BIOCHEMICAL STUDIES OF DEOXYADENOSINE IN L1210 CELLS

A Dissertation

Presented to

The Faculty of the Department of Biochemical

and Biophysical Sciences

University of Houston

In Partial Fulfillment

of the Requirement for the Degree

Doctor of Philosophy

By

Gil-Jong Kang

August, 1983

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ABSTRACT

The combination of 2'-deoxyadenosine and deoxycoformycin is known to be markedly toxic to T-lymphocyte cell lines relative to B-cell lines and this difference appears to be related to the capacity of the cells to accumulate dATP. In the presence of dipyridamole and 2'-deoxyadenosine and when adenosine deaminase was inhibited with deoxycoformycin, the L1210 leukemia cell which is a non-T, non-B cell type accumulated dATP like a T-cell type. The intracellular L1210 dATP levels using the triple combination (1.1 μM deoxycoformycin, 40 μM deoxyadenosine, 10 μM dipyridamole) reached 360 nmol/10^9 cells at which concentration ribonucleotide reductase specific activity was reduced by 80%. In addition to the increase in dATP levels, intracellular L1210 2'-deoxyadenosine levels as high as 375 nmol/10^9 cells were obtained with the triple combination.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Two major mechanisms of deoxyadenosine (dAdo)[1] toxicity have been proposed: the accumulation of dATP (Carson et al, 1979, 1977; Fox et al., 1981; Mitchell et al., 1978; Ullman et al., 1978), which is known to be a feed-back inhibitor of ribonucleotide reductase (Lin and Elford, 1980); and the inactivation of S-adenosylhomocysteine (SAH) hydrolase with subsequent inhibition of S-adenosylmethionine-dependent methylation reactions (Cass et al., 1982; Hershfield, 1979). Combinations of dAdo and adenosine deaminase (ADA) inhibitors have been reported to be more toxic to T-lymphocyte cell lines than to B-lymphocyte cell lines (Carson et al., 1979; Fox et al, 1981; Mitchell et al., 1978; Ullman et al., 1978). Biochemical studies have shown that T-cells have an increased capacity to accumulate dAdo nucleotides and that this increased capacity was associated with one form of the Severe Combined Immunodeficiency Disease (SCID) syndrome where ADA activity was deficient (Donofrio et al., 1978; Giblett et al., 1972; Simmonds et al., 1978). dCF (deoxycoformycin), a potent inhibitor of ADA, which also causes increases in intracellular dATP levels, was recently found to be active in the therapy of Acute Lymphocytic Leukemia (ALL) in phase I clinical studies (Agarwal et al., 1982; Venner et al., 1982). A commonly used laboratory model of clinical ALL is the L1210 murine leukemia. The L1210 cell has surface antigens

which indicates that it is a non-T, non-B type, a characteristic shared with the most prevalent cell type found in ALL of childhood (Holcenberg and Camitta, 1981).

We demonstrate in this dissertation that the combination of dAdo, dCF, and dipyridamole appears to convert metabolically the L1210 cell from the non-T, non-B type to a T-cell type. When the drugs were used in vitro in triple combination, intracellular L1210 dATP levels as high as 360 nmol/10^9 cells could be obtained which was correlated with an 80% decrease in ribonucleotide reductase activity. In addition to the increase in dATP levels, intracellular L1210 dAdo levels as high as 375 nmol/10^9 cells were obtained after 3 hour incubation with the triple drug combination. This finding may possibly have utility in the clinical treatment of certain forms of childhood ALL.

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[1]The abbreviations used are: dAdo, deoxyadenosine; Ado, adenosine; ADA, adenosine deaminase; dCF, deoxycoformycin; ALL, acute lymphocytic leukemia; HPLC, high-performance liquid chromatography; TCA, trichloroacetic acid; DTE, dithioerythritol; CDP, cytidine 5'-diphosphate; EHNA, erythro-9-[3-(2-hydroxynonyl)] adenine; NBMPR, nitrobenzylthioinosine; SCID, Severe combined immunodeficiency disease; dATP, 2'-deoxyadenosine 5'-triphosphate; i.p., intraperitoneal; SAH, S-adenosylhomocysteine.

A. Adenosine Deaminase (ADA)

1. Adenosine Deaminase Deficiency and Severe Combined Immunodeficiency Disease.

In 1972, Giblett et al. (1972) reported an inherited deficiency of ADA in red blood cell hemolysates of two unrelated female children with SCID. This was the first instance of an association between an enzyme abnormality and a disease of specific immunity and represented the first evidence for the necessity of an intact purine catabolic pathway for maintenance of normal immune functions. SCID is a heterogeneous syndrome in which affected children lack both cell-mediated and humoral immunity. Children with SCID suffer from repeated infections by agents of fungal, protozoan, viral or bacterial origin, and unless treated by enzyme replacement die within the first few years of life. These patients have depressed circulating lymphocyte counts and usually have greatly reduced amounts of circulating immunoglobulins (Rosen, 1975). Most cases of SCID are inherited in either an X-linked or autosomal recessive mode (Rosen, 1975) and ADA deficiency accounts for approximately one third to one half of patients with the non-X-linked form of SCID (Meuwissen et al., 1975; Ackeret et al., 1976; Cohen, 1975): ADA deficiency is inherited in an autosomal recessive fashion (Meuwissen et al. 1975). The enzyme deficiency is easily detected in hemolysates and most patients show less than 1.5% of normal enzyme activity (Hirschhorn et al., 1979).

Plasma, urine, red blood cells, and mononuclear cells from patients with ADA deficiency have been shown to have abnormally high

concentrations of Ado, dAdo, and their metabolites. It has now been firmly established that dAdo is the major abnormal urinary purine derivative (Kuttesch et al., 1978; Donofrio et al., 1978; Cohen et al., 1978; Mills et al., 1978; Simmonds et al., 1979). Erythrocytes and mononuclear cells from ADA-deficient patients were demonstrated to have 24- to 300-fold higher than normal concentrations of dATP (Donofrio et al., 1978; Cohen et al., 1978; Coleman et al., 1978) suggesting that the accumulation of dATP might be causally related to the immunodeficiency.

2. Physical and Chemical Properties of Adenosine Deaminase and Tissue Distribution.

Adenosine deaminase is an enzyme involved in the salvage of nucleosides and catalyzes the irreversible deamination of (deoxy)adenosine to (deoxy)inosine. It is widely distributed in human tissues and human ADA has been shown to exist in different molecular and electrophoretic forms. Two soluble forms have been characterized; a small form (38,000 daltons) and a large form (298,000 daltons) (Van der Weyden and Kelley, 1976). The small form of the enzyme predominates in tissues such as spleen, stomach, thymus, lymph node, and erythrocytes, while the large form predominates in kidney, liver, pancreas, and lung (Hirschhorn et al. 1978; Hirschhorn and Levytska, 1974; Hopkinson and Harris, 1969; Edwards et al., 1971; Akido et al., 1972; Osborne and Spencer, 1973; Ma and Mager, 1975). The high mole-

cular weight tissue isozymes show diminished activity and are more heat stable than the low molecular weight form of the enzyme (Hirschhorn, 1976).

Conversion of the high molecular weight form of ADA to the low molecular weight form under denaturing conditions (Akido et al., 1972; Van der Weyden and Kelley, 1976), and the absence of tissue isozymes of ADA as well as a small molecular weight form of ADA in ADA-deficient patients with SCID (Hirschhorn et al., 1973) led to the conclusion that a single genetic locus was responsible for the multiple molecular weight forms of ADA. The relationship between the high and low molecular weight forms of ADA was clarified with the discovery in tissues containing high molecular weight ADA of a protein (binding protein or conversion factor) that could convert the low molecular weight form of ADA into the high molecular weight forms with the simultaneous loss of "conversion factor" activity (Van der Weyden et al., 1976; Nishihara et al., 1973, Schrader and Stacy, 1977). In human kidney, the binding protein is present in excess of ADA, 95% of the protein being unbound (Van der Weyden and Kelley, 1976, Schrader and Stacy, 1979). This tissue, therefore, has been used as a source for purification of the ADA-binding protein (Schrader and Stacy, 1977; Daddona and Kelley, 1978). No function for the ADA binding protein has as yet been determined. It has no effect on the kinetics of the enzyme, but an inverse correlation exists between the level of binding protein in tissue and the specific activity of the ADA in those

tissues (Van der Weyden and Kelley, 1976).

3. The Role of Adenosine Deaminase in the Purine Salvage Pathway.

ADA catalyzes the irreversible hydrolytic deamination of Ado and dAdo to inosine and deoxyinosine, respectively. Adenosine can be salvaged by two alternate routes: direct rephosphorylation to AMP by adenosine kinase or the sequential action of ADA, purine nucleoside phosphorylase and hypoxanthine phosphoribosyltransferase to produce IMP (Ado → Inosine → Hypoxanthine → IMP), which can be further converted to AMP and GMP. Adenosine kinase has a high affinity for adenosine (Km = 2 ~ 4 μM) (Schneble et al., 1967), and readily converts adenosine to AMP. On the other hand, adenosine deaminase has a low affinity for adenosine (Km = 24 - 52 μM) (Agarwal et al., 1975; Daddona and Kelley, 1977), but a much higher catalytic capacity. Thus at the low concentration of adenosine generated or encountered by most cells, adenosine kinase should have preferential access to adenosine and readily convert it back to AMP. Another metabolic fate of adenosine is condensation with homocysteine to form S-adenosylhomocysteine. S-adenosylhomocysteine is catabolized by hydrolysis to adenosine and L-homocysteine in a reaction catalyzed by S-adenosylhomocysteine hydrolase (de la Haba and Cantoni, 1959).

The catabolic pathway for AMP begins with the action of either 5'-nucleotidase and the formation of adenosine, or the action of AMP deaminase and consequent IMP production. Compared to AMP catabolism,

fewer options are available for the degradation of dAMP since AMP deaminase has little capacity to deaminate dAMP. Thus 5'-nucleotidase converts dAMP to dAdo. The dAdo produced may either be rephosphorylated to dAMP by deoxyadenosine kinase or converted to IMP via deoxyinosine → hypoxanthine → IMP. Adenosine deaminase has a much higher affinity for dAdo (Km = 7 μM) (Agarwal et al., 1975) than for adenosine and is readily able to dispose of low concentrations of this potentially toxic metabolite. Deoxyadenosine kinase (human thymic extract), on the other hand, is an enzyme or relatively low affinity for deoxyadenosine with a Km of 400 μM (Carson et al., 1977) and the deoxyadenosine kinase (L1210 cells) showed a Km for deoxyadenosine of 1.25 and 0.13 mM, respectively (Chang et al., 1982). Henderson et al. (1980) have suggested that, despite the high Km values, deoxyadenosine can be an effective substrate in intact cells because of the amounts of enzymes present and the duration of their action on substrate.

B. Biochemical Properties of Deoxycoformycin.

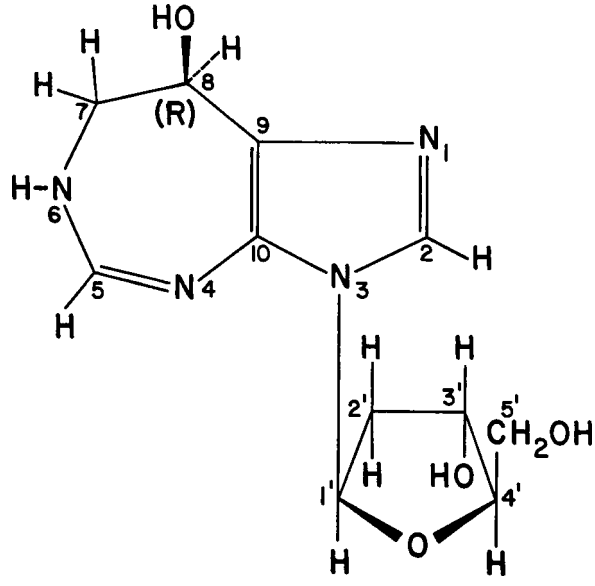
1. As an Inhibitor of Adenosine Deaminase.

dCF was isolated from the culture filtrates of S. antibioticus (NRRL 3238) (Woo et al., 1974). dCF is extremely sensitive to pH, which requires that it be isolated between pH 7 and 9.5 (Dion et al., 1977). The total chemical synthesis of dCF was accomplished by Baker and Putt (1979).

FIGURE 1

STRUCTURE OF DEOXYCOFORMYCIN

Structure of (R)-3-(2'-Deoxy-β-D-Erythro-Pentofuranosyl)-3,6,7,8-Tetrahydroimidazo[4,5-d][1,3]Diazepin-8-ol (2'-Deoxycoformycin).


HO
H
H
H
8
7
(R)
9
N1
H-N
6
10
2
H
5
N4
N3
H
H
H
2'
3'
5'
CH2OH
H
HO
1'
4'
O
H
H

Adenosine deaminase inactivates some antineoplastic agents that are analogs of adenosine. dCF is the most active adenosine deaminase inhibitor known. The Ki's for dCF with purified human erythrocytic and calf intestinal adenosine deaminase are 2.5×10^{-12} M (Agarwal et al., 1978) and 6.9×10^{-9} M (Johns and Adamson, 1976). The primary function of the deaminase inhibitor is to increase the therapeutic efficacy of adenosine analogs through the maintenance of high concentrations of these analogs. Combinations of adenosine analogs with ADA inhibitors are effective in the therapy of viral infections. These combinations also enhance the survival time of mice bearing i.p. L1210 cells (Brockman et al., 1976; Cass and Au-Yeung, 1976; LePage et al., 1976; Kimball et al., 1976; Schabel et al., 1976). Plunkett et al. (1978) have shown that a single i.p. injection of dCF into mice bearing P388 cells inhibited ara-A deamination in vivo and increased the recovery of ara-A over the control within 30 minutes. Cellular ara-ATP levels increased as did the extent and duration of inhibition of DNA synthetic capacity of P388 cells and CHO cells (Shewach and Plunkett, 1982). [^{3}H]dCF appears rapidly in the plasma following i.m. or i.v. administration (Chang et al., 1975). Rogler-Brown et al. (1978) have also demonstrated that dCF is not a substrate for purine nucleoside phosphorylase. Chang and Glazko (1976) showed that [^{3}H]ara-A is rapidly deaminated by erythrocytes to ara-HX. Addition of dCF completely prevents ara-A from enzymatic deamination and results in an increased accumulation of ara-AMP, ara-ADP, and

ara-ATP.

2. As a Tight-Binding Inhibitor.

The inhibition of ADA by dCF has been reported to be noncompetitive by Borondy et al., (1977). Cha et al. (1975, 1976) introduced a more reliable technique to study enzyme inhibition by tight-binding inhibitors. The classical methods of kinetic study based on steady-state kinetics (Lineweaver-Burk analysis) cannot be applied in the presence of tight-binding inhibitors. Agarwal et al., (1978) speculated that dCF is a competitive inhibitor of ADA. The tight-binding of dCF to ADA is presumed to be due to the presence of the tetrahedral carbon at the eight position of dCF which is an analog of the transition state compound formed during the deamination of adenosine (Evans and Wolfenden, 1970).

