2
NR[b]	nd	nd	9.34 ± 0.8
RL	33	946	9.61± 0.4
GA	148	469	10.3 ± 0.6
RA[b]	nd	nd	17.0 ± 0.9
ND	46	487	24.1 ± 1.9
MB[b]	nd	nd	30.8 ± 0.5
RR	408	nd	32.0 ± 3.5
BB	6	65	33.3 ± 3.6
SC	84	374	40.6 ± 0.8

[a]*Es* nucleoside transporter content of lymphoblasts is shown as the B_{max} values ± SEM (mean RFI) for 5-(Sx8)-F fluorescence determined in flow cytometric assays.

[b]Patients N.R., R.A. and M.B. (not previously reported in this study) were diagnosed with Pre-B ALL.

nd, not determined

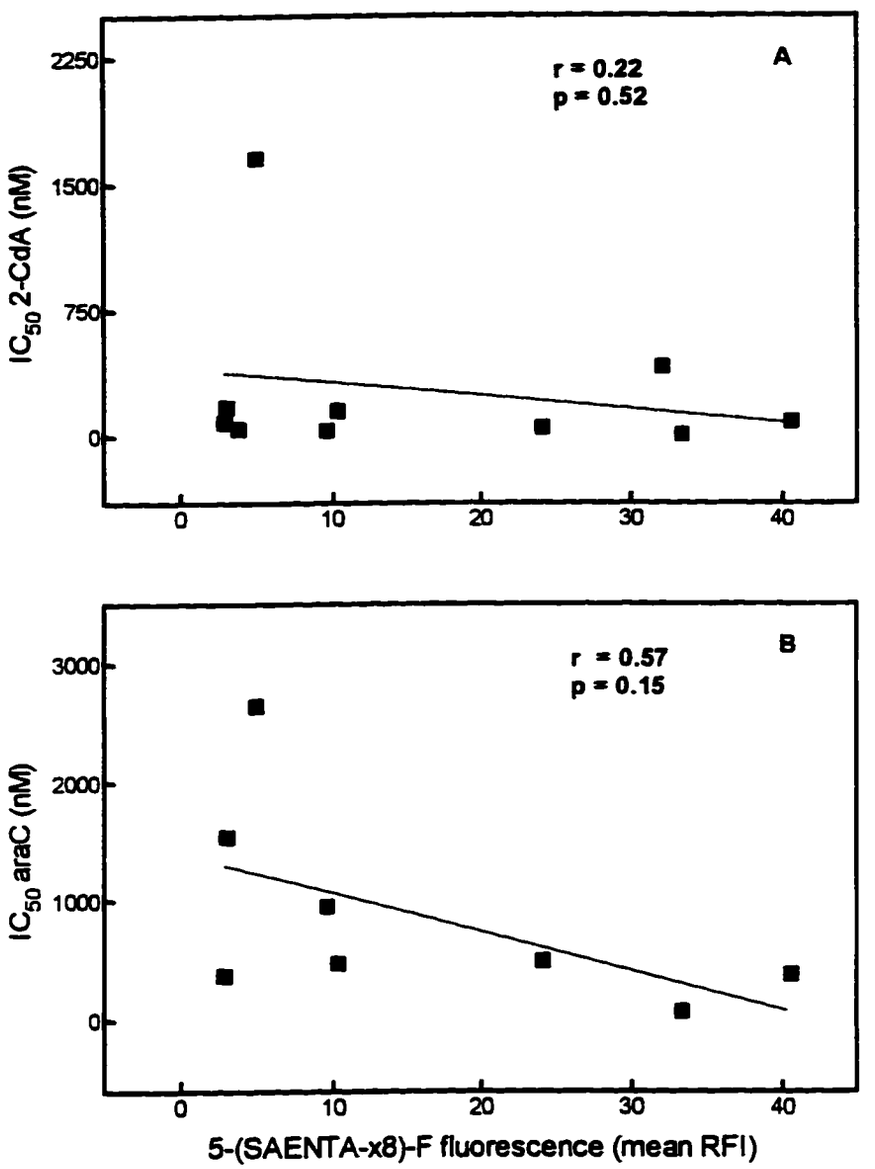

Figure 33. Correlation of 2-CdA (Panel A) and araC (Panel B) sensitivity with *es* site expression in lymphoblasts form pediatric ALL patients.
Es site content is expressed as the B_{max} (mean RFI) determined from flow cytometric analysis and IC_{50} values are the concentrations of nucleoside analogs that reduced cell viability by 50%.

significant activities of *cs*, *ei* or other NT processes. Such unrecognized complexities may explain anomalies such as the correlation that was apparent between *es* expression and araC sensitivity, but not for 2-CdA sensitivity. As well, this study has not attempted to identify other mechanisms of 2-CdA resistance that may include reduced deoxycytidine kinase levels, resulting in lower levels of the active metabolite, or reduced activity of DNA polymerases, enzymes that are targets of 2-CdA (141). Therefore, cells with high levels of *es* transporter site expression may still be resistant to 2-CdA by mechanisms further along the pathway of 2-CdA activation and not at the level of membrane transport. Further studies that identify both the type and content of the NT processes present in leukemic blasts, and examine other mechanisms of drug resistance in individual patient samples are required to evaluate and understand the importance of NT expression as a determinant in the efficacy of nucleoside analogs.

3.2.6 Cytotoxicity of 2-CdA, araC and FaraA in cultured ALL lymphoblasts

As patient samples are frequently "once only" and small in size, human lines of cultured ALL cultured cells have been used as models for fresh leukemic lymphoblasts. The sensitivities of four cultured human ALL cell lines used in this study to 2-CdA, araC and FaraA were also examined. FaraA is a purine nucleoside analog that has shown important antileukemic activity in CLL patients (135,136). However, there have been few reports on the efficacy of FaraA in treatment of acute leukemia. In cultured human T-lymphoblasts, 2-CdA was 100 times more potent than FaraA (120).

3.2.6.1 The cytotoxic effects of 2-CdA and araC are concentration- and time-dependent in cultured ALL lymphoblasts

Cultured ALL cells were exposed to graded concentrations of 2-CdA, araC and FaraA, for intervals of 24, 48 or 72 h, after which the MTT dye reduction assay was used to measure cell viability. The experiment of Fig. 34 showed that the cytotoxic effects of 2-CdA were concentration- and time-dependent in Reh-A2 cells. Significant differences were observed in the sensitivities (IC_{50} values) of cells exposed to 2-CdA for either 24 or 48 h ($p < 0.01$, Tukey Multiple comparisons test). The IC_{50} values for Reh-A2 cells exposed to 2-CdA for 24, 48 or 72 h were 400 ± 17, 62 ± 8.8 and 14.1 ± 2.9 nM, respectively. These results indicated that an exposure period of at least 48 h was necessary for the expression of significant cytotoxicity. Moreover, after the 24-h drug exposure period, approximately 35% of Reh-A2 cells remained viable in the presence of the highest concentration of 2-CdA tested (Fig. 34). These results agree with previous reports that prolonged 2-CdA exposure is necessary for maximum cell kill (266). It is possible that cells not killed in this experiment were those that had failed to incorporate 2-CdAMP into their DNA during the interval of drug exposure.

Reh-A2 cells were exposed to 2-CdA for either 24 or 48 h, following which 2-CdA was removed by washing and the cells cultured in 96-well plates for a further 48 or 24 h, respectively. Therefore, in both circumstances, cells were cultured for 72 hours in total. The IC_{50} value of 2-CdA in Reh-A2 cells exposed for 24 h followed by 48 hours drug-free incubation was, 77.6 ± 11.1 nM, whereas the IC_{50} for 2-CdA was 400 ± 17 nM for cells exposed to 2-CdA for 24 h alone (Table 17). A significant difference ($p < 0.01$) was apparent between (a) cells treated with 2-CdA for 24 h and grown in

Table 17

2-CdA cytotoxicity requires a 48-h exposure period

Reh-A2 cells were exposed to graded concentrations of 2-CdA for the indicated intervals (2-CdA exposure alone) or for the indicated intervals plus incubation in drug-free medium for a culture period totalling 72-h. IC_{50} values were determined from concentration-effect plots following exposure of Reh-A2 cells to 2-CdA under these various conditions. The MTT dye reduction assay was used to measure cell viability in the drug-treated cultures.

	2-CdA cytotoxicity (IC_{50}, nM)		
	24 h	48 h	72 h
2-CdA exposure alone	400 ± 17[1]	62.5 ± 8.8	14.1 ± 2.9
2-CdA exposure for the indicated time intervals + incubation in drug-free medium for a period totalling 72 h	77.6 ± 11.1	22.3 ± 4.8	na

na, not applicable.

[1]Values represent the mean ± SEM of three or more experiments.

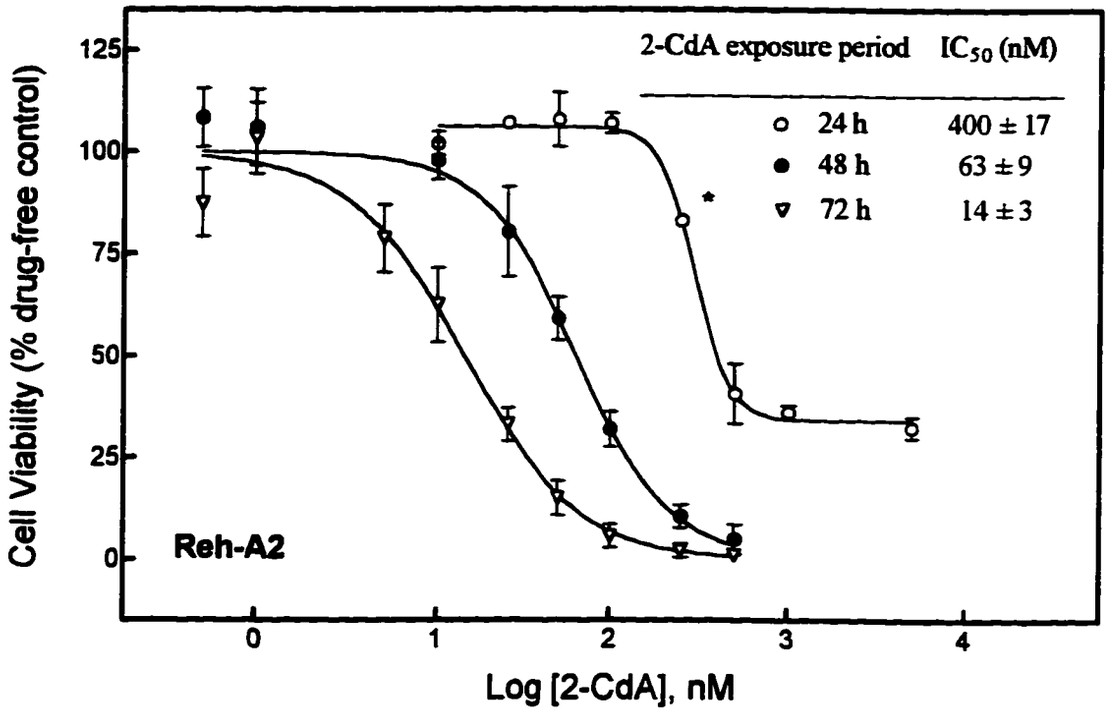

Figure 34. Concentration-and time-dependent effects of 2-CdA sensitivity in Reh-A2 cells.

Cells were incubated in multiwell culture plates in media containing graded concentrations of 2-CdA for 24 h (○), 48 h (●) or 72 h (▽); MTT dye reduction measured after these drug exposure intervals was expressed as a percentage of that in control cultures (drug-free) and provided a direct measure of cell viability in each microculture. Each data point is a mean ± SEM of data obtained from three or more experiments.

The asterisk-marked data point (*) represents the mean of six replicate determinations from a single experiment

drug-free medium for a further 48 h, and (b) cells continuously exposed to 2-CdA for 72 h. This result indicates that an exposure period of more than 24 h is necessary for significant 2-CdA cytotoxicity. However, the IC_{50} value for cells exposed to 2-CdA for 48 h followed by 24 h drug-free incubation (22.3 ± 4.8 nM) was not significantly different from the IC_{50} value in cells continuously exposed to 2-CdA for 72 h (14.1 ± 2.9 nM) (Table 17). These results indicate that a culture period of 72 h that includes a 48-h drug exposure period is required for substantial 2-CdA cytotoxicity in Reh-A2 cells.

Previous studies have also shown that in lymphocyte and myeloid progenitor cells, a 2-CdA exposure interval of 2 to 3 days was required to effect a marked inhibition of growth (84). Similarly, Petzer *et al.* (266) observed that a preincubation period of at least 48 h was required for 2-CdA inhibition of the growth of a series of progenitor cells. This delay in cytotoxicity was considered to be a function of various time-dependent metabolic changes that are a result of 2-CdA exposure.

The experiment of Fig. 35 explored the effects on araC cytotoxicity toward Reh-A2 cells of exposure of these cells (in microwell cultures) (a) to graded concentrations of araC, and (b) for exposure intervals of 24 h, 48 h and 72 h. The IC_{50} values for Reh-A2 cells exposed continuously to araC for either 24, 48 or 72 h were 387 ± 90, 16.8 ± 3.7 and 3.2 ± 0.7 nM, respectively. The 48-h exposure of the cultured Reh-A2 lymphoblasts to araC was significantly ($p < 0.05$, Tukey Multiple Comparisons test) more toxic than 24 h of exposure under the same conditions (Fig. 35). However, a significant difference was not apparent between 48- and 72-h araC exposure. Thus, it appears that, as for 2-CdA, araC requires at least a 48-h incubation period to achieve

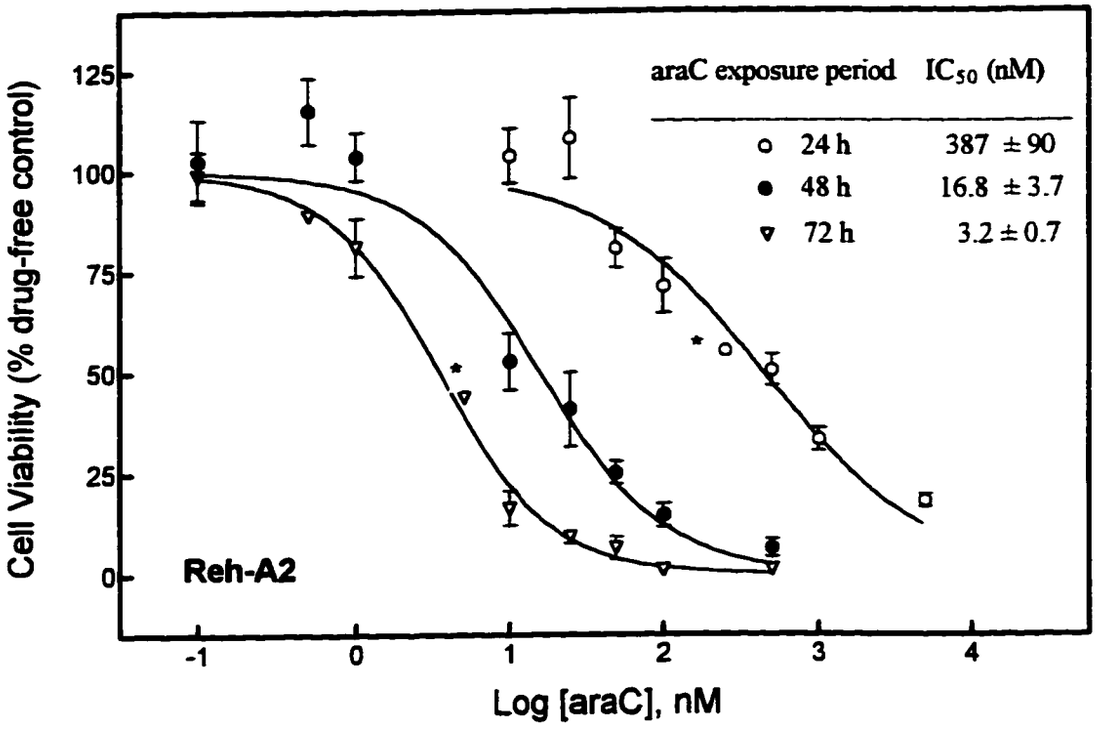

Figure 35. Concentration-and time-dependent effects of araC sensitivity in Reh-A2 cells.

Cells were incubated in multiwell culture plates in media containing graded concentrations of araC for 24 h (○), 48 h (●) or 72 h (▽); MTT dye reduction measured after these drug exposure intervals was expressed as a percentage of that in control cultures (drug-free) and provided a direct measure of cell viability in each microculture. Each point is the mean ± SEM of data obtained from three or more experiments.

The asterisk-marked data point (*) represents the mean of six replicate determinations from a single experiment.

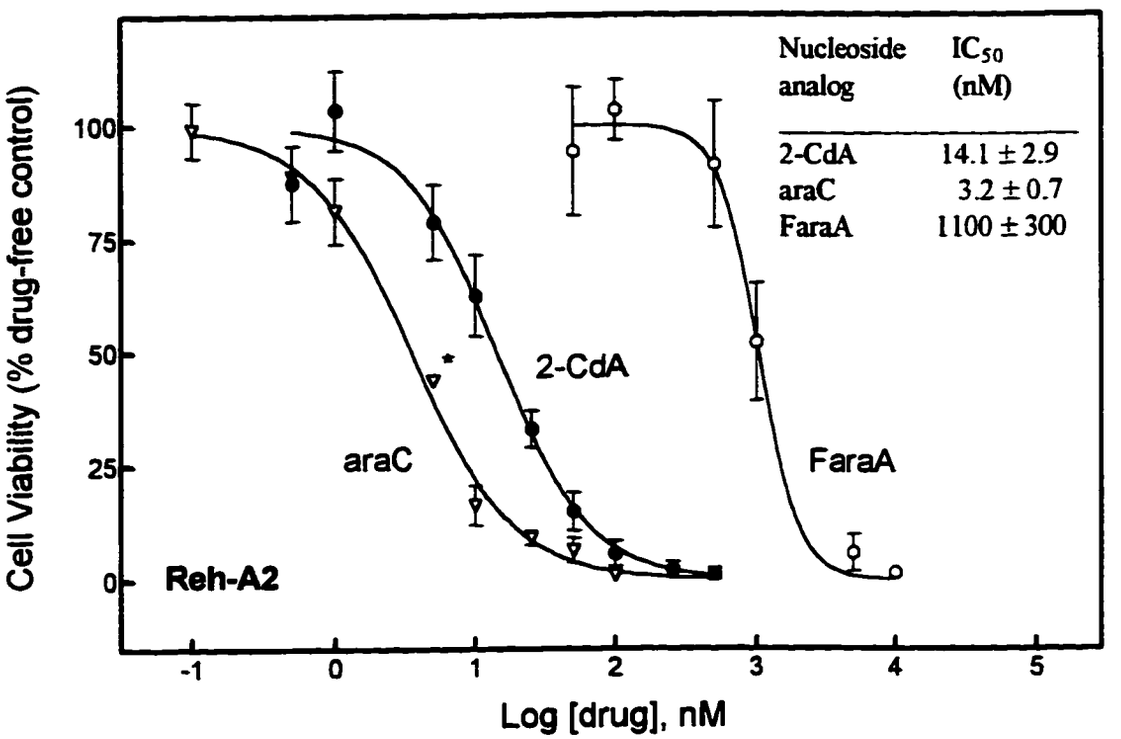

Figure 36. Concentration-effect plots of nucleoside analog sensitivity in Reh-A2 cells.

Cells were exposed to graded concentrations of 2-CdA (●), araC (▽) or FaraA (○) for 72 h in multiwell culture plates; MTT dye reduction, measured after 72 h, was expressed as a percentage of that in control wells (drug-free) and provided a direct measure of cell viability in each culture. Each point represents the mean ± SEM of three or more separate experiments.

The asterisk-marked data point (*) represents the mean of six replicate determinations from a single experiment.

substantial cytotoxicity. Finally, no statistically significant difference was seen in FaraA cytotoxicity between 48- and 72-hour exposure periods (Table 18). With the exception of Reh-A2 cells, there was a statistically significant difference between lymphoblast sensitivity to FaraA between a 24- and a 48-h exposure period. Fig. 36 shows drug concentration-effect plots following 72-h exposure of Reh-A2 cells in microwell cultures to 2-CdA, araC and FaraA. Reh-A2 cells were sensitive to both 2-CdA and araC with IC_{50} values of 14.1 ± 2.9 and 3.2 ± 0.7 nM, respectively, but were less sensitive to FaraA with an IC_{50} value of 1.1 ± 0.3 μM. The four ALL lymphoblast lines used in this study were less sensitive to FaraA, with IC_{50} (72-h drug exposure) values ranging from 1.1-6 μM.