3. Inactivation and Reactivation of Adenosine Deaminase with dCF.

Agarwal et. al. (1977, 1978) and Rogler-Brown et al. (1978) have demonstrated that the erythrocyte membrane plays a key role in the association of dCF with ADA, and that the nucleoside transport system found in the cell membranes of erythrocytes and other tissues plays a significant role in the influx of dCF. The apparent K_1 values (the second-order rate constant) in intact erythrocytes are about 300 to 500-fold lower than the k_1 values determined either with hemolyzed human erythrocytes or with partially purified human erythrocytic ADA.

In intact erythrocytes only slight reactivation (< 10 per cent) of the inhibited ADA (EI complex) was detectable over 24 hours, whereas with hemolysates about 50 per cent reactivation of the inhibited ADA was observed in about 25 hours. Therefore ADA does not undergo permanent inactivation when inhibited by dCF even over prolonged periods.

4. Phosphorylation of Deoxycoformycin.

Venner and Glazer (1979) demonstrated that dCF is metabolized by L1210 cells only to the dCF monophosphate and this metabolite accounts for 3-16% of the total intracellular concentration of dCF. This anabolic process presumably occurs via deoxyadenosine kinase.

C. Increased Toxicity of Deoxyadenosine in the Presence of Deoxycoformcycin.

Lapi and Cohen (1977) have demonstrated that low concentrations of dAdo are not inhibitory to L (mouse fibroblast) cells. However, dAdo, at these same concentrations, kills L cells that have been exposed to dCF, even when dCF is removed from the culture medium. Henderson et al. (1977) reported that dCF potentiates the toxicity of adenine in cultured mouse lymphoma cells. Lowe et al. (1977) showed that low concentrations of dAdo in the presence of dCF, inhibited the growth of cultured L 5178Y cells and also caused inhibition of DNA synthesis and accumulation of cells in G_1 or early S phase. Since then, the introduction of drugs inhibiting ADA, in particular dCF (which is currently

under trial in the management of T-cell acute lymphoblastic leukemia) has stimulated interest in the mechanisms of cytotoxicity of dAdo (Prentice et al., 1980; Siaw et al., 1980). It has been reported that cultured human T- and null-cell leukemic lymphocytes in the presence of an ADA inhibitors are extremely sensitive to growth inhibition by dAdo (Carson et al., 1979; Fox et al., 1980; Wortmann et al., 1979). By contrast EBV-transformed B-cell, lines were quite resistant (Fox et al. 1981).

D. Possible Mechanisms of Deoxyadenosine Toxicity.

1. Inhibition of Ribonucleotide Reductase by dATP Accumulation.

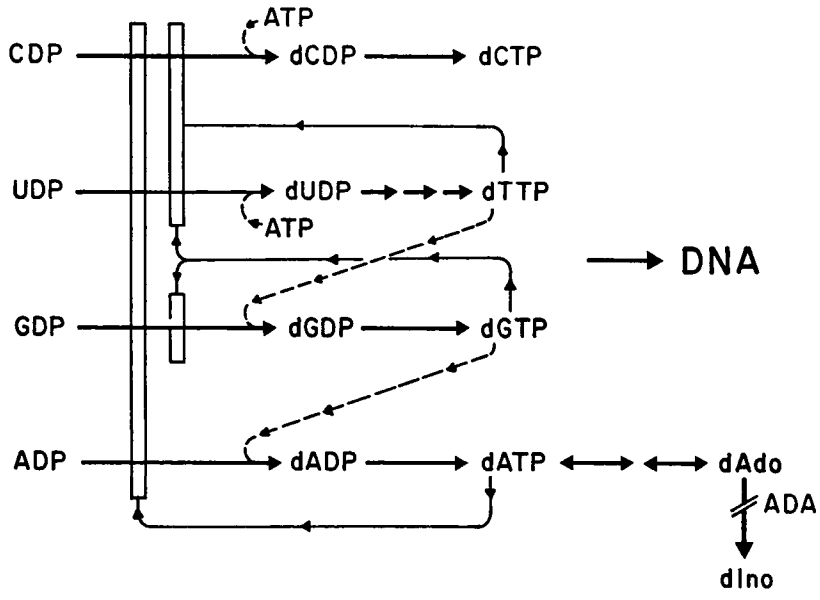
Carson et al. (1977) suggested that the dAdo toxicity might be caused by the "trapping" of dAdo in nucleotide form in lymphoid tissues through the action of deoxynucleoside kinases. Since the Km of ADA for dAdo is 7 μM (Agarwal et al., 1975) while that of deoxyadenosine kinase is 400 μM (Carson, et al., 1977), when ADA is present, dAdo is likely to be deaminated rather than phosphorylated at low substrate concentrations.

The enzyme ribonucleotide reductase catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates. It is the only known route for the de novo synthesis of deoxyribonucleotides from ribonucleotides in mammalian cells. Therefore this enzyme is one of the targets for anticancer agents. In eucaryotic systems, isotope experiments with regenerating rat liver (Larsson and

FIGURE 2

REGULATION OF DEOXYRIBONUCLEOTIDE SYNTHESIS

The broken arrows stand for positive effects, the open bars for negative effects, dIno; deoxyinosine.


ATP
CDP
dCDP
dCTP
UDP
dUDP
dTTP
ATP
DNA
GDP
dGDP
dGTP
ADP
dADP
dATP
dAdo
ADA
dIno

Neiland, 1966) strongly suggested that all DNA precursors were derived via the ribonucleotide reductase pathway. Deoxyribonucleotide synthesis begins with the reduction of CDP and UDP by an ATP-activated enzyme, proceeds to GDP reduction via a dTTP-activated enzyme, and finally, reaches ADP reduction by a dGTP-activated enzyme (Reichard, 1978). The activity of the enzyme for each of its four substrates is inhibited by dATP, with Ki's in the range of 4-20 μM, (Moore and Hulburt, 1966). Earlier workers established that high concentrations of dAdo (1-5 mM), without ADA inhibitors, cause the accumulation of dATP and inhibition of DNA synthesis in Ehrlich ascites cells (Klenow, 1959, 1962; Langer, 1960; Munch-Paterson, 1960; Overgaard-Hansen, 1961; Prusoff, 1959), chick embryo extracts (Maley and Maley, 1960), bovine liver cells (Wanka, 1974), and normal human peripheral blood lymphocytes stimulated with phytohemagglutinin (PHA) (Tattlersall et al., 1975).

In the presence of EHNA or dCF, dAdo was found to be more potent than Ado as an inhibitor of growth of peripheral blood lymphocytes (Carson et al,. 1977; Hirschhorn et al., 1979; Simmond et al., 1978) and as an inhibitor of growth of S49 mouse T-cell lymphoma (Ullman et al., 1978) and human leukemic cell line of T-cell origin (Mitchell et al., 1978; Fox et al., 1981). Deoxyadenosine in combination with EHNA or dCF caused the accumulation of dATP in S49 cells (Ullman et al., 1978), human leukemic cell line of T-cell origin (Mitchell et al., 1978; Fox et al., 1981), and HeLa cells (Lin and Elford, 1980). A

mutant of S49 mouse T-cells deficient in deoxyadenosine kinase was resistant to the growth-inhibitory effect of low concentrations of dAdo (Ullman, et al., 1978). This result indicated that intracellular phosphorylation of dAdo was required for the lethal effect against those cell lines. Furthermore, in S49 cells the accumulation of dATP induced by dAdo and EHNA was accompanied by a depletion of intracellular concentrations of dCTP, dGTP, dTTP (Ullman et al., 1978). dAdo toxicity in the presence of dCF or EHNA was reversed by addition of deoxycytidine to S49 cells (Ullman et al., 1978) and to a human leukemic cell line of T-cell origin (Mitchell et al., 1978; Fox et al., 1981). Deoxycytidine may be acting to inhibit dAdo phosphorylation either by inhibiting transport of dAdo or the activity of a deoxynucleoside kinase. This mechanism is supported by the finding that human leukemic T-cells treated with dAdo and deoxycytidine showed a 68% decrease in intracellular dATP when compared to cells treated with dAdo alone (Mitchell et al., 1978). However, there has been no direct measurement of ribonucleotide reductase activity in the increased intracellular dATP state except the measurement of ribonucleotide reductase by Lin and Elford (1981). They showed that ribonucleotide reductase activity was reduced to one-half of the control value in cells treated either with enough dAdo to inhibit HeLa cell growth or with a combination of EHNA and dAdo. Removal of dATP from those extracts increased ribonucleotide reductase activity to several-fold higher than control values. In spite of the evidence in support of

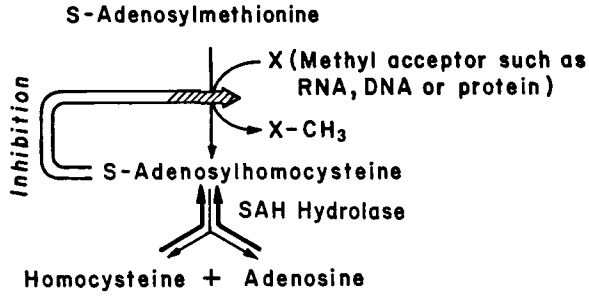
dATP accumulation and inhibition of ribonucleotide reductase, recently, dAdo toxicity of nondividing human lymphoid cells has been reported to occur by a mechanism which does not involve the inhibition of ribonucleotide reductase by dATP (Kefford and Fox, 1982).

In the course of studying dAdo toxicity in a variety of different cell culture systems, dAdo proved to be far more toxic to human lymphoblasts of T-cell origin than B-cell origin (Mitchell et al., 1978; Carson et al., 1978; Ullman et al., 1978). The increased sensitivity of human leukemic T-cell lines to dAdo as compared to B-lymphoblast lines appears to correlate with the lower activity of ecto-5'-nucleotidase in T-cell lines and a decreased ability to degrade deoxynucleoside triphosphates rather than to an increased capacity to synthesize dATP (Carson et al., 1979; Wortmann et al., 1979, 1980). It seems unlikely, however, that an enzyme located on the outside surface of the cell could regulate intracellular dAMP dephosphorylation. Carson et al. (1981) reported that human lymphoblasts also contain a soluble deoxynucleotidase activity that is distinguishable from the plasma membrane enzyme. In multiple human lymphoblastoid cell lines of varying origin and phenotype, soluble deoxynucleotidase correlated significantly with sensitivity to deoxyadenosine toxicity.

2. Inactivation of S-adenosylhomocysteine Hydrolase with Subsequent Inhibition of S-adenosylmethionine-dependent Methylation Reactions.

FIGURE 3

ROLE OF ADENOSINE IN S-ADENOSYLMETHIONINE-DEPENDENT METHYLATION REACTIONS.


S-Adenosylmethionine
X (Methyl acceptor such as RNA, DNA or protein)
X-CH3
Inhibition
S-Adenosylhomocysteine
SAH Hydrolase
Homocysteine + Adenosine

dAdo has recently been shown to be a potent, irreversible inhibitor of S-adenosylhomocysteine hydrolase by an apparent "suicidelike" mechanism (Hershfield, 1979). S-adenosylhomocysteine hydrolase catalyzes the reversible hydrolysis of S-adenosylhomocysteine (Richard et al., 1978). S-adenosylhomocysteine, a product of reactions catalyzed by S-adenosylmethionine dependent methyltransferases, is a potent methyltransferase inhibitor (Borchadt, 1980). Several recent reports have provided evidence of the importance of catabolism of S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase in the regulation of methylation of cellular components (Bader et al., 1978; Chiang and Cantoni, 1979; Hershfield, 1979; Hershfield and Kredich, 1978, 1980; Kredich and Hershfield, 1979; Kredich and Martin, 1977). Inhibition of S-adenosylhomocysteine hydrolase in intact cells by dAdo, ara-A, or 3-deazaadenosine results in elevated levels of S-adenosylhomocysteine (Bader et al., 1978; Chiang and Cantoni, 1979; Zimmerman et al. 1980; Helland and Ueland, 1982; Cass et al., 1982). S-Adenosylhomocysteine hydrolase is thus a potential biochemical target for anticancer or antiviral agents designed to interfere with the methylation of macromolecules (DNA, RNA, protein) (Borchadt, 1980; Chiang and Cantoni, 1979; Zimmerman et al., 1980). Intracellular levels of S-adenosylhomocysteine were elevated in cultured cells treated with L-homocysteine thiolactone, adenosine, and an inhibitor of ADA under conditions that resulted in inhibition of cellular proliferation and of methylation of DNA (Kredich and Hershfield, 1979; Kredich and Martin, 1977).

Ara-A, which is an antiviral and anticancer agent, inactivated S-adenosylhomocysteine hydrolase by pseudoirreversible binding to the enzyme like dAdo (Hershfield, 1979). Cass et al. (1982) demonstrated that S-adenosylhomocysteine hydrolase in crude extracts of L1210 leukemia cells was inactivated by ara-A and dAdo when these agents were protected from deamination. Clinically, inhibition of an S-adenosylhomocysteine hydrolase was observed in patients during ara-A therapy and this could contribute to the side effects and antiviral activity of ara-A (Sacks et al., 1982).

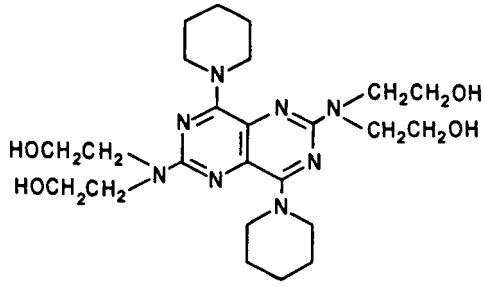
E. Dipyridamole

Dipyridamole (Persantin), or 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido(5,4-d)pyrimidine is a smooth muscle relaxant used as a coronary vasodilator; it also increases cardiac oxygen consumption. It decreases nucleotide breakdown, causes an accumulation of adenosine in hypoxic heart muscle, and increases blood adenosine concentrations by decreasing its uptake by erythrocytes (Henderson and Paterson, 1973). Dipyridamole inhibited the uptake of nucleosides and deoxynucleosides in chick fibroblasts (this inhibition of uptake of a given nucleoside can be reversed only by such nucleosides which are phosphorylated by the same nucleoside kinase) (Scholtissek, 1968), and in rat hepatoma cells (Plagemann, 1971), and inward and outward movement of deoxycytidine by murine leukemia cells unable to metabolize deoxycytidine (Kessel and Hall, 1970). Low concentrations of dipyri-

FIGURE 4

STRUCTURE OF DIPYRIDAMOLE

Structure of 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido (5,4-d) pyrimidine (Dipyridamole).


N
N
N
N
N
N
N
N
CH2CH2OH
CH2CH2OH
HOCH2CH2
HOCH2CH2

damole inhibited inward transport of phosphate, fucose, deoxycytidine and adenosine, and high concentrations of dipyridamole inhibited outward transport of these substances from pre-loaded cells (Kessel and Dodd, 1972). The transport of all deoxyribonucleosides were inhibited to about the same extent by dipyridamole in cultured Novikoff hepatoma cells (Ki = 1-2 μM) (Plagemann and Erbe, 1974). This multiplicity of effects is difficult to interpret since measurements using many different cell types were involved under varying conditions. Also, these experiments indicate that dipyridamole can modify activity of a variety of membrane transport systems. Recently Paterson et al. (1980) found that dipyridamole competed with NBMPR (Ki = 30 nM) for binding to the high affinity inhibitor sites on the nucleoside transport protein of HeLa cells, but they suggested that occupancy of a site on the transport system by either NBMPR or dipyridamole might not have the same ultimate consequences with respect to transport activity because of differences in the chemical structures of the two inhibitors.