3.2.6.2 Sensitivity of cultured ALL lymphoblasts to the nucleoside analogs, 2-CdA, araC and FaraA

Table 18 summarizes the sensitivities of the four lines of cultured ALL lymphoblasts to the three nucleoside analogs. In these cell lines, a significant difference ($p < 0.05$, Tukey Multiple comparisons test) was observed in the IC_{50} values for 2-CdA exposure and for araC exposure after 24- and 48-h drug exposure intervals. However, no significant difference was observed between 48- and 72-h exposure periods for the two agents (the exception includes Reh-A2 cells in which a significant difference ($p < 0.001$) was apparent between IC_{50} values for 2-CdA cytotoxicity between a 48- and a 72-h exposure period). As found with patient samples, cultured cells from lymphoblast lines were heterogenous in their responses to 2-CdA, with IC_{50} values (72-h drug exposure) ranging from 14.1-64.2 nM. Fig. 37 illustrates the range of 2-CdA sensitivity

Table 18

Cytotoxicity of 2-CdA, araC and FaraA in four lines of cultured ALL lymphoblasts

IC_{50} values were determined from concentration-effect plots following exposure of ALL cell lines to the nucleoside analogs for the indicated time intervals. The MTT dye reduction assay was used to measure cell viability in the drug-treated cultures.

	FaraA cytotoxicity (IC_{50}, μM)			araC cytotoxicity (IC_{50}, nM)			2-CdA cytotoxicity (IC_{50}, nM)		
Cell line	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Reh-A2	11.9 ± 1.6[1]	2.2 ± 0.5	1.1 ± 0.3	387 ± 90	16.8 ± 3.7	3.2 ± 0.7	400 ± 17	62.5 ± 8.8	14.1 ± 2.9
SB	113 ± 33.1	8.1 ± 1.9	6.0 ± 1.2	423 ± 48	12.5 ± 0.8	2.6 ± 0.9	2810 ± 770	184 ± 48.8	60.6 ± 10
CEM	74.3 ± 14.3	7.5 ± 1.6	4.5 ± 0.4	203 ± 45	8.9 ± 1.5	5.6 ± 0.7	1790 ± 290	178 ± 7.9	64.2 ± 0.9
KM3-2B	47.7 ± 11.1	5.7 ± 0.9	4.5 ± 0.8	117 ± 14.3	3.8 ± 0.8	2.2 ± 0.2	739 ± 73	53.0 ± 7.9	45.9 ± 8.2

[1]Values represent the mean ± SEM of three or more experiments.

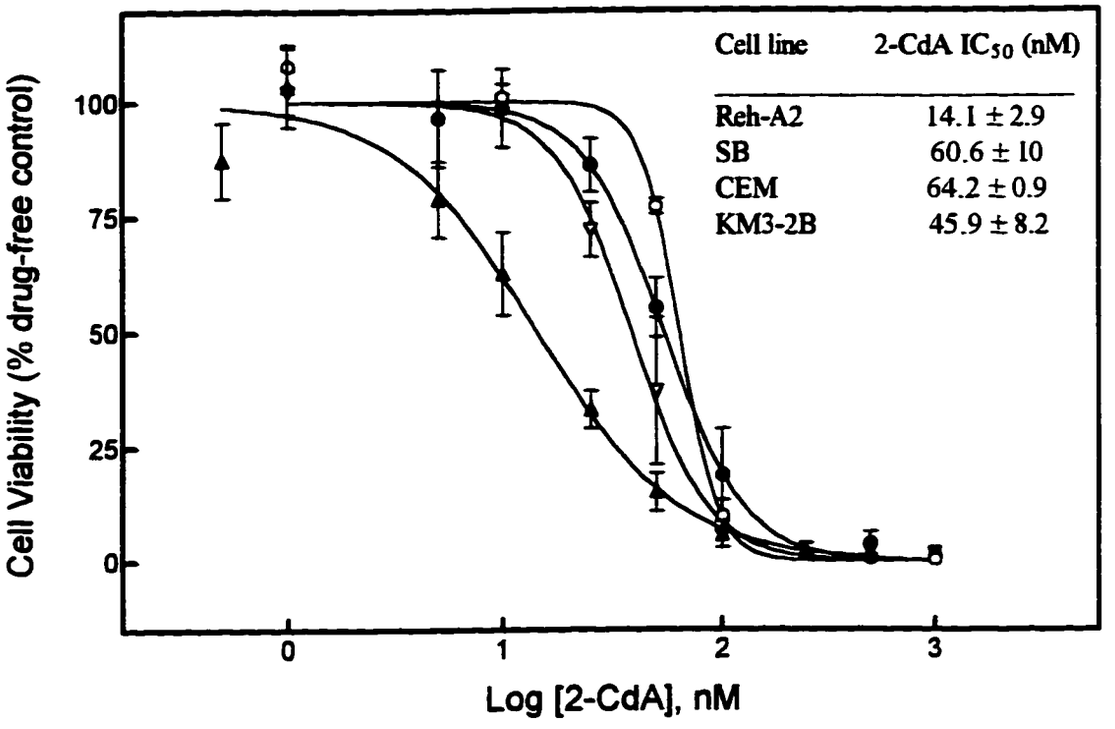

Figure 37. Concentration-effect plots for 2-CdA sensitivity in cultured ALL lymphoblasts.

Reh-A2 (▲), SB, (●), CEM (○) and KM3-2B cells (▽) were exposed to graded concentrations of 2-CdA for 72 h; MTT dye reduction was expressed as a percentage of that in control cultures (drug-free) and provided a direct measure of cell viability in each microculture. Data are means ± SEM of three or more experiments.

in the four lines of cultured cells. Reh-A2 cells were significantly more sensitive ($p < 0.05$, Tukey Kramer Multiple comparisons test) to 2-CdA than were the other cell lines. Cell lines were significantly ($p < 0.01$) more sensitive to araC than to 2-CdA, whereas blast cells from patients were more sensitive to 2-CdA than to araC (Table 15). Importantly, no correlation was observed between 2-CdA and araC sensitivity in the cell lines studied. The small number of cell lines used in this study and the small variability in sensitivity to araC may account for the latter finding. These observations question the suitability of human ALL cell lines as model systems for the study of nucleoside analog cytotoxicity in fresh leukemic lymphoblasts.

3.2.6.3 Sensitivity to 2-CdA and araC in ALL cell lines and the relevance of **es** NT expression

This study asked whether membrane transport is a major determinant of the sensitivity of ALL cells to nucleoside analogs. To answer this, IC_{50} values were determined from concentration-effect plots following 72-h exposure to either 2-CdA or araC and were compared with ***es*** site content and 2-CdA transport efficiency (Table 19). There was no apparent correlation between nucleoside analog sensitivity and ***es*** site abundance. On the contrary, Reh-A2 cells that were most sensitive to 2-CdA, had the lowest ***es*** site content and lowest 2-CdA transport efficiency. This finding does not agree with reports by King *et al.* (102,103), which showed that cytotoxicity of 2-CdA correlated with efficiency of transport in cultured leukemic cells. A possible explanation for this may include the fact that both human and murine cell lines were used in that study.

Table 19

Comparison of sensitivity to 2-CdA and araC with *es* NT expression and 2-CdA transport efficiency in ALL cell line

IC_{50} values were determined from concentration-effect plots following 72-h exposure to the nucleoside analogs. ***Es*** nucleoside transporter content was determined by equilibrium binding of [^{3}H]NBMPR. 2-CdA transport efficiency (V_{max}/K_m) was determined from kinetic analysis of 2-CdA transport as described in Section 3.1.8.

	Sensitivity (IC_{50} in MTT assay) of ALL cell lines to nucleoside analogs		*es* nucleoside transporter content	2-CdA transport efficiency
Cell line	2-CdA (nM)	araC (nM)	(sites/cell)	(V_{max}/K_m)
Reh-A2	14.1 ± 2.9[1]	3.2 ± 0.7	4.8 ± 0.7 × 10^4	0.08
SB	60.6 ± 10.1	2.6 ± 0.9	17.2 ± 2.3 × 10^4	0.26
	64.2 ± 0.9	5.6 ± 0.7	15.2 ± 3.0 × 10^4	0.27
KM3-2B	45.9 ± 8.2	2.2 ± 0.2	19.1 ± 1.3 × 10^4	0.27

[1]Values represent mean ± SEM of three or more experiments.

The results presented in this section indicate that membrane transport may not be a major determinant of sensitivity to 2-CdA in either fresh lymphoblasts or cultured ALL lymphoblasts. It was not possible to correlate in lines of cultured ALL cells sensitivity to araC with *es* expression because of the similarity in the sensitivity of the four cultured cell lines to this drug. However, results described in Section 3.3.3 of this chapter suggest that deoxycytidine kinase (dCK) activity may be a determinant of the sensitivity of cultured lymphoblasts to 2- CdA. This finding agrees with a recent study by Orr *et al.* (267) that showed dCK to be a major determinant of 2-CdA resistance in both murine and human lymphoid cell lines. It remains to be determined whether impaired phosphorylation is a determinant of the sensitivity to both 2-CdA and araC in fresh lymphoblasts.

3.3 Enhancement of retention and cytotoxicity of 2-CdA by NBMPR and DPM in ALL cell lines

Intracellular 2-CdA levels and its metabolites have been shown to decline rapidly when cells are placed in 2-CdA-free medium. Santana *et al.* (130) reported, half-lives of 1.29 and 2.47 hours for 2-CdAMP and 2-CdATP, respectively, in leukemic blasts from pediatric acute leukemia patients that were "loaded" with 2-CdA. It has also been reported in leukemic cells from CLL patients, that cellular levels of 2-CdA and metabolites thereof decayed in a biexponential manner (268). In that study, when the 2-CdA-loaded cells were suspended in 2-CdA-free medium a significant reduction in 2-CdA efflux was observed in the presence of 0.5 μM NBMPR in cells from 13 out of the 19 samples examined. Because the decay in 2-CdA levels was blocked in the presence

of NBMPR it was reasoned that the *es* transporter-mediated efflux process was responsible for the decay. The inability to inhibit efflux in some patients in that study may be due in some part to the presence of NT processes that were insensitive to NBMPR.