It is not sufficient merely to determine the elevated dATP concentrations in cells treated with dAdo in the presence of ADA inhibitors in order to verify the ribonucleotide reductase hypothesis. It is necessary to evaluate the relevance of these elevations with respect to cell toxicity. It was, therefore, the purpose of this research to:

1. determine the relationship between intracellular dATP con-

centrations and the toxicity.

2. determine the direct relationship between intracellular dATP concentrations and inhibitions of ribonucleotide reductase activity.

CHAPTER II

MATERIALS AND METHODS

A. Drugs and Reagents

dCF was obtained from Drug Research and Development, National Cancer Institute, Bethesda, MD. The Ki value (6×10^{-11} M) for dCF was obtained using calf intestinal adenosine deaminase by the method of Cha *et al.* (Cha *et al.*, 1975; Agarwal *et al.*, 1977). dAdo, dipyridamole, nucleotide and nucleoside standards for HPLC, DTE, calf intestinal adenosine deaminase, apyrase, and alkaline phosphatase were purchased from Sigma Chemical Company, St. Louis, MO. Dowex-1-borate resin was prepared from Dowex-1-Chloride (X-8, 200-400 mesh) resin also obtained from the Sigma Chemical Company. [8-^{3}H]dAdo (specific activity, 13.5 Ci/mmole) was purchased from ICN pharmaceuticals Inc., Irvine, CA. [2,8-^{3}H]Ado (specific activity, 31.0 Ci/mmole) and [^{14}C]CDP (trisodium salt, specific activity, 476 mCi/mmole) were purchased from New England Nuclear, Boston, MA. Fischer's medium, horse serum, and penicillin-streptomycin solution (10,000 units/ml, 10,000 mcg/ml) were purchased from K.C. Biological, Inc. (Lenexa, KS). Trypan blue stain (0.4%) was purchased from GIBCO (Grand Island Biological Company, Grand Island, NY.).

dAdo was dissolved in a 0.9% NaCl solution, gently heated, and injected i.p. when cool for *in vivo* studies. dAdo purity after heating gently was checked by paper chromatography (n-butanol - gla-

cial acetic acid - water; 50:25:25).

B. L1210 Cells in Suspension Culture

L1210 cells were a gift from Dr. D.H.W. Ho of the University of Texas Cancer Center, M.D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston, Texas. L1210 cells for culture were prepared with cells from ascitic fluid from a DBA/2 mouse 6 days after implantation with 10^5 *in vivo*-passaged cells in December 1980. Stocks of vials containing 2 x 10^6 cells/ml in Fischer's medium with 10% horse serum and 10% dimethylsulfoxide were frozen and stored in liquid nitrogen.

L1210 cells were routinely maintained in 5% CO_2 - 95% air at 37°C in static suspension cultures in loosely capped bottles in a incubator (Model 329S, Forma Scientific, Marietta, Ohio). Cultures were diluted at 2 day intervals to 1 x 10^5 cells/ml with fresh growth medium composed of Fischer's medium containing 10% horse serum with penicillin (100 units/ml) and streptomycin sulfate (100 µg/ml).

C. *In Vitro* Inhibition Studies with Drugs

Duplicate 20 ml cultures containing about 1 x 10^5 cells/ml in growth medium supplemented with antibiotics were incubated in the presence and absence of drugs. The drugs were dissolved in saline and sterilized by filtration through a 0.22 µm pore diameter HA membrane (Millex filter units, Millipore Corp., Bedford, MA) immediately prior

to addition to cells. Cell numbers were determined at regular intervals using a Coulter counter, model ZBI (Coulter Electronics, Hialeah, FL). Cell viability was determined in a hemocytometer using the trypan blue dye exclusion method (Merchant et al., 1965).

D. Recovery of L1210 Cell Growth after Drug Treatment

L1210 cells were incubated with drugs for 72 hours at which time the cells were centrifuged at 1,800 g for 3 minutes and resuspended in drug-free medium. Cell numbers and viability were determined at regular intervals.

E. HPLC of Acid Soluble Metabolites

1. Extraction of Acid Soluble Metabolites

L1210 cells in 500 ml of Fischer's medium (0.7-1 x 10^6 cells/ml) were incubated with various concentrations of drugs. dCF was incubated with cells 1 hour before adding dAdo and/or dipyridamole in order to inhibit ADA completely. Nucleotides and other acid-soluble metabolites were extracted from the cells with 0.3 ml of perchloric acid at 0°C. After 30 minutes, the extracts were centrifuged at 10,000 g for 3 minutes and the acid-soluble extracts were neutralized with 7 N and 1 N KOH solutions. The $KClO_4$ precipitates were removed by centrifugation. These extracts were stored in liquid nitrogen until analysed.

2. HPLC of Intracellular Nucleotide Metabolites

HPLC analyses of acid soluble extracts of cells were performed on a Beckman Model 334 gradient liquid chromatography system with a model 421 system controller (Beckman Instruments, Inc., Berkeley, CA) employing Ultrasil-NH_2 columns. Samples (20 µl) were eluted at room temperature starting with 100% 5 mM $NH_4H_2PO_4$ (pH 2.8) and 0% 750 mM $NH_4H_2PO_4$(pH 2.6) for 1 min, and increasing to linearly 40% 750 mM $NH_4H_2PO_4$ at 81 min. The flow rate was 2 ml/min and the effluent was detected at 254 nm with a Hitachi spectrophotometer, model 100-10 (Hitachi, Ltd., Tokyo, Japan). Nucleotides were identified by their respective retention times and coelution with known standards. Quantitation was done with a Hewlett Packard Integrator, model 3390A (Hewlett Packard, Avondale, PA) or by a graphical method for small peaks that were not measurable with the integrator. HPLC scans were performed with the following parameters of the recorder held constant (Figure 5).

Peak capacity = 1,159

Zero = 0, -0.6

Attenuation 2 = -2

Chart speed = 0.5 cm./minute

Peak width = 0.16

Threshold = 0

Area rejection = 0

3. HPLC of Intracellular Nucleoside Metabolites

HPLC analysis of acid soluble extracts of cells were performed under the same conditions as outlined above except for flow rate (1 ml/minute), column, and buffer conditions. Samples (20 µl) were eluted at room temperature starting with 100% 0.5 M CH_3COONH_4 (pH 6.5) and 0% CH_3OH (50%) for 10 minutes, and increasing to 50% CH_3OH (50%) at 50 minutes using a Bio-Sil ODS-10 column (Bio-Rad Lab. Richmond, CA) (Figure 6).

4. Measurement of [^{3}H]dAdo Incorporation by HPLC

Measurement of [^{3}H]dAdo incorporation was carried out by treating cells with [^{3}H]dAdo and drugs as above. [^{3}H]dAdo metabolites were collected in fractions of 1 or 2 ml using an LKB 211 fraction collector (LKB Produkter AB, Bromma, Sweden) and the sample radioactivity was determined in a Beckman LS 9000 scintillation counter (Beckman Instruments, Inc. Fullerton, CA).

F. Incorporation of [^{3}H]dAdo and [^{3}H]Ado into Whole Cells and Acid Insoluble Materials

Suspensions of cells were treated with labeled Ado and dAdo as indicated in the appropriate experiments. Duplicate samples (1 ml) of cell suspensions at various times were analysed for radioactivity in total cell and acid insoluble materials. For the determination of total radioactivity, the cells were collected by centrifugation at

FIGURE 5

HPLC ANALYSIS OF STANDARD NUCLEOTIDES

Separation of 2 nmole each of standards using an Ultrasil-NH_2 column. Conditions for chromatography were as given in text.

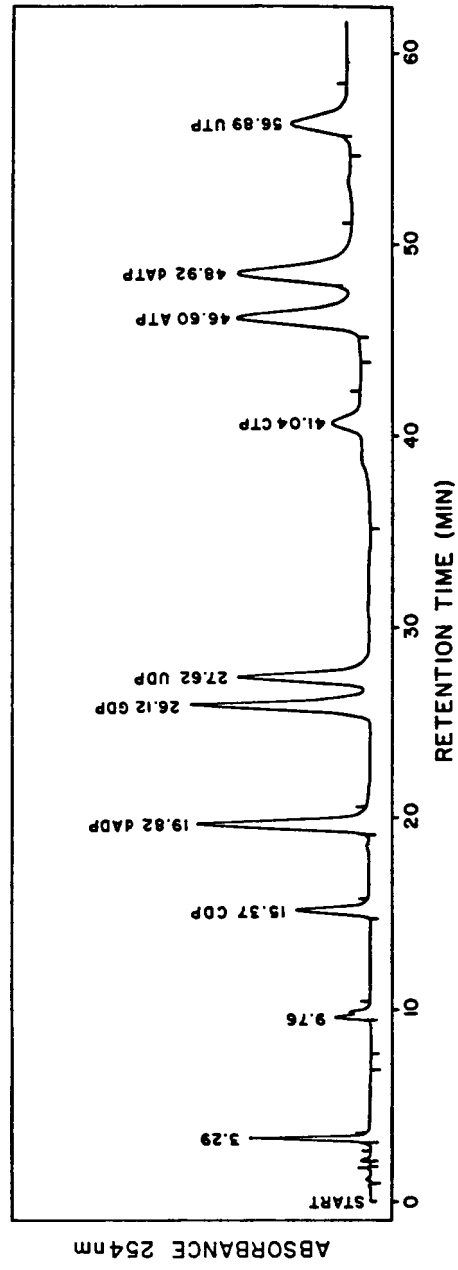
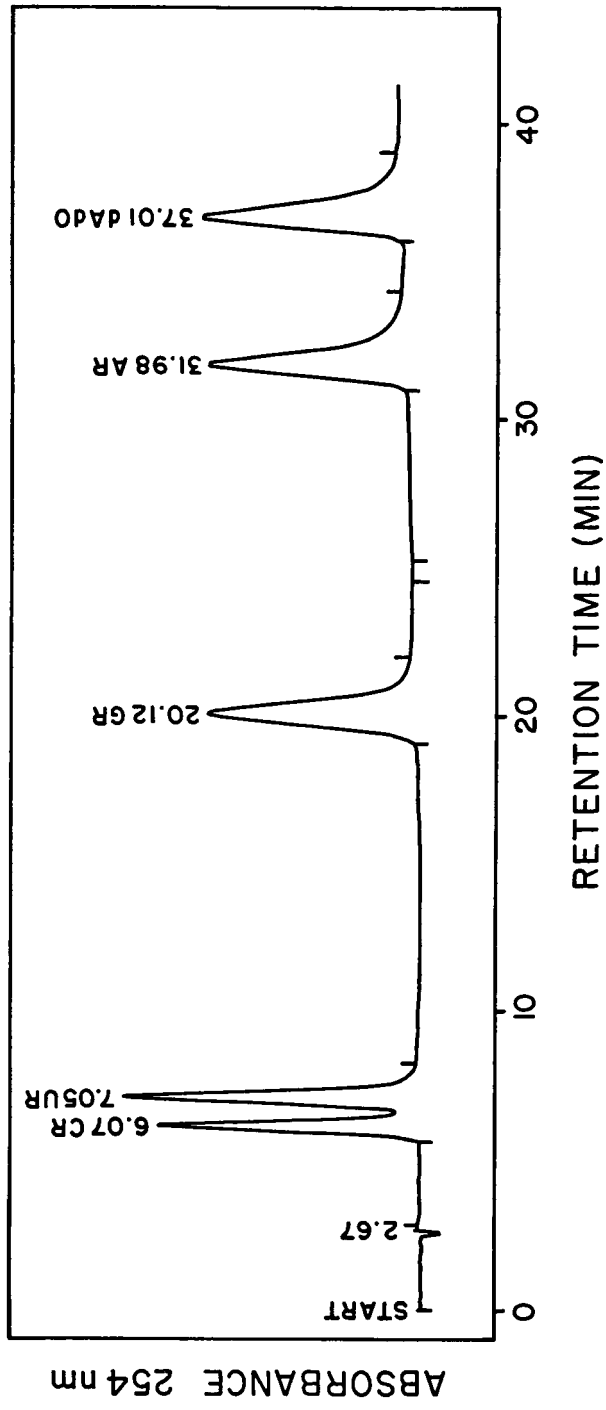

ABSORBANCE 254nm
START
3.29
9.76
15.37 CDP
19.82 dADP
26.12 GDP
27.62 UDP
41.04 CTP
46.60 ATP
48.92 dATP
56.89 UTP
0
10
20
30
40
50
60
RETENTION TIME (MIN)

FIGURE 6

HPLC ANALYSIS OF STANDARD NUCLEOSIDES

Separation of 1 nmole each of standards using an ODS-10 column. Conditions for chromatography were as given in text.


ABSORBANCE 254 nm
START
2.67
6.07 CR
7.05UR
20.12 GR
31.98 AR
37.01 dAdO
0
10
20
30
40
RETENTION TIME (MIN)

1800 g for 3 minutes at 4°C and the cells were rapidly washed with 5 ml of ice-cold saline solution and then suspended in 0.2 ml of 0.5 N TCA. This mixture was then heated at 70°C for 30 minutes and then counted for radioactivity. For the determination of radioactive acid-insoluble material only, other replicate samples were mixed with 1 ml of 1 N perchloric acid at 0°C, and the cellular precipitates collected by centrifugation at 1800 g for 3 minutes at 4°C. The precipitates were washed twice with 5 ml of cold 0.5 N perchloric acid and once with 5 ml of cold 0.5 N TCA. The precipitates were suspended in 0.1 ml of 0.5 N TCA and heated at 70°C for 30 minutes and counted for radioactivity after adding ACS II with a Beckman scintillation counter (Plagemann and Roth, 1969).

G. Ribonucleotide Reductase Assay

1. Preparation of Cell Extracts

L1210 cells from 100 ml of medium (0.6-0.8 x 10^6 cells/ml) were homogenized at 4°C in 0.3 ml of buffer (0.05 M Tris buffer, pH 7.5, containing 0.025 M DTE) using a Potter-Elvejhem homogenizer fitted with a power-driven teflon pestle. These homogenates were centrifuged at 100,000 g for 1 hr in a Beckman ultracentrifuge (Model L5-65) with a Beckman 50 Ti angle head rotor at 4°C. The extracts were used for the ribonucleotide reductase assays immediately after preparation.

2. Protein Assay

Protein concentrations were determined by Bradford's method (Bradford, 1976) using bovine gamma globulin as the standard.

3. Preparation of Dowex-1-borate Column

One Kg of Dowex-1-Chloride (X-8, 200-400 mesh) was washed extensively with 1 M HCl and the resin was rinsed with water until the pH of the washing solution was neutral. The Dowex-1-Chloride was then mixed with a saturated solution of sodium borate (1.5 l) and stirred for approximately 48 hours with several changes of fresh saturated sodium borate solutions. The resin was filtered, rinsed with water, and stored as a slurry in water.