It has been shown previously in human leukemic blasts that intracellular araCTP levels may be increased by blocking efflux of araC with DPM (269). Earlier studies had demonstrated (a) a correlation between *in vitro* retention of araCTP by AML cells and the duration of remission of patients receiving araC therapy (239), and (b) a relationship between the levels of araCTP incorporation into DNA and the cytotoxic potential of this agent (240). Therefore, increasing intracellular levels of 2-CdA and its phosphorylated metabolites by blocking 2-CdA efflux would likely increase the cytotoxic potential of 2-CdA. An aim of the present study was to decide if the decay of intracellular 2-CdA and its metabolites might be impeded at the level of 2-CdA membrane transport. It was hypothesized that the "retention tactic", that is, inhibition of 2-CdA efflux with NT inhibitors such as NBMPR or DPM would, through blockade of 2-CdA efflux, sustain cellular levels of 2-CdA and its active metabolites, and thereby enhance the cytotoxicity of 2-CdA exposure.

3.3.1 Modulation of the intracellular pharmacokinetics of 2-CdA by inhibitors of nucleoside transport

Time courses for 2-CdA loss from the four lines of cultured ALL lymphoblasts are presented in Fig. 38. Cells were "loaded" with 2-CdA for 1 hour; the rational behind this approach was based on the observation that 2-CdAMP and 2-CdATP reached

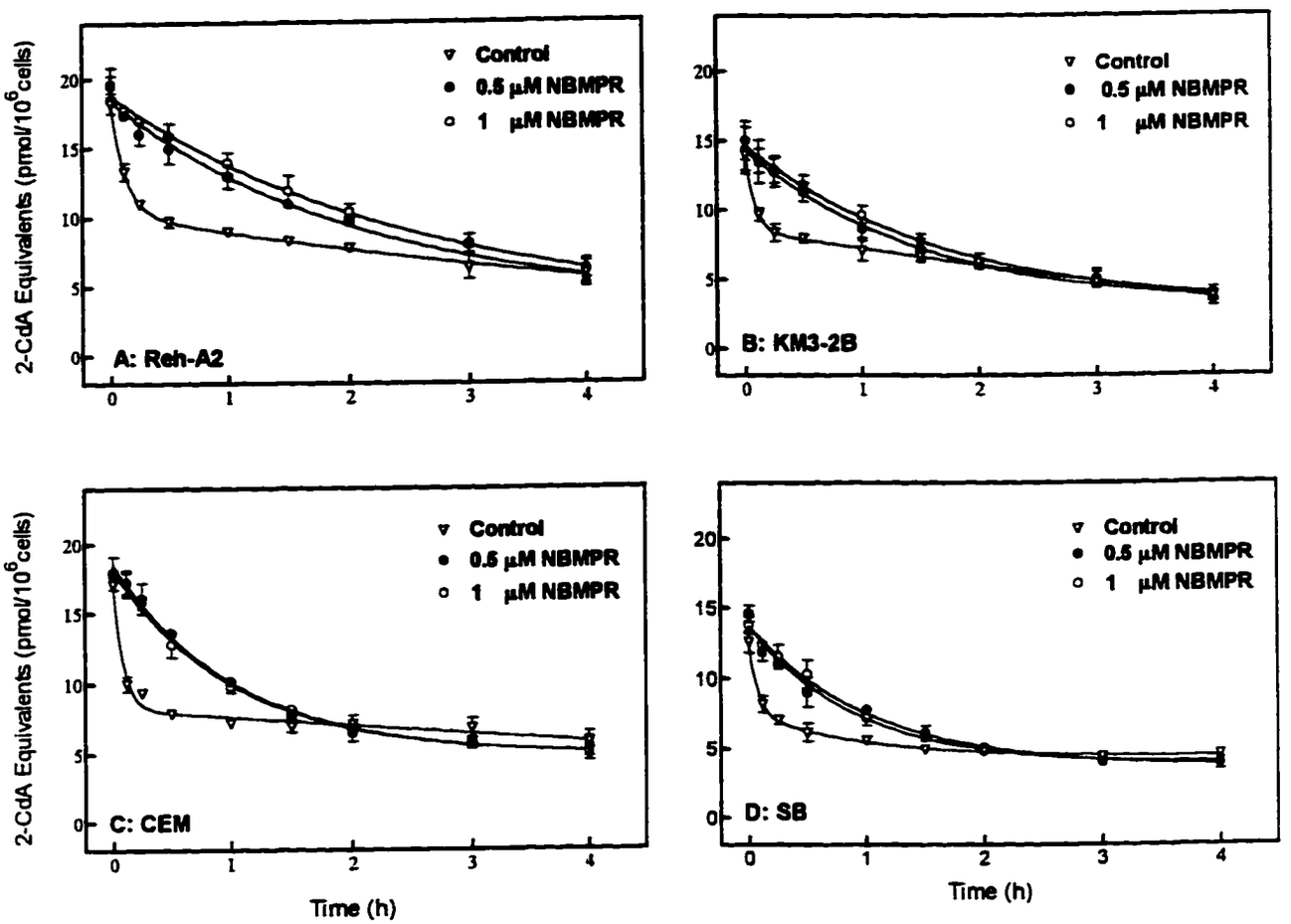

Figure 38. Retention of 2-CdA in cultured ALL lymphoblasts by NBMPR. Cells were loaded with 1 μM [^{3}H]2-CdA for 1 h at 37°C, cell suspensions were then diluted 10-fold with DPBS medium without additives (control) (∇), or with 0.5 μM NBMPR (●), or 1 μM NBMPR (○). Incubation at 37°C was continued, with samples (10^6 cells) taken for measurement of cellular ^{3}H-content (2-CdA and its phosphate metabolites) at the indicated intervals. Means ± SEM of three separate experiments are shown except for the experiment that employed DPBS medium with 0.5 μM NBMPR; in those instances, the data shown are the means ± the ranges of two separate experiments.

concentrations close to steady-state levels within 1 hour in pediatric leukemia cells (130). Earlier studies in this laboratory identified intracellular metabolites of 2-CdA as the mono, di and triphosphate equivalents, and showed 2-CdA to be the only efflux product detectable in CLL cells (268). In the four cell lines, a biexponential decline in cellular 2-CdA equivalents took place when reaction mixtures were diluted 10-fold with drug-free medium. Initial half-lives ($t_{1/2\alpha}$) ranged from 0.04-0.09 h, with a mean value of 0.06 ± 0.02 h. However, terminal half-lives varied significantly, with values ranging from 0.6-58.3 h. The long terminal half-life is probably due to a pool of stable 2-CdA phosphate metabolites (268,270). In contrast, cellular 2-CdA equivalents followed a monoexponential decline in cell suspensions diluted with medium containing NT inhibitors, NBMPR or DPM. The mean half-lives were 0.97 ± 0.3 h in the presence of 0.5 μM NBMPR, 1.11 ± 0.43 h in the presence of 1 μM NBMPR and 1.38 ± 0.47 h in the presence of 1 μM DPM. Table 20 summarizes the effects of NT inhibitors on the pharmacokinetic profile of 1 μM 2-CdA in the four ALL cell lines. A similar effect was seen when lower concentrations of 2-CdA (0.1 and 0.5 μM) were used to load cells (Fig. 39).

To follow changes with time in the cellular content of 2-CdA in cultured ALL lymphoblasts, the areas under the curves (AUC) of the time course data illustrated in Fig. 38 were determined (as described in Materials and Methods, 2.3.4). As the inhibitor effect was most pronounced in the early stages of the time course, and because of the high variability in the terminal half-lives, AUC of time courses were analyzed from 0-2 h ($AUC_{(0\text{-}2h)}$) for comparative purposes. The Tukey multiple comparisons test (252) performed on the $AUC_{(0\text{-}2h)}$ values showed a significantly ($p < 0.001$) lower content of 2-CdA equivalents in control cells relative to cells treated with inhibitors. The $AUC_{(0\text{-}2h)}$

Table 20

Cellular pharmacokinetic parameters for 2-CdA efflux in cultured human ALL lymphoblasts in the absence and presence of the NT inhibitors, NBMPR and DPM

Cellular decay of 2-CdA and its metabolites followed a biexponential decline when 2-CdA loaded cells were diluted 10-fold with drug-free medium (control). However, cellular loss of 2-CdA and its metabolites followed a monoexponential decline when 2-CdA loaded cells were diluted 10-fold with medium containing NT inhibitors, NBMPR or DPM.

	$t_{1/2\alpha}$ (h)[a]	$t_{1/2\beta}$ (h)	$t_{1/2}$ (h)		
Cell line	Control		0.5μM NBMPR	1μM NBMPR	1μM DPM
Reh-A2	0.09	4.2	1.49	1.79	2.21
SB	0.05	0.6	0.60	0.73	0.85
CEM	0.04	0.6	0.73	0.73	1.00
KM3-2B	0.06	58.3	1.06	1.20	1.47
Mean ± SD	**0.06 ± 0.02**	**15.9 ± 24.5**	**0.97 ± 0.34**	**1.11 ± 0.39**	**1.38 ± 0.47**

[a] $t_{1/2}$ values were calculated from time course data of 2-CdA equivalents *versus* time (Fig. 38) as described in Materials and Methods, 2.3.4.

Table 21

Retention of 2-CdA in cultured ALL lymphoblasts following exposure to NBMPR or DPM

$AUC_{(0\text{-}2h)}$ values were obtained from time course data for the decay of cellular 2-CdA and its metabolites (Fig. 38). The $AUC_{(0\text{-}2h)}$ values for the inhibitor-treated groups were significantly higher ($p < 0.001$, Tukey multiple comparisons test) than the corresponding values for the untreated control group.

	$AUC_{(0\text{-}2h)}$(pmol·h/10^6 cells)			
Cell line	Control	0.5 μM NBMPR	1 μM NBMPR	1 μM DPM
Reh-A2	18.9	26.5	28.0	28.7
SB	11.4	15.3	15.9	17.6
CEM	15.6	21.9	21.6	24.5
KM3-2B	14.9	18.4	19.3	19.1
Mean ± SD	**15.2 ± 3.1**	**20.5 ± 4.8**	**21.2 ± 5.1**	**22.5 ± 5.1**

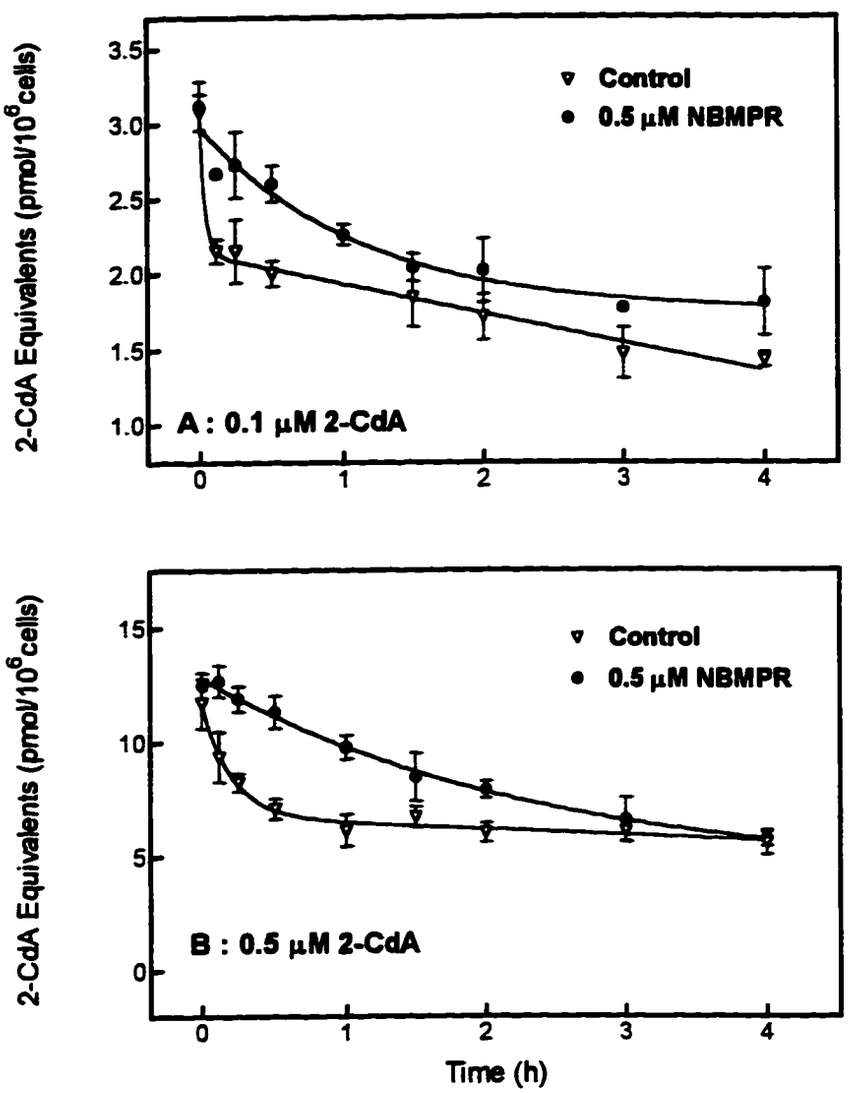

Figure 39. Effect of NBMPR on 2-CdA retention in Reh-A2 cells.
Cells were loaded with either 0.1 μM 2-CdA (Panel A) or 0.5 μM 2-CdA (Panel B) for 1 h at 37°C, cell suspensions were then diluted 10-fold with DPBS without additives (control) (▽), or with 0.5 μM NBMPR (●). Incubation at 37°C was continued, with samples (1×10^6 cells) taken for measurement of cellular ^{3}H-content (2-CdA and its phosphate metabolites) at the indicated intervals. Each point represents the means ± SEM of triplicate determinations from a single experiment.

values for control cells (15.2 ± 2.5 pmol·h/10^6cells) were significantly less than those for cells treated with 0.5 μM NBMPR (20.5 ± 4.1 pmol·h/10^6cells), 1 μM NBMPR (21.2 ± 4.4 pmol·h/10^6cells), or 1 μM DPM (22.5 ± 4.4 pmol·h/10^6cells) (Table 21). There was no significant difference between the cellular retention of 2-CdA induced by NBMPR and by DPM.

In conclusion, NBMPR and DPM significantly reduced the loss of 2-CdA equivalents from four cultured ALL lymphoblast lines by slowing the very rapid initial phase of 2-CdA efflux. As *es* was the only NT processes expressed in these cells and loss of 2-CdA equivalents was reduced by NT inhibitors, it appears that the *es* transporter may have contributed significantly to the loss of 2-CdA, particularly in the initial phase of efflux. These findings provide a rationale for the use of NBMPR or DPM as retentive agents in chemotherapy with nucleoside analogs.

Because *es* appears to be the only NT process expressed in these cells, exploitation of the efflux process may be less complex than if other nucleoside transporters were coexpressed with *es*. For example, the presence of the *cif* concentrative NT process, which is insensitive to NBMPR, would presumably increase the retentive effect, by allowing uptake of the nucleoside analog even in the presence of NBMPR. Alternatively, the presence of *ei* would likely reduce the retentive effect, by permitting 2-CdA efflux in the presence of NBMPR. The level of plasma membrane expression of these transporters that would influence the ability of NBMPR to block significant 2-CdA efflux is unknown at present.

3.3.2 Modulation of 2-CdA cytotoxicity by NBMPR

A prerequisite for the cytotoxicity of nucleoside drugs is the expression of functionally active nucleoside transport proteins in target cells. Therefore, the possibility exists to modulate nucleoside analog toxicity at the level of the transporter by means of the very potent NT inhibitors that are available today. One approach to increasing the cytotoxicity of nucleoside drugs is to block the efflux processes that participate in the loss of these agents from leukemic cells; this approach is referred to here as the "retention tactic".

3.3.2.1 Enhancement of 2-CdA cytotoxicity in the presence of NBMPR in Reh-A2 and CEM cell lines

In the present study the effect of a delayed, secondary exposure to NBMPR on the cytotoxicity of 2-CdA was examined in Reh-A2 and CEM cells, using the MTT dye reduction assay. As described for the "retention" experiments (described above), cells were loaded with 2-CdA and then cell suspensions were diluted 10-fold with 2-CdA-free medium to initiate the efflux process either in the presence or absence of 0.5 μM NBMPR. After a 2-h incubation period, cells were washed and resuspended in a drug-free medium and incubated for a further 69 h. The use of 0.5 μM NBMPR in the cytotoxicity assays was based upon three observations: (a) there was no significant difference between the ability of NBMPR or DPM to slow 2-CdA efflux, (b) a 0.5 μM plasma concentration of NBMPR may be clinically achievable (268), and (c) DPM is highly bound to plasma proteins (271,272), reducing available DPM concentrations in plasma significantly, and therefore its potential utility in clinical protocols.

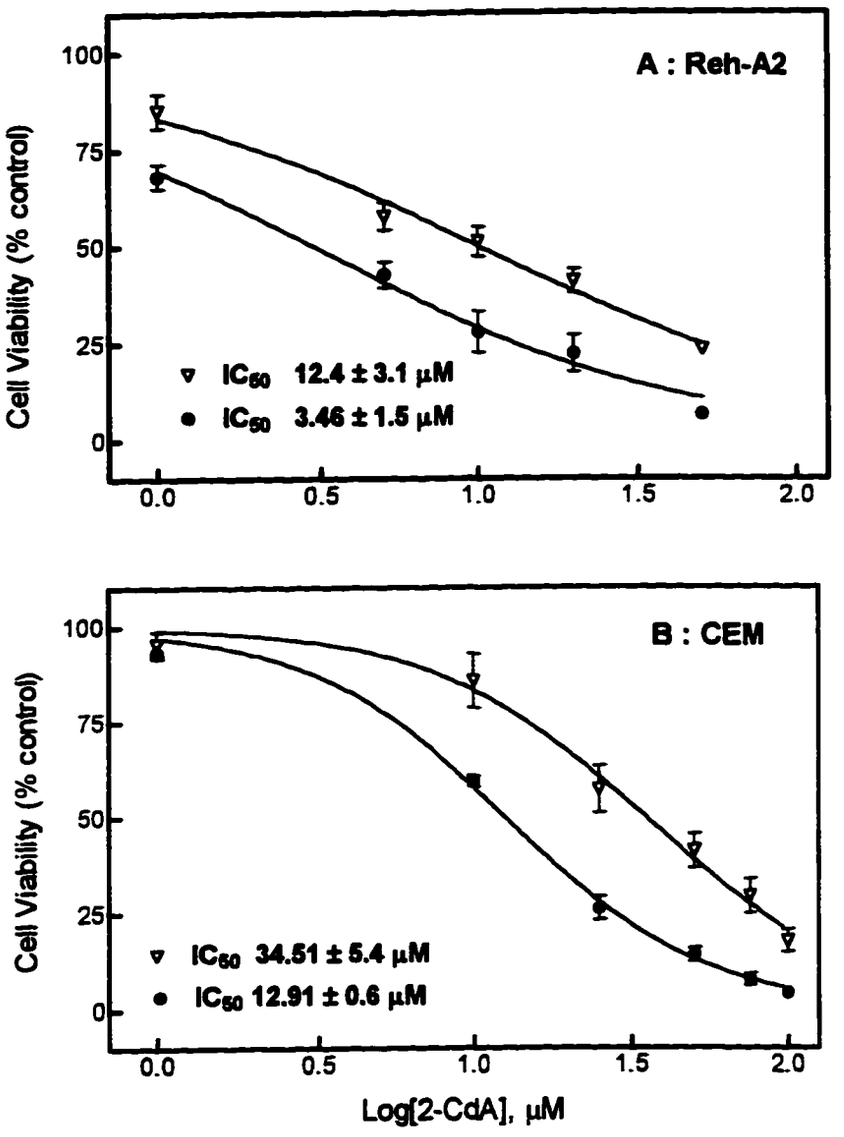

Figure 40. Enhancement of 2-CdA cytotoxicity by 0.5 μM NBMPR in Reh-A2 (Panel A) and CEM (Panel B) cells.

Cells were loaded with the indicated concentrations of 2-CdA for 1 h, cell suspensions were then diluted 10-fold with culture medium without (▽), or with 0.5 μM NBMPR (●). After 2 h, cells were washed twice and resuspended in culture medium without drug or inhibitor, and incubated for a further 69 h. The cytotoxicity of the 2-CdA exposure then was measured in a tertrazolium dye reduction assay (MTT) for cell viability. The data are means ± SEM obtained from 2-5 experiments. The IC_{50} values are the means ± SEM of values obtained from these experiments.