4. CDP Reductase Assay

CDP reduction was assayed by the method of Chang and Cheng 1980) which is based on Steeper and Steuart's (1970) method. Dowex-1-borate ion exchange chromatography was used to separate CDP from dCDP. The assay mixture contained [^{14}C]CDP (0.2 µCi, 0.15 M), DTE (3 mM), $MgCl_2$ (6 mM), ATP (5 mM), and 30 µl of crude enzyme extract in a final volume of 0.2 ml. Incubations were carried out for 1 hour at 37°C. The reaction was stopped by placing the tubes in a boiling water bath for 2 minutes. After cooling the reaction tubes, 0.2 µmoles of deoxycytidine (10 µl) was added, and the nucleotides were converted to nucleosides by the sequential action of potato apyrase (40 µg/20 µl)

for 15 minutes and alkaline phosphatase (0.2 units/25 µl) for 90 minutes at 38°C. The tubes were heated for 2 minutes in boiling water. After centrifuging down the precipitated protein, the supernatant solution was applied to a Dowex-1-borate column (0.5 x 7.5 cm) that had been washed with 20 ml of saturated sodium borate solution followed by 20 ml of water immediately prior to use. Each reaction tube was then rinsed with 0.5 ml of water, which was also applied to the column, and allowed to absorb. An additional 1 ml of water was allowed to run through the column, after which 4 ml of water was applied and the effluent collected in a glass scintillation vial. ACS II (17 ml) was added to the vial, and the radioactivity determined in a Beckman liquid scintillation counter.

H. Survival Studies

Leukemia L1210 cells were maintained by weekly serial passage of 1 x 10^5 cells/mouse implanted i.p. in DBA/2 female mice, 18-20 g, purchased from Jackson Laboratory (Bar Harbor, Maine). Survival studies were carried out in female BDFI (C57BL x DBA/2) hybrid mice obtained from the Jackson Laboratory. BDFI mice, 18-20 g, were each implanted i.p. with 10^5 L1210 cells. Mice were randomized and selected into groups of 6 mice each such that the weights of mice within a group were ±1 g. Drugs in saline were given i.p. in 0.2 ml volumes. dCF, 0.5 mg/kg, was given once daily or every 12 hours for 2 doses on days 1, 4, 7. The dAdo was given at a 25, 50, 100, 125, or

150 mg/kg dose every 3 hours for 8 doses on days 1, 4, 7 (Skipper et al., 1967). Mice surviving more than 30 days were calculated as surviving only 30 days (Schabel et al., 1977). Mice that survived for 50 days were considered long term survivors. Daily records were kept of the average group weights and deaths resulting from drug toxicity were also noted.

CHAPTER III

RESULTS

A. L1210 Cytotoxic Effects of dCF, dAdo, and Dipyridamole Triple Combination Correlated with dATP Levels.

1. Effects of dCF and dAdo on the Growth of L1210 Cells *in vitro*

When L210 cells were incubated with either dCF (1.1 μM) or dAdo (40 μM) alone, there were no effects by either drug on cell growth. However, when the two drugs were added together, no growth occured. The cytotoxic effects of dCF and dAdo were markedly synergistic (Figure 7).

2. DATP Accumulation Dependency on dAdo Concentration

Since the dCF and dAdo combination gave consistent results *in vitro*, HPLC experiments were conducted to see to what extent L1210 cells could phosphorylate dAdo to dATP. Figure 14 shows that L1210 cells treated with dCF (1.1 μM) at various concentrations of dAdo and incubated *in vitro* for 3 hours converted dAdo to dATP. The dATP accumulation was dependent on the concentrations of dAdo. The level of dATP in cells incubated with dCF (1.1 μM) only for 3 hours was less than 10 nmole/10^9 cells and the level of dATP resulting from incubation with dAdo (40 μM) only was less than 15 nmol/10^9 cells. This experiment showed that L1210 cells had the capacity to synthesize and accumulate dATP when supplied with dAdo in the presence of dCF *in*

FIGURE 7

IN VITRO INHIBITORY EFFECTS OF dCF AND dAdo ON THE GROWTH OF L1210 CELLS

Cells were treated with dCF for 1 hour and then were incubated with dAdo. Conditions for cell growth were as described in text.

● Control

O dAdo (40 μM)

Δ dCF (1.1 μM)

▲ dCF (1.1 μM) + dAdo(40 μM)

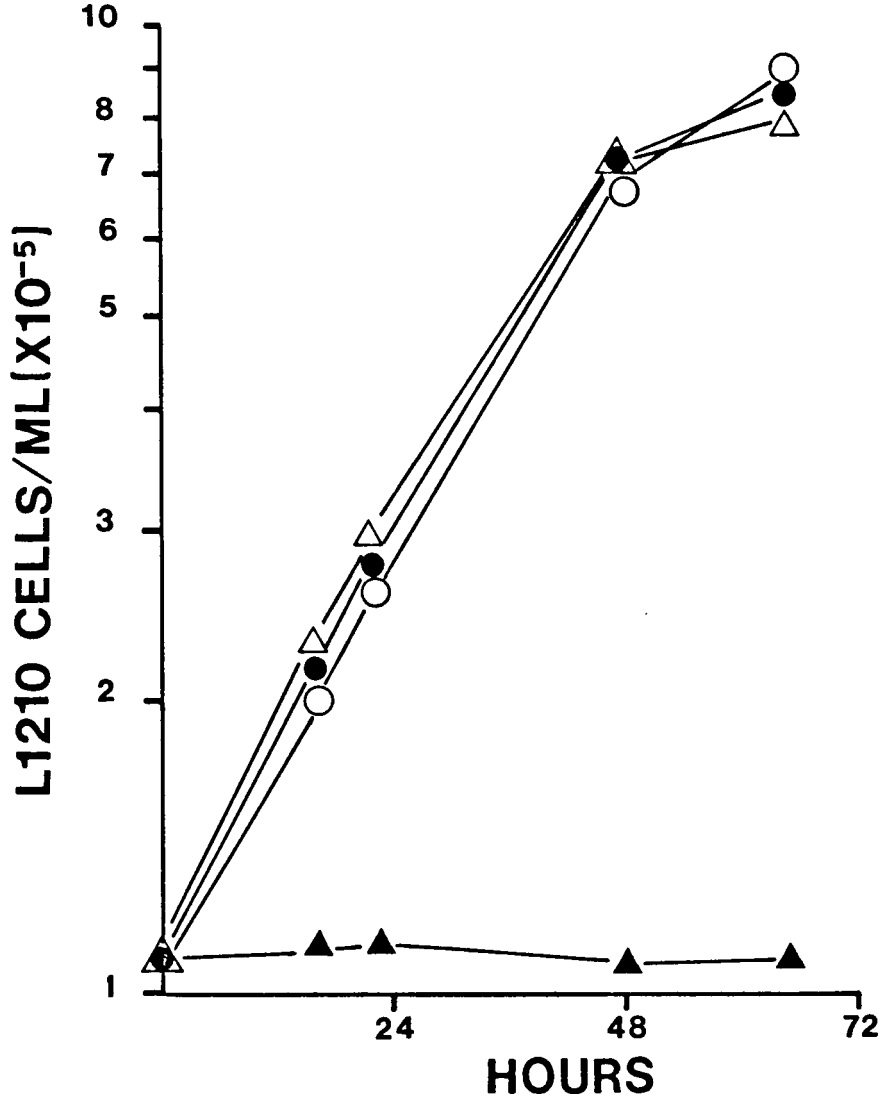
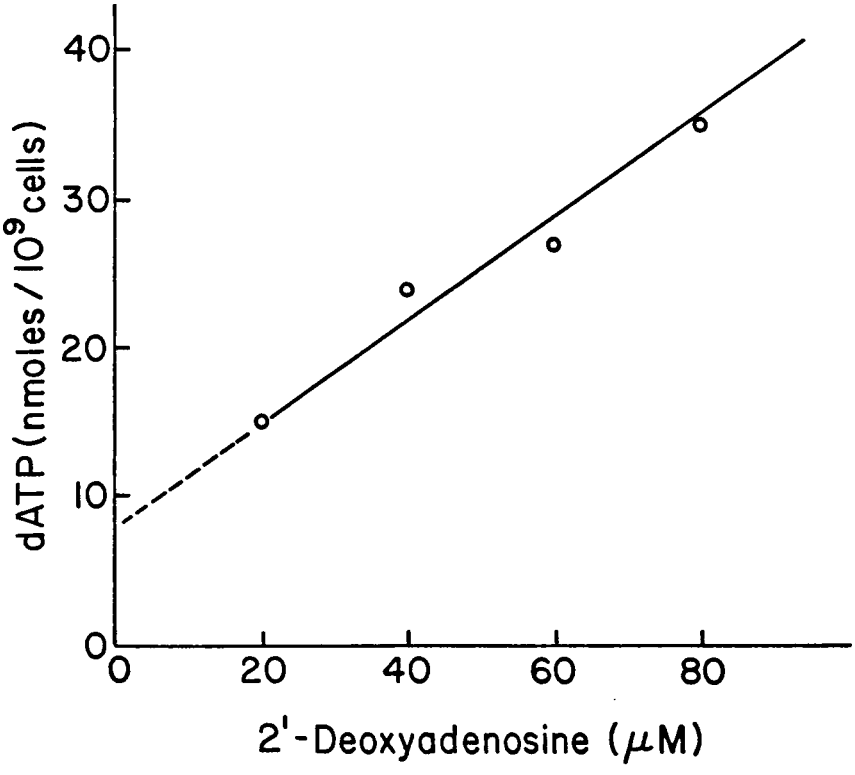

10
8
7
6
5
3
2
1
L1210 CELLS/ML[X10⁻⁵]
24
48
72
HOURS

FIGURE 8

dATP ACCUMULATION IN L1210 CELLS

Cells were treated with dCF (1.1 μM) for 1 hour and then incubated with various concentrations of dAdo for 24 hours.


40
30
20
10
0
dATP (nmoles / 10^9 cells)
0
20
40
60
80
2'-Deoxyadenosine (μM)

vitro. The dATP amounts in L1210 cells treated with dCF (1.1 μM) at various concentration of dAdo for 24 hours (Figure 8) were almost the same as in those treated for 3 hours (Figure 14).

3. Time Dependency of dATP Accumulation.

Figure 9 shows the result of a typical experiment in which cells were incubated with dCF (1.1 μM) and dAdo (40 μM) for various times. dATP accumulation increased progressively for 3 hours and remained relatively constant for up to 15 hours.

4. Effect of Various Concentrations of dCF, dAdo, and Dipyridamole

Dipyridamole is a known competitive inhibitor of deoxyribonucleoside transport (Plagemann and Erbe, 1974). Mitchell et al. (1978) obtained decreases in cytotoxicity and dATP amounts in T-lymphoblast cultures in which dipyridamole was added to EHNA and dAdo in combination. We expected similar results with dipyridamole in L1210 cells since it might also have inhibited dAdo uptake in L1210 cells. However, we found an increase in cytotoxicity and dATP amounts when L1210 cells were incubated with dCF, dAdo, and dipyridamole in triple combination. Figure 10 shows the in vitro effects of dCF and dAdo with dipyridamole. Dipyridamole alone had no significant effects on the growth of L1210 cells at concentrations up to 10 μM. dAdo in the presence of dCF (1.1 μM) had no effect on the growth of L1210 cells at concentrations up to 10 μM and only showed 50% inhibition of

FIGURE 9

TIME DEPENDENCY OF dATP ACCUMULATION

L1210 cells were treated with dCF (1.1 μM) for 1 hour and then incubated with dAdo (40 μM) for various times.

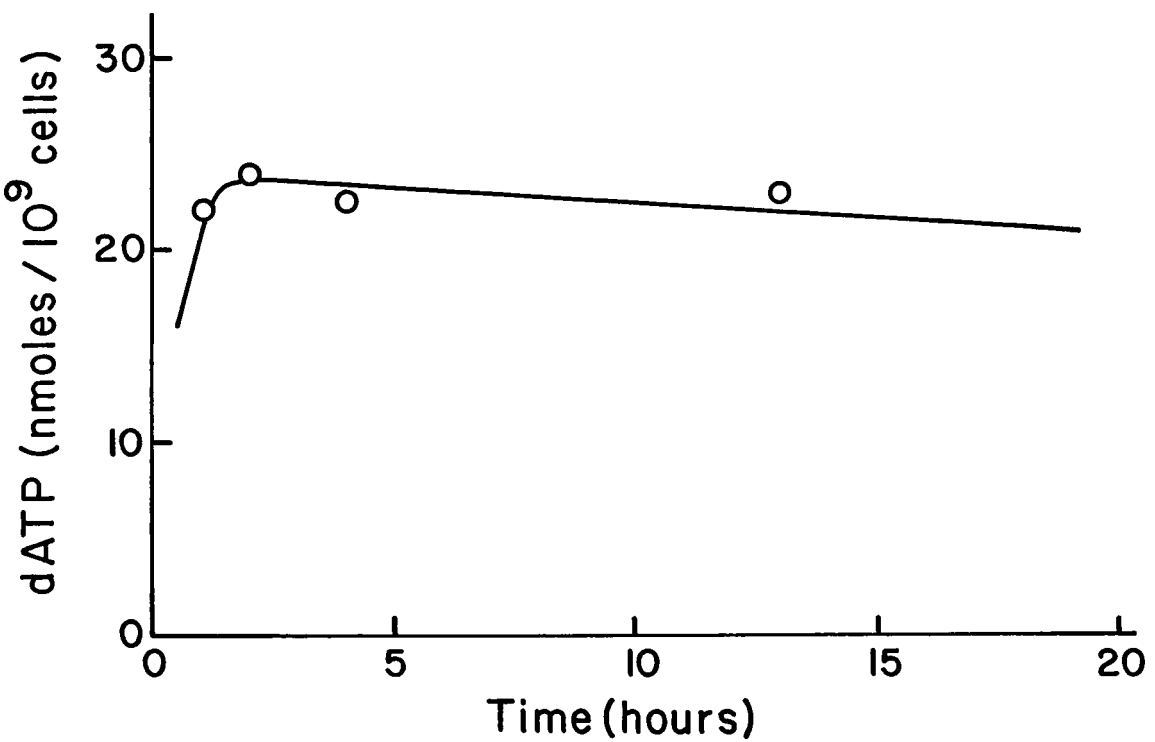

dATP (nmoles / 10^9 cells)
30
20
10
0
0
5
10
15
20
Time (hours)