Figs. 40A and 40B show concentration-effect plots for Reh-A2 and CEM cells exposed to 2-CdA alone or to the 2-CdA/NBMPR sequence. There was a statistically significant ($p < 0.01$; Student's Paired t test) enhancement of cytotoxicity in both cell lines when the efflux of 2-CdA was slowed by NBMPR. This increase in cytotoxicity was probably the result of an increase in the cellular levels of 2-CdATP, the metabolite believed to be responsible for 2-CdA cytotoxicity. There was an approximately 3-fold reduction in the IC_{50} values for 2-CdA in cells exposed to the drug-inhibitor sequence (IC_{50} values for Reh-A2 and CEM cells exposed to the drug-inhibitor sequence were 3.5 ± 1.5 μM and 12.9 ± 0.6 μM, respectively) relative to cells exposed to 2-CdA alone under the conditions of these experiments (IC_{50} values for Reh-A2 and CEM cells exposed to 2-CdA alone were 12.4 ± 3.1 μM and 34.5 ± 5.4 μM, respectively). The concentrations of 2-CdA producing a 50% reduction in cell viability could not be determined exactly, because of the 10-fold dilution of the suspension of 2-CdA-loaded cells. The IC_{50} value estimates (Fig. 40) were based on the 2-CdA concentrations to which the cells were exposed to prior to the 10-fold dilution. Despite this, the IC_{50} values obtained demonstrate the enhancement of 2-CdA cytotoxicity arising from the delayed secondary exposure of the 2-CdA loaded cells to NBMPR. This effect was seen with both Reh-A2 and CEM lymphoblasts. NBMPR itself had no cytotoxic effect on either of the cell lines (data not shown).

3.3.2.2 Protection against 2-CdA cytotoxicity in Reh-A2 cells by NBMPR

NBMPR is a potent inhibitor of 2-CdA influx with IC_{50} values for inhibition of 2-CdA uptake ranging from 1-10 nM for the four cell lines (Table 10). The ability of

NBMPR to block both the influx and efflux of many nucleoside analogs currently used in the clinic, may complicate application of the pharmacologic manipulation of NT processes. For example, if cells were exposed to a 2-CdA/NBMPR combination for prolonged periods, uptake of 2-CdA may also be inhibited, potentially reducing 2-CdA cytotoxicity. To test this idea, 2-CdA-loaded cells (one-hour loading) were cultured with or without 0.5 μM NBMPR for 72 hours without washing out either drug or inhibitor. Fig. 41 shows that, under these conditions, the presence of NBMPR in the medium reduced by 5-fold the cytotoxicity of 2-CdA in Reh-A2 cells ($p < 0.005$; Student's Paired t-test). The IC_{50} value for cells exposed to 2-CdA alone was 0.14 ± 0.02 μM, compared with 0.66 ± 0.05 μM for cells exposed to the 2-CdA/NBMPR combination. As noted above, these IC_{50} values are based on the 2-CdA concentrations that cells were exposed to prior to the 10-fold dilution step. It appears that over time, the ability of NBMPR to block the influx of 2-CdA reduces its cytotoxicity.

In summary, it has been shown that low concentrations of NBMPR (0.5 μM) can significantly reduce the efflux of 2-CdA from cultured ALL cells, suggesting participation of the *es* NT process in the loss of 2-CdA from these cells. Associated with the NBMPR-induced 2-CdA retention was an enhancement in the cytotoxicity of 2-CdA.

3.3.3 Effect of NBMPR on the intracellular levels of 2-CdA and its metabolites in Reh-A2 and CEM cells

It is hypothesized that enhancement in 2-CdA cytotoxicity to lymphoblasts resulting from a delayed secondary exposure to NBMPR (as in the experiment of Fig.

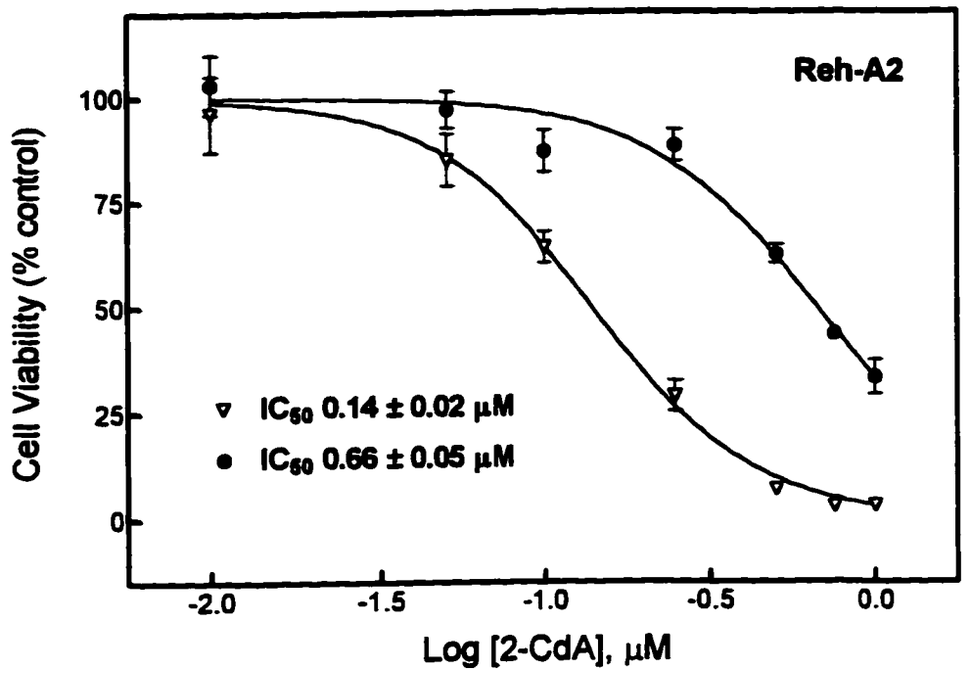

Figure 41. Reduction of 2-CdA cytotoxicity by 0.5 μM NBMPR in Reh-A2 cells. Cells were loaded with the indicated concentrations of 2-CdA for 1 h, cell suspensions were then diluted 10-fold with culture medium without (▽), or with (●) 0.5 μM NBMPR and incubated for a further 69 h. Each point represents the mean ± SEM from three separate experiments; the IC_{50} values are the means ± SEM of values obtained from these experiments.

40) is due to cellular retention of 2-CdA and its metabolites, particularly. Levels of 2-CdA and its metabolites were measured in Reh-A2 and CEM cells exposed to 2-CdA and in cells exposed to the 2-CdA/NBMPR sequence. As described for retention experiments, cells were loaded with 1 μM 2-CdA for one hour at 37°C after which the cell suspension was diluted 10-fold to initiate the efflux process. After a 2-h incubation period cells were cooled on ice, and metabolite levels determined by methanol extraction and HPLC analysis as described in Materials and Methods, 2.6.

HPLC analysis of cellular 2-CdA and its metabolites in methanolic extracts of 2-CdA-loaded Reh-A2 and CEM cells revealed the presence of three major products besides the parent drug (Figs. 42 and 43). The products appeared to be the mono-, di-, and triphosphate metabolites of 2-CdA because their HPLC retention times were similar to the retention times of dAMP, dADP and dATP (Table 22). Table 23 lists the levels of 2-CdA and its metabolites found in [^{3}H]2-CdA-loaded Reh-A2 and CEM cells after 2 h of incubation at 37°C with and without 0.5 μM NBMPR. In Reh-A2 cells, a significant increase in the intracellular mono-, di, and triphosphate metabolites of 2-CdA ($p < 0.05$) was apparent in cells exposed to 0.5 μM NBMPR in the medium when compared with cells exposed to 2-CdA alone. An increase in intracellular 2-CdA levels was also observed in Reh-A2 cells exposed to NBMPR ($p < 0.05$), supporting the hypothesis that NBMPR enhances 2-CdA retention by blocking efflux via the *es* nucleoside transport protein. The presence of NBMPR in the growth medium did not alter the percentage of each metabolite formed (data not shown). Fig. 42 shows the enhanced retention by NBMPR of 2-CdA and its metabolites in Reh-A2 cells. Similar results (Fig. 43, Table 23) were observed in CEM cells, however, in these cells only 2-

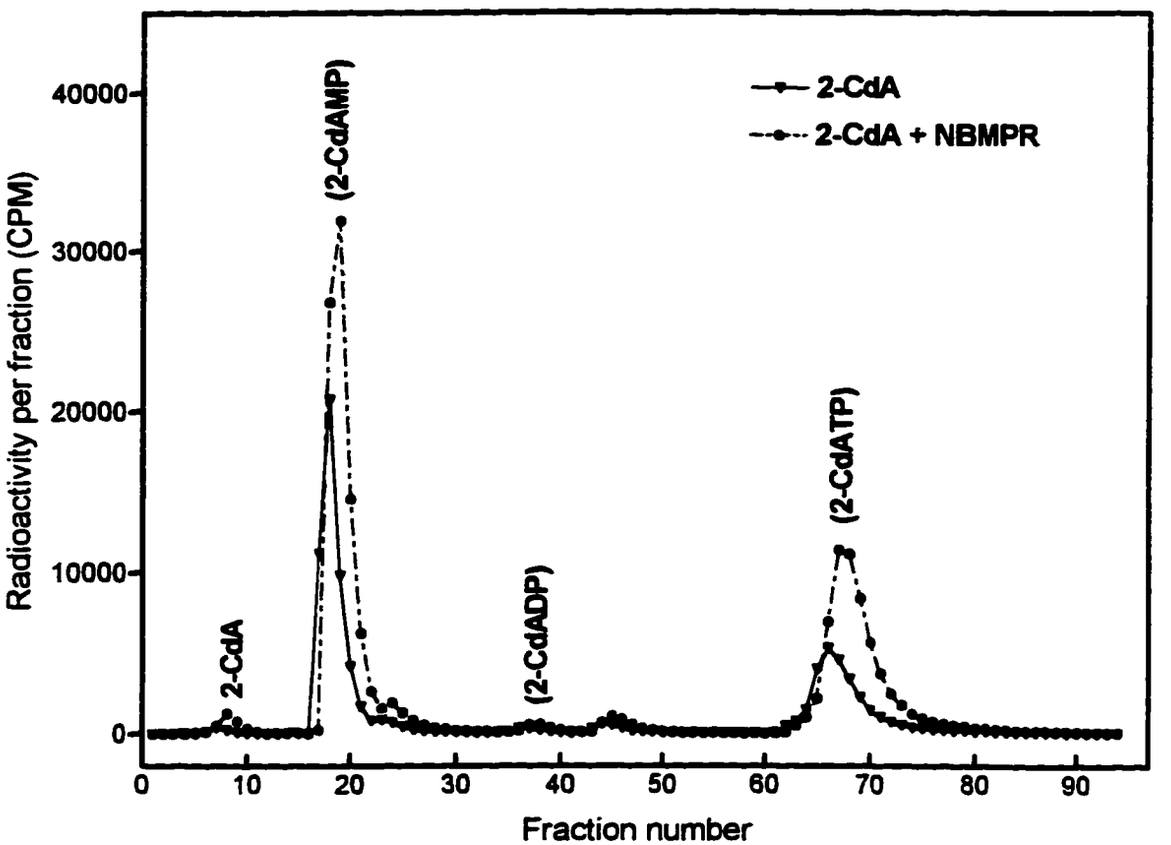

Figure 42. HPLC elution profile of labelled 2-CdA and its metabolites in methanolic extracts of Reh-A2 cells.

Cells were loaded with 1 μM [^{3}H]2-CdA for 1 h at 37°C, cell suspensions were then diluted 10-fold with RH10F medium without (– ∇ –) or with (---● ---) 0.5 μM NBMPR. Incubation at 37°C continued for 2 h, after which time 2-CdA cells were extracted with 70% methanol. Those extracts were chromatographed on a Partisil 10-SAX HPLC column and fractions were collected every 20 sec as described in Materials and Methods 2.6. The data presented here are representative of three experiments each performed in duplicate.

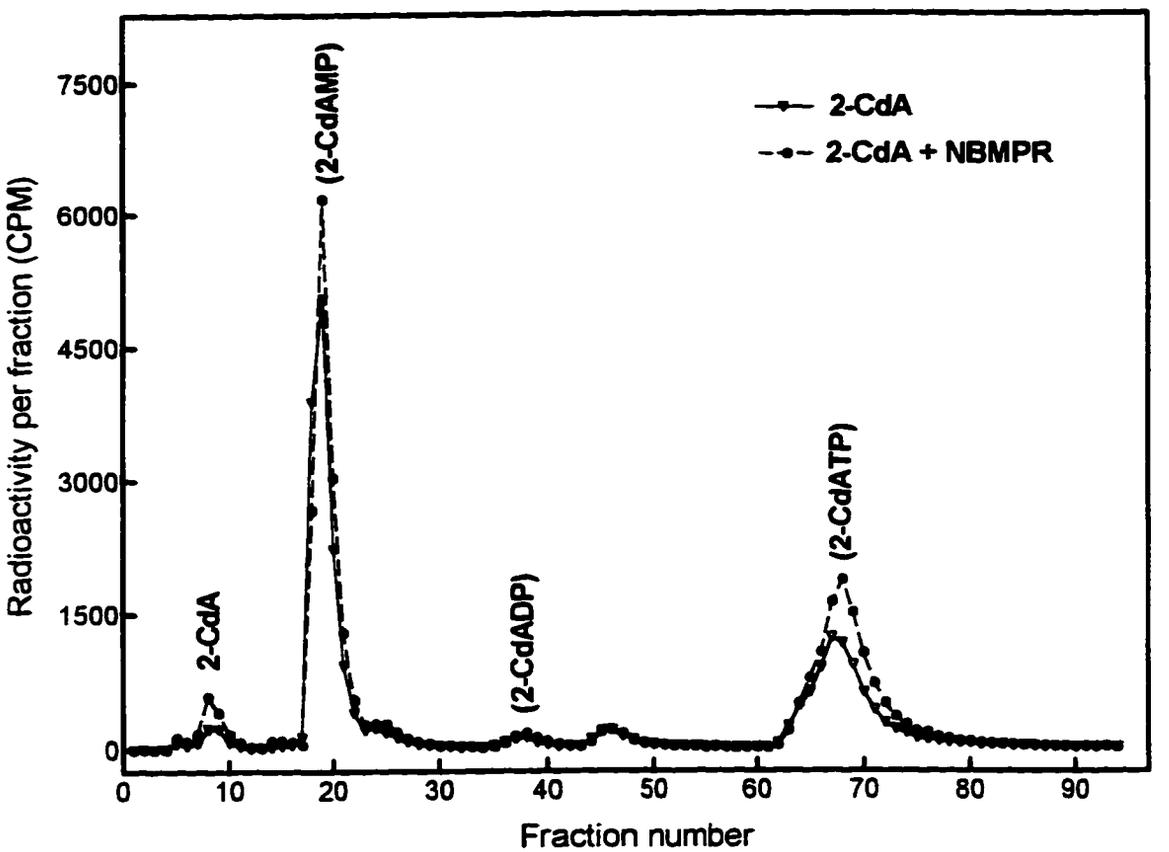

Figure 43. HPLC elution profile of labelled 2-CdA and its metabolites in methanolic extracts of CEM cells.
Cells were loaded with 1 μM [^{3}H]2-CdA for 1 h at 37°C, cell suspensions were then diluted 10-fold with RH10F medium without (– ▽ –) or with (---● ---) 0.5 μM NBMPR. Incubation at 37°C continued for 2 h, after which time cells were extracted with 70% methanol. Those extracts were chromatographed on a Partisil 10-SAX HPLC column and fractions were collected every 20 sec as described in Materials and Methods 2.6. The data presented here are representative of two experiments each performed in duplicate.

Table 22

HPLC retention times of 2-CdA and its metabolites

Cell extracts obtained as described in Section 2.6, and standards were chromatographed by HPLC on a Partisil 10-SAX anion exchange column as described in Materials and Methods.

Nucleoside	Retention time (min)
Standards	
2-CdA	2.1
dAMP	6.5
dADP	13.3
dATP	23.4
Cell extracts	
2-CdA	2.4
2-CdAMP	6.3
2-CdADP	12.8
2-CdATP	22.6

Table 23

Enhanced retention of 2-CdA and its metabolites in Reh-A2 and CEM cells by NBMPR

Cells loaded for 1 h with [^{3}H]2-CdA (1 μM) were diluted 10-fold in the presence or absence of NBMPR to initiate 2-CdA efflux. After a 2-h period cells were pelleted and methanol extracts prepared for HPLC analysis of the cellular content of 2-CdA and its metabolites.

		pmol/10^6 cells		Intracellular concentration (μM)	
Cell line	Metabolite	-NBMPR	+NBMPR	-NBMPR	+NBMPR
Reh-A2	2-CdA	0.03 ± 0.01[1]	0.05 ± 0.01	0.04 ± 0.01	0.10 ± 0.02*
	2-CdAMP	1.40 ± 0.22	2.54 ± 0.32	2.03 ± 0.32	3.73 ± 0.48*
	2-CdADP	0.02 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.07 ± 0.10*
	2-CdATP	0.50 ± 0.12	1.29 ± 0.31	0.77 ± 0.21	1.91 ± 0. 46*
CEM	2-CdA	0.05 ± 0.01	0.07 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
	2-CdAMP	0.86 ± 0.09	0.93 ± 0.12	0.55 ± 0.06	0.60 ± 0.07
	2-CdADP	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
	2-CdATP	0.36 ± 0.03	0.71 ± 0.06	0.23 ± 0.02	0.39 ± 0.05*

[1]Values represent the mean ± SEM of 4-6 determinations.

*Statistically different from intracellular metabolite levels in the absence of 0.5 μM NBMPR, $p < 0.05$.

CdATP levels were significantly ($p < 0.05$) increased in the presence of 0.5 μM NBMPR. The results presented in this section indicate that the increased sensitivity of Reh-A2 and CEM cells to the 2-CdA/NBMPR sequence was probably the result of an increase in the intracellular levels of the proposed active metabolite, 2-CdATP.

One recognized mechanism for 2-CdA or araC resistance is impaired phosphorylation (105,264). To decide if the difference in sensitivity to 2-CdA between Reh-A2 and CEM cells was due in part to different phosphorylation capacities, the levels of 2-CdA and its metabolites (-NBMPR, Table 23) were compared with IC_{50} values of 2-CdA cytotoxicity for these cell lines (Table 18). Reh-A2 cells were 5-fold more sensitive to 2-CdA relative to CEM cells. In Reh-A2 cells, the levels of 2-CdAMP and 2-CdATP were 4-fold and 3-fold higher, respectively, than those seen in CEM cells, despite the fact that intracellular 2-CdA levels were similar. These findings suggest that phosphorylation of 2-CdA was a major determinant in the cytotoxicity of this agent in Reh-A2 and CEM cells.

CONCLUSIONS

1. A large interindividual variation exists in the sensitivity to 2-CdA and araC of lymphoblasts from pediatric ALL patients.
2. Leukemic cells from pediatric ALL patients showed significantly ($p < 0.005$) greater sensitivity to 2-CdA compared with araC.
3. A linear relationship between sensitivity to 2-CdA and araC was observed in the ALL samples, suggesting similar or overlapping mechanism(s) of action and of resistance for these agents.
4. Patient-to-patient differences exist in the nucleoside transport processes expressed in fresh leukemic lymphoblasts.
5. The cellular abundance of the *es* transporter is not a major determinant of the sensitivity to 2-CdA of leukemic lymphoblasts from pediatric ALL patients.
6. In cultured lymphoblasts from four pediatric ALL cell lines, the *es* NT process accounted for most of the inward flux of 2-CdA.
7. In contrast to leukemic lymphoblasts from patients, cultured lymphoblasts were significantly ($p < 0.01$) more sensitive to araC than to 2-CdA.
8. Cultured leukemic lymphoblasts may not be an appropriate model for fresh lymphoblasts.
9. NBMPR and DPM significantly reduced the loss of 2-CdA and its metabolites from cultured lymphoblasts, suggesting participation of the *es* NT process in the loss of 2-CdA from these cells.
10. Sequential exposure of cultured lymphoblasts to 2-CdA and NBMPR (in that order) enhanced the cellular retention of 2-CdA and 2-CdA metabolites, and

potentiated the cytotoxicity of 2-CdA probably as the result of an increase in the intracellular levels of 2-CdATP.