FIGURE 10

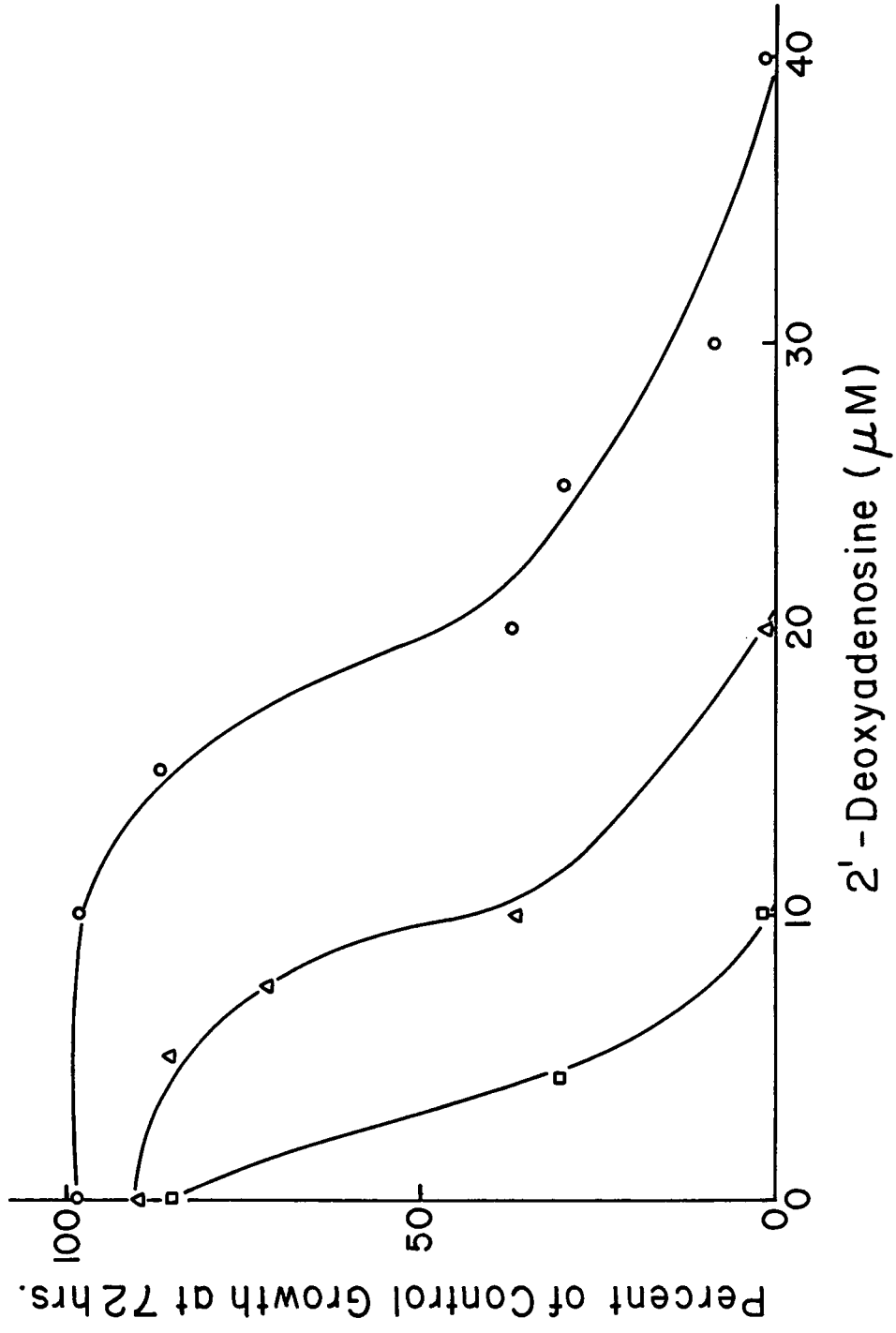
EFFECTS OF VARIOUS CONCENTRATIONS OF DIPYRIDAMOLE AND dAdo ON THE GROWTH OF L1210 CELLS

Cells were treated with dCF (1.1 μM) for 1 hour and then incubated with dAdo and/or dipyridamole. Results are expressed as the percent of control growth defined as the percent of viable control cell number at 72 hours.

O Without Dipyridamole

Δ Dipyridamole (1 μM)

□ Dipyridamole (5 μM)


Percent of Control Growth at 72 hrs.
100
50
0
0
10
20
30
40
2'-Deoxyadenosine (μM)

growth at 72 hour (IC_{50}) at a concentration of 20 μM. However, in the presence of 1 or 5 μM dipyridamole, the IC_{50} for dAdo in the presence of dCF(1.1 μM) was reduced to 10 and 2.5 μM, respectively, in L1210 cells.

5. Dependence of dATP Accumulation on Dipyridamole Concentration

dATP accumulation in the cells treated with dCF (1.1 μM) and dAdo (40 μM) increased with increasing concentration of dipyridamole (Figure 11). The highest concentration of dATP obtained was about 360 nmol/10^9 cells when dipyridamole was used at 10 μM.

6. dATP Accumulation with Time

dATP accumulations in cells treated with dCF (1.1 μM), dAdo (40 μM), and dipyridamole (10 μM) increased to a maximum of about 500 nmol/10^9 cells at 7 hour and slowly decreased thereafter (Figure 12).

7. Dependence of dATP Accumulation on dCF Concentration

dATP accumulation in cells treated with dAdo (40 μM) and dipyridamole (1 μM) with different concentrations of dCF (0.4 μM - 3.7 μM) did not increase with increasing concentration of dCF up to 3.7 μM dCF, 1.1 μM, was sufficient to inhibit maximally the adenosine deaminase activity in L1210 cells (Figure 13).

FIGURE 11

EFFECT OF DIPYRIDAMOLE ON dATP LEVELS IN L1210 CELLS

Cells were treated with dCF (1.1 μM) for 1 hour and then incubated with dAdo (40 μM) and different concentrations of dipyridamole for 3 hours.

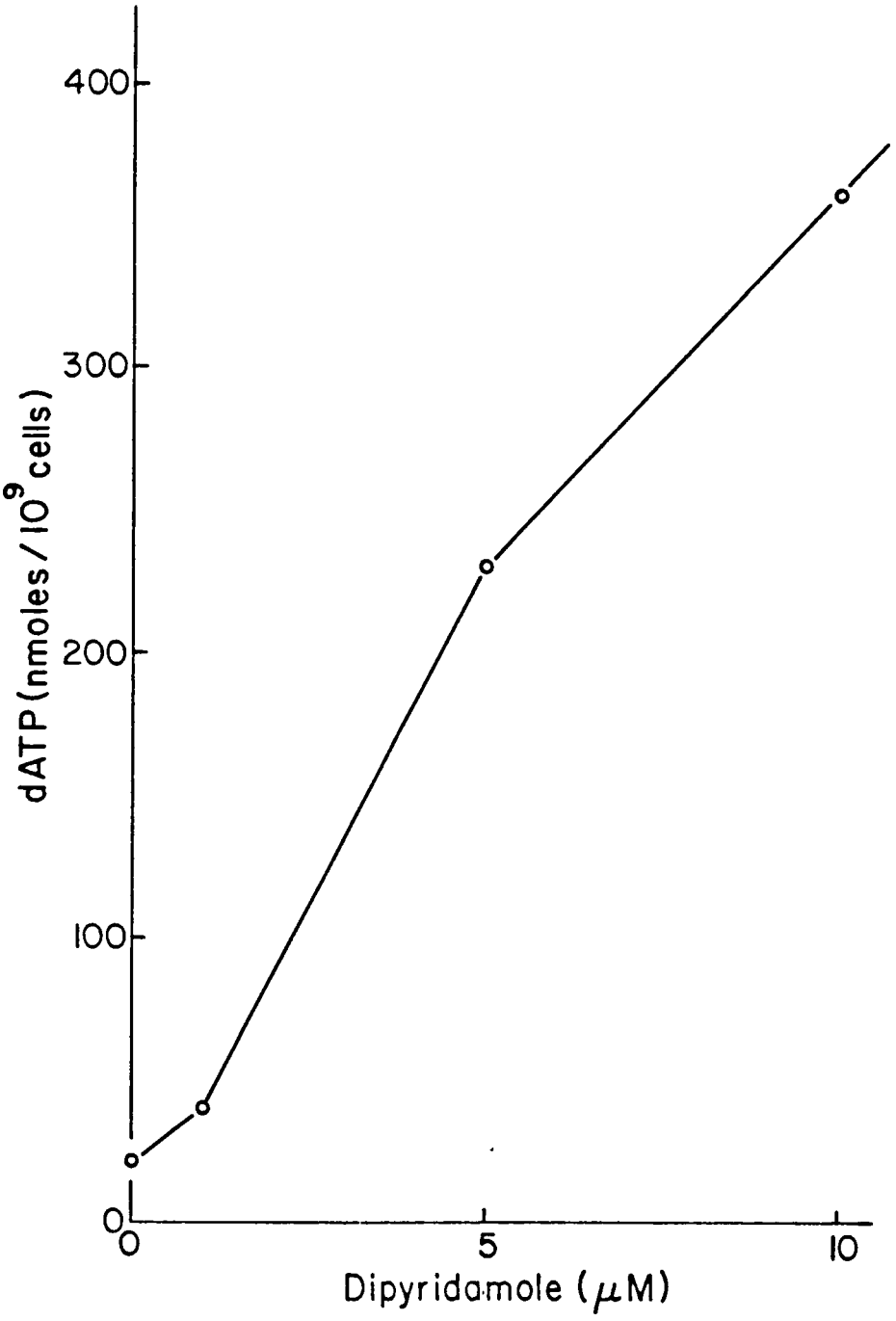

400
300
200
100
0
dATP (nmoles / 10^9 cells)
0
5
10
Dipyridamole (μM)

FIGURE 12

dATP ACCUMULATION WITH TIME IN THE PRESENCE OF DIPYRIDAMOLE

L1210 cells were treated with dCF (1.1 μM) for 1 hour and then incubated with dAdo (40 μM) and dipyridamole (10 μM) for various times.

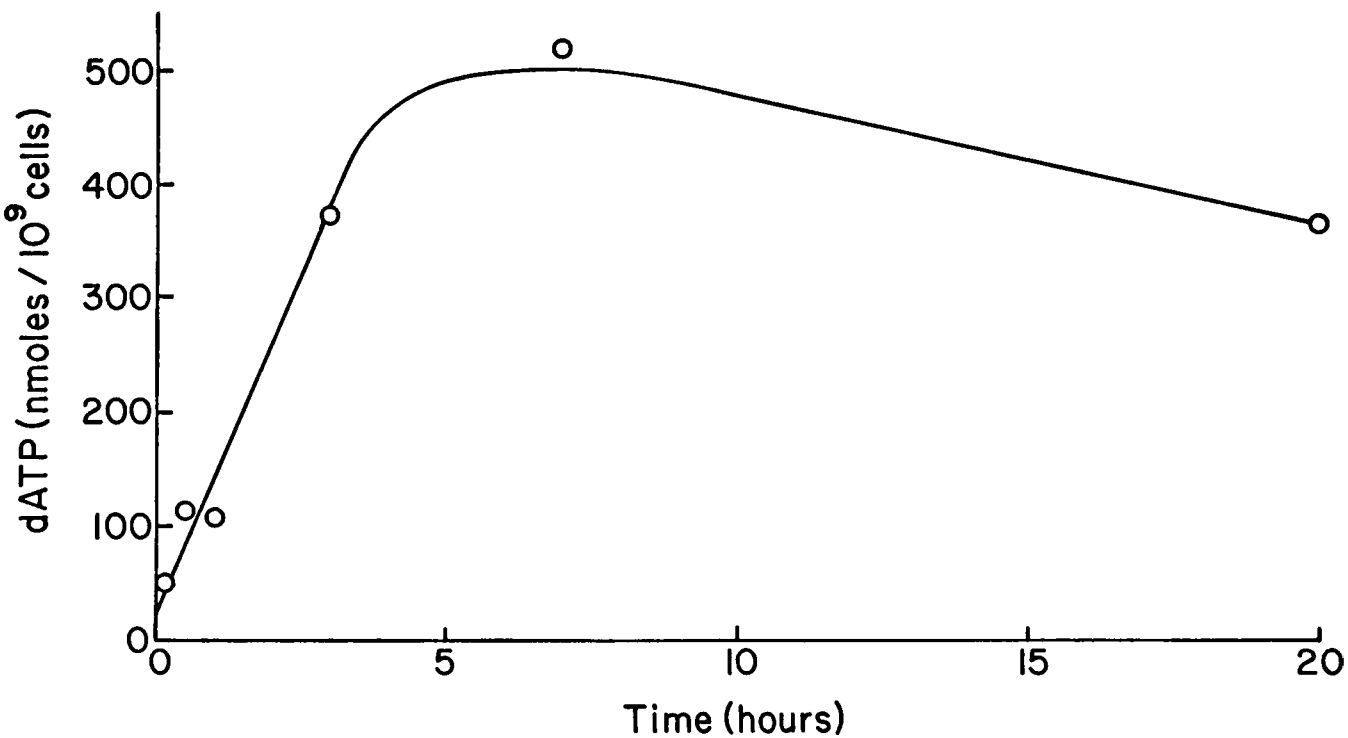

dATP (nmoles / 10^9 cells)
500
400
300
200
100
0
0
5
10
15
20
Time (hours)

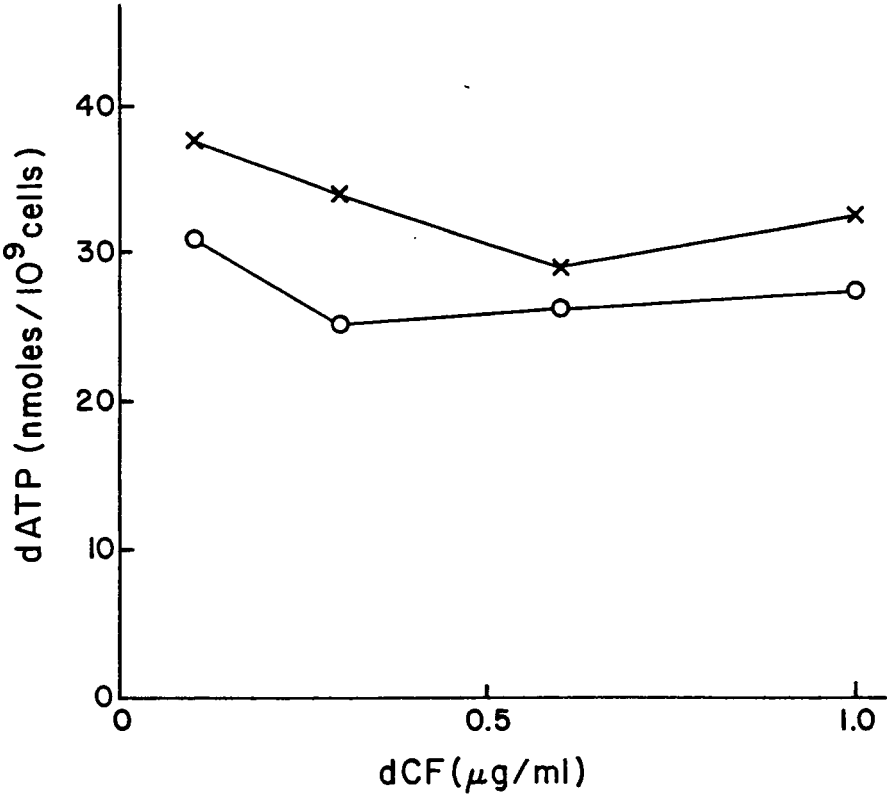
FIGURE 13

EFFECT OF dCF ON dATP LEVELS IN L1210 CELLS

Cells were treated with various concentrations of dCF for 1 hour and then were incubated with dAdo (40 μM) and/or dipyridamole.

O Without dipyridamole

X With dipyridamole (1 μM)


40
30
20
10
0
0
0.5
1.0
dATP (nmoles / 10^9 cells)
dCF (μg/ml)

8. Comparison of dATP Amounts between dCF and dAdo Combinations and dCF, dAdo, and Dipyridamole Triple Combinations

There was a correlation between dATP concentration and inhibition of cell growth. In the presence of 1 μM dipyridamole, the dAdo concentration could be reduced by one half to get the same intracellular dATP concentration and inhibition of cell growth (Figure 14).

9. Recovery of L1210 Cell Growth after Drug Treatment

Since we had observed a correlation between the triple combination (dCF, dAdo, and dipyridamole) effects on cytotoxicity and dATP amounts, the recovery of growth of cells treated with the triple combination was studied after resuspending the cells in fresh medium. The dATP amounts in the cells treated with the double combination for 3 hours was 23-25 nmol/10^9 cells and the cell growth recovered at 118 hour after resuspension in drug-free medium. The dATP amounts in the cells treated with 5 μM or 10 μM dipyridamole in the presence of dCF(1.1 μM) and dAdo (40 μM) for 3 hours were 210-250 nmol/10^9 cells and 330-380 nmol/10^9 cells, respectively. These dATP concentrations appeared to be too toxic for cell growth to recover. We can conclude from these data that a correlation appears to exist between cytotoxicity and dATP amounts and that there is a limit of intracellular dATP concentrations beyond which the cells probably can not recover (Figure 15).

FIGURE 14

COMPARISONS OF dATP ACCUMULATIONS BETWEEN DOUBLE COMBINATION AND TRIPLE COMBINATION-TREATED CELLS

L1210 cells were first treated with dCF for 1 hour and then incubated 3 hours with a range of dAdo concentration in the absence or presence of dipyridamole.

O Without dipyridamole

X With dipyridamole (1 μM)

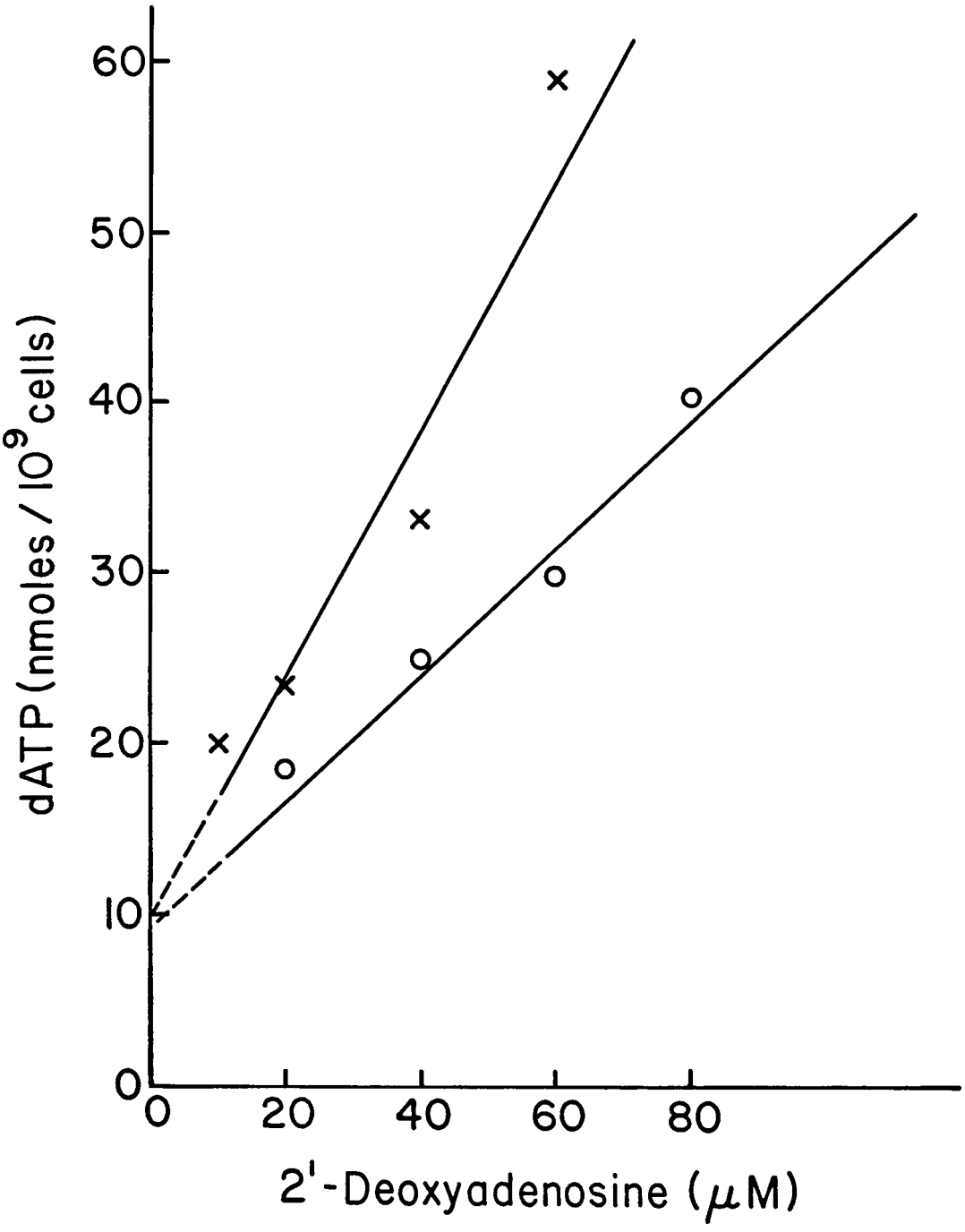

60
50
40
30
20
10
0
0
20
40
60
80
dATP (nmoles / 10^9 cells)
2'-Deoxyadenosine (μM)

FIGURE 15

RECOVERY OF L1210 CELL GROWTH OF CELLS TREATED WITH VARIOUS CONCENTRATION OF DRUGS

L1210 cells were incubated with drugs for 72 hours at which time the cells were centrifuged down and resuspended in drug-free medium. dCF was added 1 hour before dAdo and/or dipyridamole.

O Control

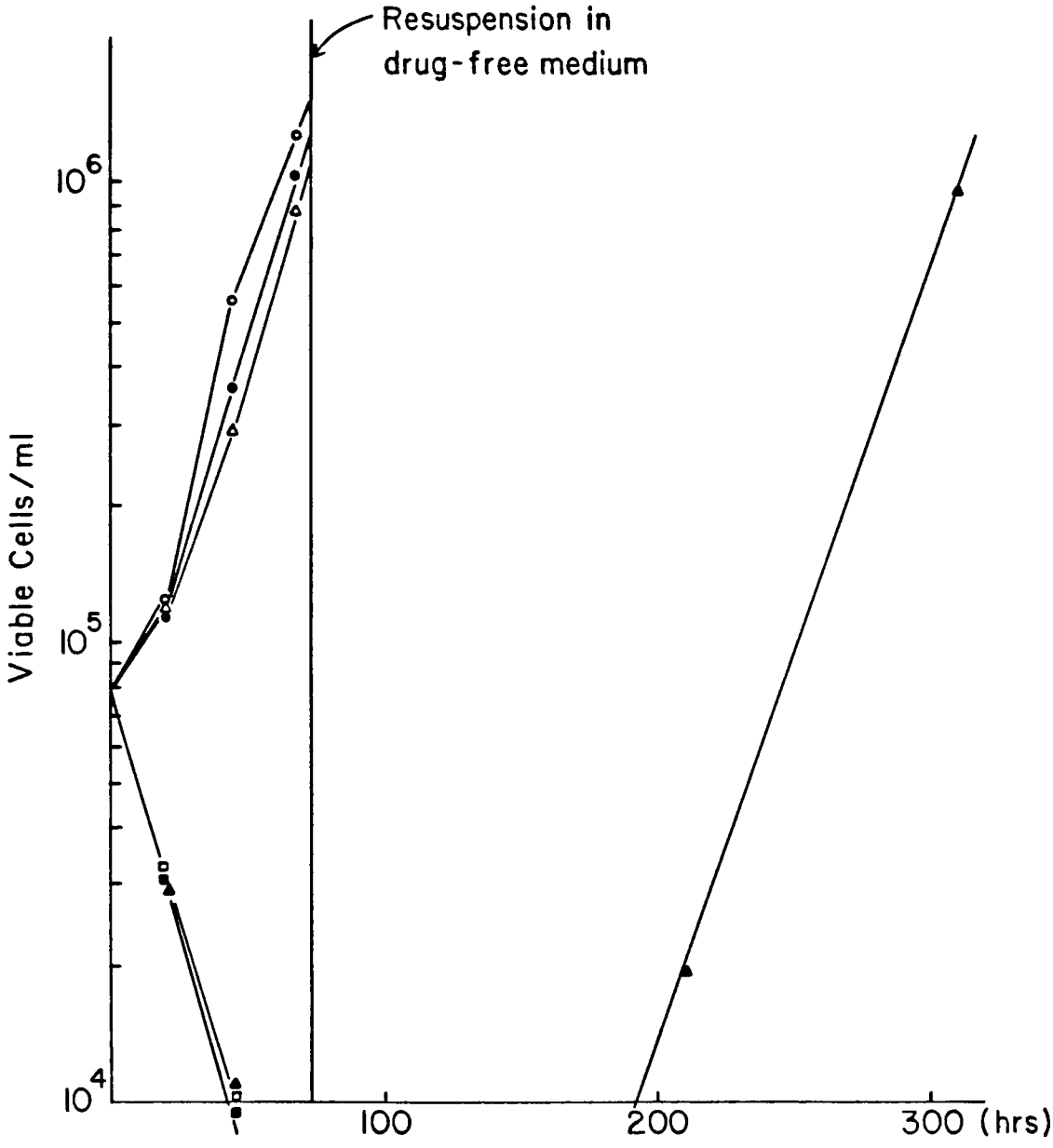
● Dipyridamole (10 μM)

Δ dCF (1.1 μM) + dipyridamole (10 μM)

▲ dCF (1.1 μM) + dAdo (40 μM)

□ dCF (1.1 μM) + dAdo (40 μM) + dipyridamole (5 μM)

■ dCF (1.1 μM) + dAdo (40 μM) + dipyridamole (10 μM)


Resuspension in
drug-free medium
Viable Cells/ml
10^6
10^5
10^4
100
200
300 (hrs)

10. Effects of Drugs on [^{3}H]dAdo and [^{3}H]Ado Uptake into Intracellular Metabolites

Generally, cellular uptake has been an important factor for the biological activity of nucleoside analogs since natural nucleosides and their analogs must be taken up and phosphorylated to active metabolites in the cell. Dipyridamole, like NBMPR, is a potent inhibitor of adenosine uptake. All deoxyribonucleoside transport systems are inhibited to about the same extent by dipyridamole, and the inhibition of deoxynucleoside transport results in apparent competitive inhibitions of their incorporation into nucleic acids in cultured Novikoff Rat Hepatoma cells (Ki = 1-2 μM) (Plagemann and Erbe, 1974). Mitchell et al. (1978) have shown that dipyridamole was effective in preventing deoxyadenosine-induced cytotoxicity and the elevation of dATP levels in the presence of 5 μM EHNA in T-lymphoblast cultures. In spite of the fact that the uptake of all nucleosides was inhibited by dipyridamole, the block on a given nucleoside could be reversed by such nucleosides that are phosphorylated by the same nucleoside kinase in chick fibroblast cells (Scholtissek, 1968). The data presented here indicate that [^{3}H]dAdo incorporation in the presence of 40 μM dAdo was not inhibited significantly in whole cell materials by either dCF (1.1 μM) alone or a combination of dCF (1.1 μM) and dipyridamole (10 μM) for 2 hours, but incorporation into the acid insoluble materials was inhibited by about one third. [^{3}H]Ado incorporation in the presence of dAdo (40 μM) was inhibited markedly in whole cell materials by dCF

FIGURE 16

EFFECTS OF dCF, dAdo AND DIPYRIDAMOLE ON THE INCORPORATION OF [^{3}H]dAdo INTO TOTAL CELL MATERIAL (A) AND ACID-INSOLUBLE MATERIAL (B).

O dAdo (40 µM)

Δ dCF (1.1 µM) + dAdo (40 µM)

□ dCF (1.1 µM) + dAdo (40 µM) + dipyridamole (5 µM)

X dCF (1.1 µM) + dAdo (40 µM) + dipyridamole (10 µM)

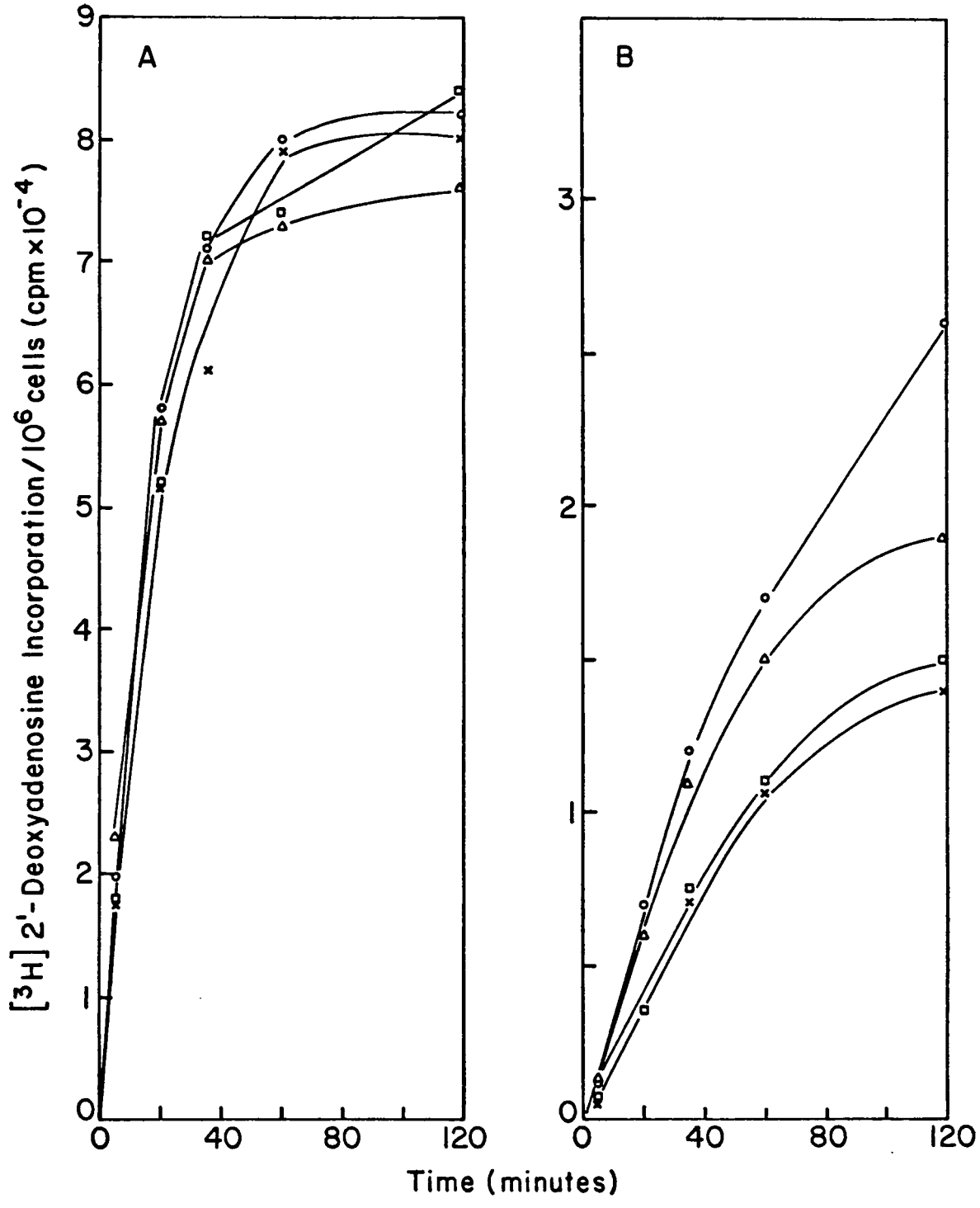

A
B
[3H] 2'-Deoxyadenosine Incorporation/10^6 cells (cpm x 10^-4)
Time (minutes)
0
40
80
120
1
2
3
4
5
6
7
8
9