SUMMARY and FUTURE DIRECTIONS

At present, a number of nucleoside drugs have useful clinical applications as antineoplastic and antiviral chemotherapeutic agents. Much effort has been aimed at relating the intracellular metabolism of the nucleoside drugs with their activity in the treatment of various disease states. However, few studies have examined the role of NT processes in the cytotoxicity of these agents, despite the fact that most nucleoside drugs require transporter-mediated entry into cells.

In the present study, the role of NT processes in the cytotoxicity of 2-CdA toward ALL cells was examined. 2-CdA is a purine nucleoside analog that has shown important activity in treatment of a number of lymphoid malignancies, but as yet, has not been extensively tested in the treatment of ALL. A goal of this study was to measure the *in vitro* sensitivity to 2-CdA of blast cells from previously untreated pediatric ALL patients, and to determine if the level of *es* nucleoside transporter expression in these cells was a factor in the cytotoxicity of this agent. The cytotoxicity of 2-CdA towards leukemic blasts was compared with that of araC, a pyrimidine nucleoside drug used in induction and consolidation protocols for treatment of ALL.

A large interpatient variation in sensitivity of ALL blasts to the nucleoside analogs was observed in this study with IC_{50} values for 2-CdA that ranged from 6 nM to > 5 μM, and from 65 nM to > 5 μM for araC after 72-h exposure periods (Table 15). The leukemic cells were significantly more sensitive ($p < 0.005$) to 2-CdA than to araC. In about one-half of the patient samples tested, IC_{50} values were similar to clinically achievable plasma levels of 2-CdA (141) and araC (156), which are approximately 23-85

nM and 60-800 nM, respectively. This result suggests that 2-CdA may be useful in the treatment of some pediatric ALL patients, whereas for others such treatment may be of little benefit.

In the present study, a linear correlation between the sensitivity of the leukemic cells to 2-CdA and araC was observed (Fig. 32), suggesting that the two agents may have a similar mode of action, or possibly a common mechanism of resistance. Therefore, in future studies to assess the potential usefulness of 2-CdA in the treatment of pediatric ALL, it would be meaningful to compare the cytotoxicity of 2-CdA and araC in lymphoblasts from relapsed patients who had previously been exposed to araC.

Previous studies have shown that the cellular *es* site content was a determinant of araC influx (194), araCTP formation (265) and araC efficacy in acute leukemia (236). The present study compared the *in vitro* sensitivity to 2-CdA and araC and the *es* site content of lymphoblasts from pediatric ALL patients. It was hypothesized that *es*-mediated inward flux of 2-CdA and araC would be a determinant of their cytotoxicity, and that *es* site abundance may be a predictor of sensitivity or resistance to these agents. A flow cytometric assay, employing an *es*-specific ligand, 5-(SAENTA-x8)-fluorescein, was used to determine the cellular content of the *es* transporter in lymphoblasts from pediatric ALL patients. No direct relationship was apparent between sensitivity to 2-CdA and *es* site content in cells from patients (Fig. 33A and Table 16), suggesting that *es* site abundance may not be the rate-limiting determinant in the response to 2-CdA. In contrast, when araC sensitivity in blast cells was correlated with *es* site content, a linear relationship, although not statistically significant, was apparent (Fig. 33B and Table 16).

Confounding factors in this study may include the presence of other NT

processes that are not identified by this flow cytometric probe. For example, in the present study, in 3 out of 5 samples tested at least two NT processes were identified, including *es* and *cs* transporters. Thus, although cells may have a low *es* site content, they may express other NT processes which participate in the uptake of 2-CdA. In this context, it is noted that the present study did not attempt to measure other mechanisms of 2-CdA resistance, such as reduced deoxycytidine kinase levels or reduced activity of DNA polymerases (141). Further studies that identify the type and cell content of NT processes expressed in leukemic blasts, and that measure deoxycytidine kinase levels, are required to understand the relative importance of nucleoside transport in the efficacies of 2-CdA and araC.

As the accrual rate of patient samples was low in this study and samples were of a "once-only" nature, model systems were perceived to be important for characterizing NT processes and for assessing nucleoside drug sensitivity in ALL cells. Four pediatric ALL cell lines (Reh-A2, CEM, SB and KM3-2B) were chosen as model systems for the study of NT processes in ALL cells. Kinetic studies of NT identified the *es* transporter as the only NT process expressed in these cell lines. In contrast, at least two NT processes were identified in patient lymphoblasts, including, *es* and *cs* transport processes, suggesting that patient-to-patient differences probably exist in the uptake mechanisms for 2-CdA to leukemia cells (Figs. 18-20). Other differences between lymphoblasts from patient samples and cultured lymphoblasts included the following: (a) inward fluxes of 2-CdA were approximately 10 to 20-fold lower in fresh pediatric ALL lymphoblasts than in cultured ALL lymphoblasts, (b) cultured ALL lymphoblasts had a 5 to 20-fold greater abundance of *es* sites than patient lymphoblasts, and (c) in contrast to

blast cells from patient samples, cultured lymphoblasts were significantly ($p < 0.01$) more sensitive to araC than to 2-CdA. Moreover, there was little difference in sensitivity to araC among the cell lines.

Although cells from only four cell lines were examined in this study, the differences between the fresh leukemic cells and the cultured leukemic cells in nucleoside analog sensitivity and in rates of inward nucleoside transport raises the question of the suitability of cultured lymphoblasts as models for fresh lymphoblasts from pediatric ALL patients. However, cultured lines of lymphoblasts are still a valuable experimental tool in that they provide a means of studying many biochemical aspects of drug action.

Rustum and Preisler (239) showed a significant correlation between *in vitro* retention of araCTP in acute myelocytic leukemia cells and the duration of remission of patients receiving araC treatment. A later study by Kufe *et al.* (240) demonstrated a relationship between the cellular levels of araCTP, incorporation of araCTP into DNA and the cytotoxic potency of this agent. Therefore, increasing intracellular levels of 2-CdA and its metabolites through blockade of 2-CdA efflux would likely increase the cytotoxicity of 2-CdA. An aim of the present study was to decide if decay of cellular levels of 2-CdA and its metabolites might be impeded at the level of 2-CdA membrane transport. Alessi-Severini *et al.* (268) reported a biexponential decay in cellular levels of 2-CdA and phosphate derivatives thereof in leukemic cells from CLL patients. That decay process proceeded when 2-CdA-loaded cells were transferred to 2-CdA-free medium and was in part attributable to an NBMPR-sensitive, transporter-mediated efflux process. In the present study, NBMPR and DPM significantly reduced the loss of 2-CdA and its metabolites from four ALL cell lines. In the presence of *es* inhibitors,

rates of decline in the cellular levels of 2-CdA and metabolites were much reduced, but still proceeded with monoexponential kinetics (Fig. 38). Because the ***es*** transporter was the only NT process detected in these cells, and the loss of 2-CdA and its metabolites was reduced by NT inhibitors, it appears that the ***es*** nucleoside transporter contributes significantly to the efflux of 2-CdA in cultured ALL lymphoblasts.

The operation of multiple NT processes in leukemic cells would likely influence attempts to pharmacologically manipulate transport fluxes of nucleoside drugs by means of NT inhibitors. For example, the expression of the ***cib*** NT process or other Na^+-dependent systems that are insensitive to NBMPR would probably increase cellular retention of 2-CdA and that of other nucleoside analogs by allowing influx of nucleoside analogs even in the presence of NT inhibitors, which would block the ***es*** transporter-mediated efflux process. Alternatively, expression of the ***ei*** NT process would likely reduce the retentive effect by permitting 2-CdA efflux even in the presence of NBMPR.

The blockade by NBMPR of transporter-mediated uptake of many nucleoside analogs complicates the potential usefulness of the retention tactic in the clinic. For example, if 2-CdA-loaded cells were exposed to NBMPR for prolonged periods, uptake of 2-CdA would be inhibited, presumably with reduction of 2-CdA cytotoxicity. This situation would likely be encountered with *in vivo* treatment of the 2-CdA/NBMPR combination, if these agents were administered in that order, as NBMPR would be tightly bound by ***es*** sites on erythrocytes and endothelial cells, and therefore would be released into the circulation slowly with time. In the present study, 2-CdA-loaded cells were cultured with or without 0.5 μM NBMPR for 72 h without washing out either drug or inhibitor. Under these conditions the presence of NBMPR in the medium

reduced by 5-fold the cytotoxicity of 2-CdA in Reh-A2 cells (Fig. 41).

The cytotoxic effects of 2-CdA toward ALL lymphoblasts following a 1-h exposure to 2-CdA with a further 2-h exposure to NBMPR was examined in cultured Reh-A2 and CEM cells (Fig. 40). Under these conditions, a significant enhancement ($p < 0.01$) of 2-CdA cytotoxicity was observed in the presence of 0.5 μM NBMPR, suggesting a potential therapeutic tactic for NBMPR induced-cellular retention of nucleoside analogs. HPLC analysis of cellular levels of 2-CdA and its metabolites were determined in 2-CdA loaded Reh-A2 and CEM cells after 2 h of dilution-induced efflux. In the presence of 0.5 μM NBMPR, a significant increase ($p < 0.05$) in 2-CdATP levels occurred in both cell lines (Figs. 42, 43 and Table 23), suggesting that the enhancement by NBMPR of the cytotoxic effects of 2-CdA is attributable to increased retention of a cytotoxic metabolite of 2-CdA.

These findings suggest that a clinical application of the 2-CdA/NBMPR combination in that sequence would probably have to consist of single treatments with 2-CdA followed by NBMPR, with time allowed for NBMPR clearance from the circulation so that uptake by the target cells of a subsequent 2-CdA dose would not be impaired by NBMPR. Alternatively, the 5- or 7-day schedules for administration of 2-CdA currently used in the clinic might be ended by the addition of an NBMPR dose to enhance 2-CdA retention. The probability of increasing cellular drug levels in dose-limiting tissues is a potential problem associated with the use of NBMPR as a retentive agent. An understanding of how various NT processes at sites of dose-limiting toxicity and at leukemic cells, affect the ability of NBMPR to enhance the cytotoxicity of nucleoside analogs should be an important component of future studies.

In conclusion, although cellular *es* site abundance may not be the major determinant in the response of lymphoblasts from pediatric ALL patients to 2-CdA, an understanding of the pharmacological manipulation of nucleoside transport processes may enhance the use of nucleoside analogs, such as 2-CdA, in the treatment of the leukemias.

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