FIGURE 17

EFFECTS OF dCF, dAdo AND DIPYRIDAMOLE ON THE INCORPORATION OF 1 μM [^{3}H]Ado INTO TOTAL CELL MATERIAL (A) AND ACID INSOLUBLE MATERIAL (B)

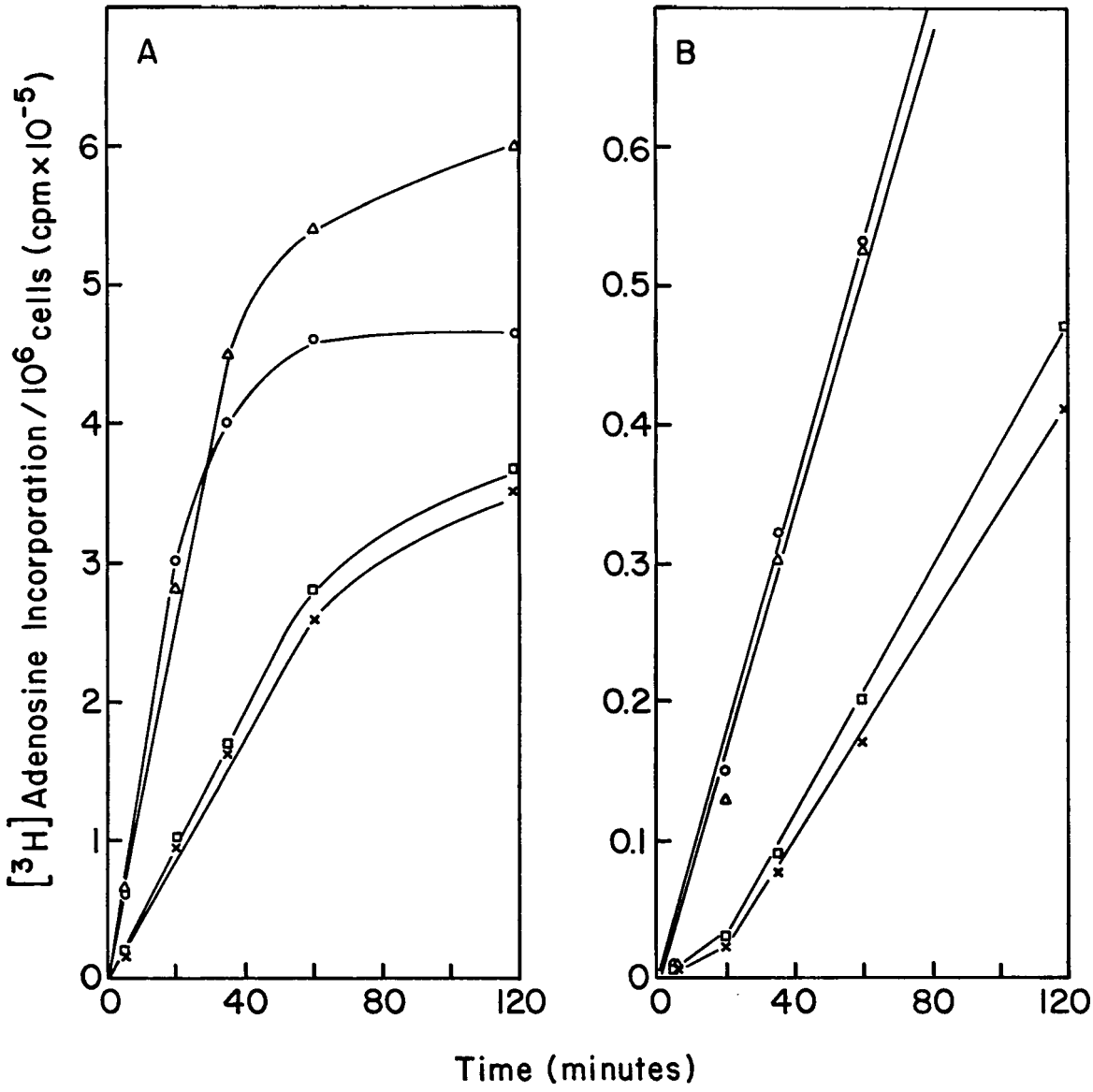
[^{3}H]Ado (1 μCi/ml medium, specific activity: 1 μCi/0.032 nmol) was used. dCF was treated for 1 hour before dAdo and/or dipyridamole treatment.

O dAdo (40 μM)

Δ dCF (1.1 μM) + dAdo (40 μM)

□ dCF (1.1 μM) + dAdo (40 μM) + dipyridamole (5 μM)

X dCF (1.1 μM) + dAdo (40 μM) + dipyridamole (10 μM)


A
B
[3H] Adenosine Incorporation / 10^6 cells (cpm x 10^-5)
0
1
2
3
4
5
6
0.1
0.2
0.3
0.4
0.5
0.6
0
40
80
120
Time (minutes)

(1.1 μM) plus dipyridamole (10 μM) in combination for 2 hours (Figures 16, 17).

11. Effect of dCF, dAdo, and Dipyridamole on Ribonucleotide Reductase Activity in L1210 Cells

a. 3 hour Incubation

Cells grown in the presence of dCF (1.1 μM) alone showed about a 20% inhibition of CDP reduction. The ribonucleotide reductase specific activity was inhibited about 60% with dAdo (40 μM) alone. The cells grown with dAdo (40 μM) in combination with dCF (1.1 μM) did not appear to be inhibited to any further extent. The inhibition of ribonucleotide reductase specific activity increased to 80% using the triple combination of dCF (1.1 μM), dAdo (40 μM), and dipyridamole (10 μM) (Table 1).

b. 16 Hour Incubation

A 25% increase in the specific activity of ribonucleotide reductase was found in extracts of L1210 cells grown in the presence of either dCF (1.1 μM) or dAdo (40 μM) compared to control. The cells grown with dAdo (40 μM) in combination with dCF (1.1 μM) showed about a 2.8-fold increase in ribonucleotide reductase activity. In contrast, ribonucleotide reductase specific activity was approximately one-half the control value in L1210 cells grown in the presence of a triple combination of dCF (1.1 μM), dAdo (40 μM), and dipyridamole (10

TABLE 1

EFFECT OF dCF, dAdo, AND DIPYRIDAMOLE ON CELL GROWTH AND RIBONUCLEOTIDE REDUCTASE ACTIVITY IN L1210 CELLS

One unit of specific activity is defined as the conversion of 100 cpm of [^{3}H]CDP to [^{3}H]dCDP in 1 hour at 37°C using 1 mg of supernatant protein. Values represent the mean values from triplicate determinations.

TABLE 1. EFFECTS OF dCF, dAdo, AND DIPYRIDAMOLE ON CELL GROWTH AND RIBONUCLEOTIDE REDUCTASE ACTIVITY IN L1210 CELLS

Drug Combination	Viable Cell Number ($\times 10^5$) 0 hr	3 hr	Ribonucleotide Reductase Specific Activity	% Inhibition
Control	8	8	8.43	0
dCF (1.1 μM)	8	8	6.65	21
dAdo (40 μM)	8	8	3.25	61
Dipyridamole (10 μM)	8	8	2.99	65
dCF (1.1 μM) + dAdo (40 μM)	8	8	3.49	59
dCF (1.1 μM) + dAdo (40 μM)+dipyridamole (10 μM)	8	8	1.59	81

TABLE 2

EFFECTS OF dCF, dAdo, and DIPYRIDAMOLE ON CELL GROWTH AND RIBONUCLEOTIDE REDUCTASE ACTIVITY IN L1210 CELLS

One unit of specific activity is defined as the conversion of 100 cpm of [^{3}H]CDP to [^{3}H]dCDP in 1 hour at 37°C using 1 mg of supernatant protein. Values represent the mean values from triplicate determinations.

TABLE 2. EFFECTS OF dCF, dAdo, AND DIPYRIDAMOLE ON CELL GROWTH AND RIBONUCLEOTIDE REDUCTASE ACTIVITY IN L1210 CELLS

Drug Combination	Viable Cell Number ($\times 10^5$)		Ribonucleotide Reductase Specific Activity	% Inhibition
	0 hr	16 hr		
Control	6.2	8.6	7.9	0
dCF (1.1 μM)	6.2	8.6	9.9	-25
dAdo (40 μM)	6.2	9.7	9.9	-25
Dipyridamole (10 μM)	6.2	8.1	7.9	0
dCF (1.1 μM) + dAdo (40 μM)	6.2	7.0	22.2	-181
dCF (1.1 μM) + dAdo (40 μM)+dipyridamole (10 μM)	6.2	4.3	3.8	51

μM) (Table 2).

12. The HPLC Analysis of Intracellur Acid-Soluble Metabolites Using [^{3}H]dAdo

The HPLC profiles of [^{3}H]dAdo metabolites in acid soluble extracts from L1210 cells that were exposed to dCF only, a combination of dCF and dAdo, and the triple combination of dCF, dAdo, and dipyridamole, are shown in Figure 18. The HPLC pattern shown in Figure 18(A) was obtained using extracts from cells that were treated with dAdo alone. The dATP peak was too small to be quantitated (less than 15 nmol/10^9 cells), but it was detectable compared to the control peak. The HPLC pattern in Figure 18(B) was obtained with cells incubated with dAdo (40 μM) in combination with dCF (1.1 μM). Incubation with this combination resulted in a small increase in dATP pools and showed a dADP shoulder which could not be quantitated. The HPLC pattern in Figure 18(C) was obtained with cells incubated with the triple combination [dCF (1.1 μM), dAdo (40 μM), and dipyridamole (10 μM)]. This analysis revealed a large increase in dATP and dADP pools. Despite the marked increase in dATP and dADP pools, the ATP pool was almost constant.

FIGURE 18

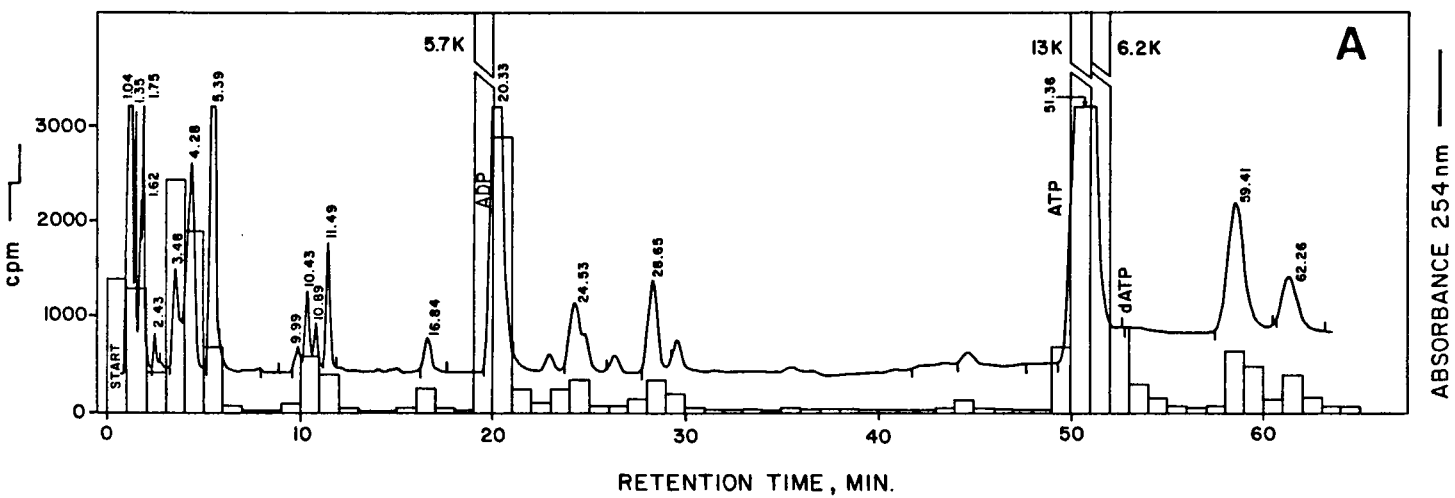
HPLC ANALYSIS OF ACID SOLUBLE EXTRACTS FROM L1210 CELLS TREATED WITH [^{3}H]dAdo (NUCLEOTIDES)

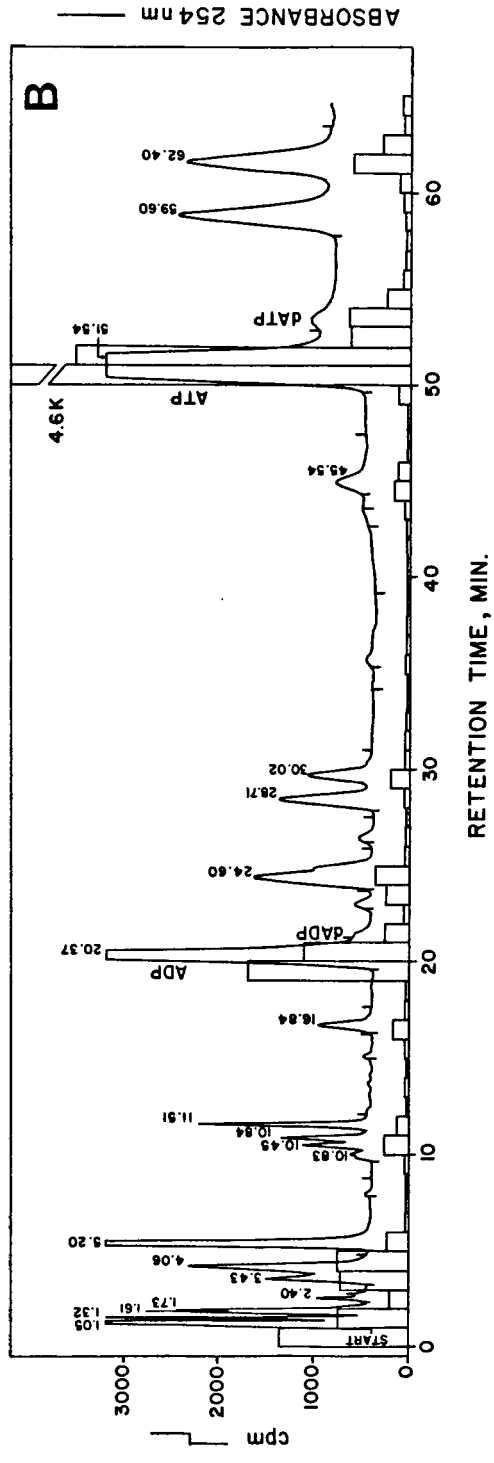
[^{3}H]dAdo (0.4 µCi/ml, specific activity: 1 µCi/0.07 nmol) was used.

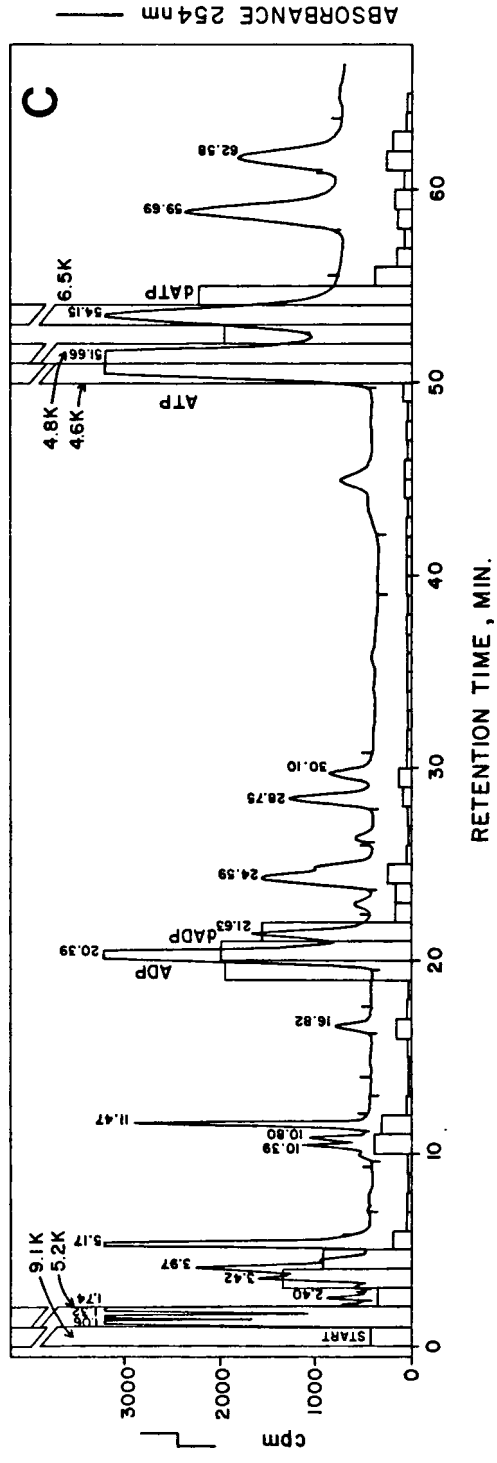
A) Cells (0.8 x 10^6/ml, 500 ml) were treated with dAdo (40 µM) for 3 hours.

B) Cells (0.6 x 10^6/ml, 500ml) were treated with dCF (1.1 µM) for 1 hour and then were incubated with dAdo (40 µM) for 3 hours.

C) Cells (0.85 x 10^6/ml; 500 ml) were treated with dCF (1.1 µM) for 1 hour and then incubated with dAdo (40 µM) and dipyridamole (10 µM) for 3 hours.


5.7K
13K
6.2K
A
cpm
3000
2000
1000
0
START
1.04
1.35
1.75
1.62
2.43
3.48
4.28
5.39
9.99
10.43
10.89
11.49
16.84
ADP
20.33
24.53
28.65
51.36
ATP
dATP
59.41
62.26
0
10
20
30
40
50
60
RETENTION TIME, MIN.
ABSORBANCE 254nm


ABSORBANCE 254 nm
B
62.40
59.60
dATP
51.54
ATP
4.6K
45.54
30.02
28.71
24.60
dADP
20.37
ADP
16.84
11.51
10.84
10.45
10.83
9.20
4.06
3.43
2.40
1.73
1.61
1.32
1.05
START
3000
2000
1000
0
cpm
0
10
20
30
40
50
60
RETENTION TIME, MIN.


ABSORBANCE 254nm
C
cpm
3000
2000
1000
0
START
1.74
2.40
3.42
3.97
5.17
9.1K
5.2K
10.39
10.80
11.47
16.82
ADP
20.39
dADP
21.63
24.59
28.75
30.10
4.8K
4.6K
ATP
51.66
54.15
dATP
6.5K
59.69
62.58
0
10
20
30
40
50
60
RETENTION TIME, MIN.

B. L1210 Cytotoxic Effects of dCF, dAdo, and Dipyridamole Triple Combination Correlated with High dAdo Levels

1. dAdo Accumulation Dependency on dAdo Concentration

Since inhibition of S-adenosyl-L-homocysteine hydrolase has been suggested as contributing to the cytotoxic effects of dAdo (Hershfield, 1979; Cass et al., 1982), dAdo amounts in L1210 cells were examined after treating the cells with dCF (1.1 μM) at various concentration for 3 and 24 hours in vitro. The level of dAdo in the cells treated with dCF (1.1 μM) increased with increasing concentration of dAdo after 3 hours of incubation. In contrast to the small increase in dAdo levels after a 3 hour incubation, a large increase in dAdo level was shown after a 24 hour incubation with drugs (Figure 19). When L1210 cells were incubated with either dCF (1.1 μM) or dAdo (40 μM) alone, the level of dAdo could not be detected by HPLC.

2. dAdo Accumulation with Time

dAdo accumulations in cells treated with dCF (1.1 μM) and dAdo (40 μM) increased with incubation time up to 24 hours (Figure 20).

3. Dependence of dAdo Accumulation on dAdo and Dipyridamole

Figure 21 shows that L1210 cells treated with dCF (1.1 μM) at various concentrations of dAdo for 3 hours accumulated dAdo. The dAdo accumulation was dependent on the concentrations of dAdo and 1 μM

FIGURE 19

COMPARISON OF dAdo ACCUMULATIONS BETWEEN 3 HOUR AND 24 HOUR INCUBATED L1210 CELLS

Cells were first treated with dCF for 1 hour and then incubated for 3 hours and 24 hour respectively with a range of dAdo.

X 3 hour incubation

O 24 hour incubation

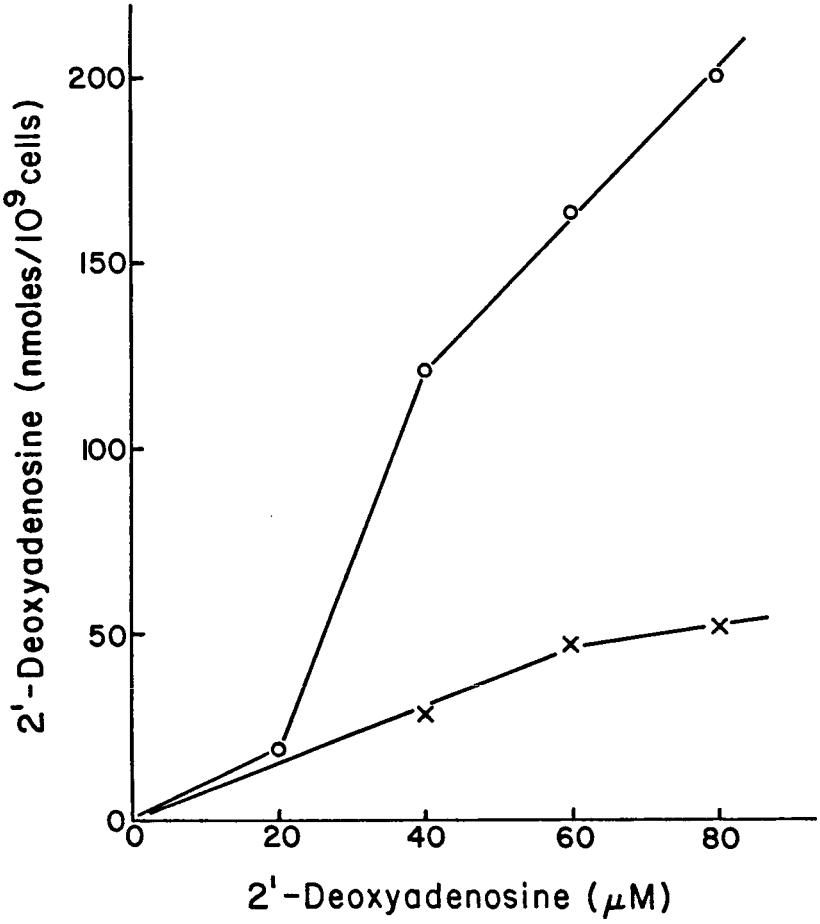

2'-Deoxyadenosine (nmoles/10^9 cells)
200
150
100
50
0
0
20
40
60
80
2'-Deoxyadenosine (μM)

FIGURE 20

dAdo ACCUMULATION WITH TIME IN L1210 CELLS

L1210 cells were treated with dCF (1.1 μM) for 1 hour and then incubated with dAdo (40 μM) for various times.

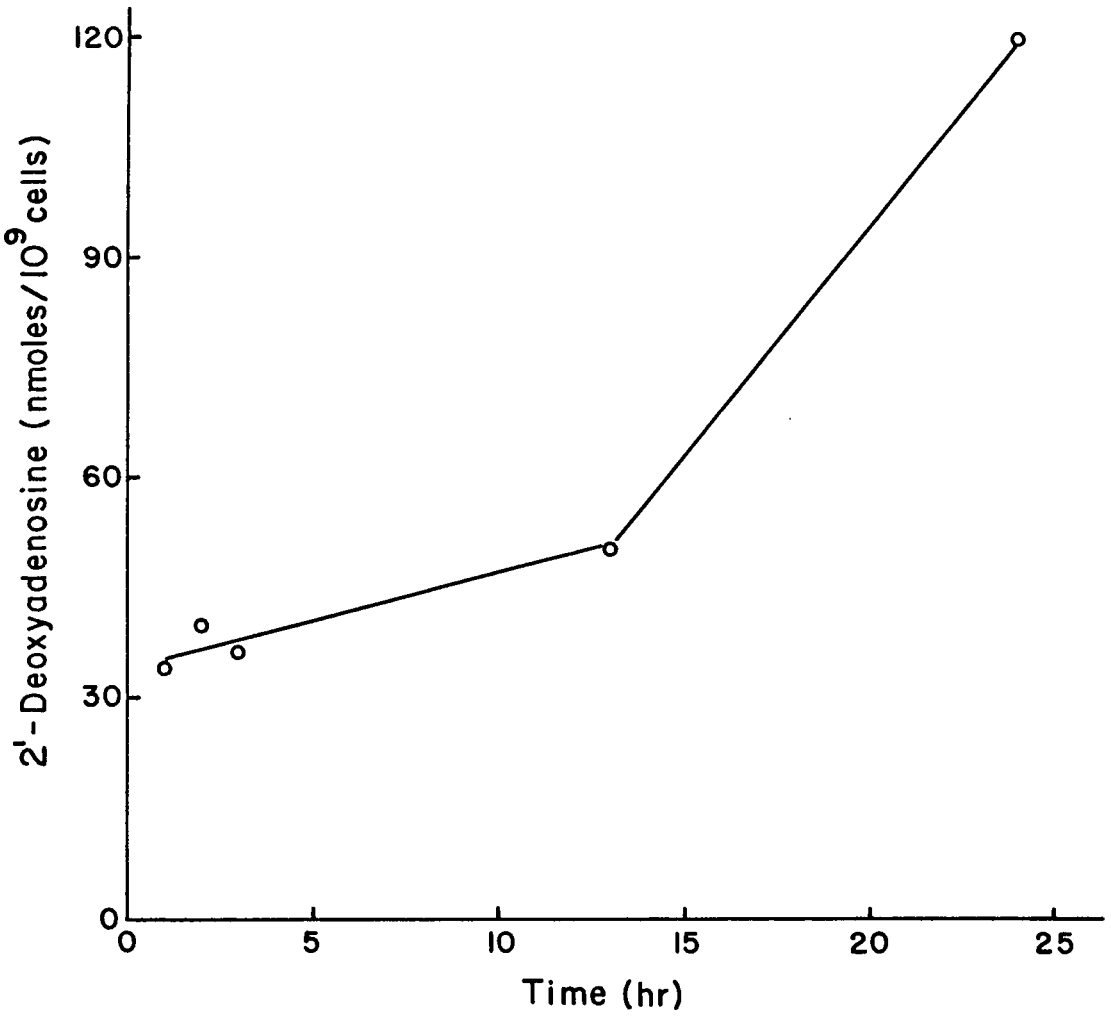

120
90
60
30
0
2'-Deoxyadenosine (nmoles/10^9 cells)
0
5
10
15
20
25
Time (hr)

FIGURE 21

COMPARISONS OF dAdo ACCUMULATIONS BETWEEN DOUBLE COMBINATION AND TRIPLE COMBINATION-TREATED CELLS

L1210 cells were first treated with dCF for 1 hour and then incubated 3 hours with a range of dAdo concentration in the absence or presence of dipyridamole.

O Double combination: dCF (1.1 μM) + dAdo

X Triple combination: dCF (1.1 μM) + dAdo + Dipyridamole(1 μM).

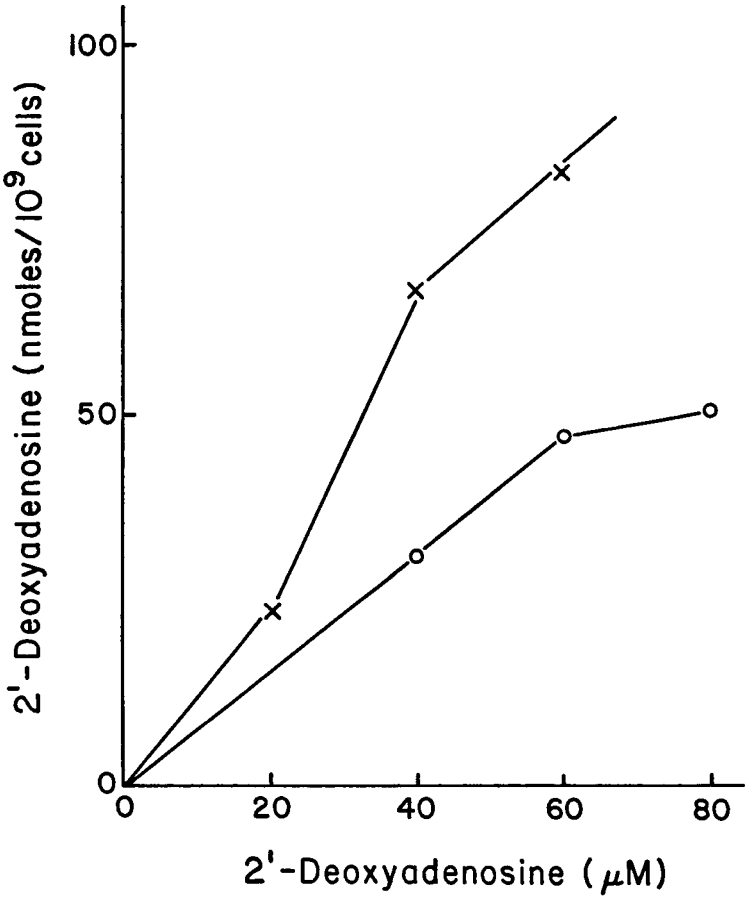
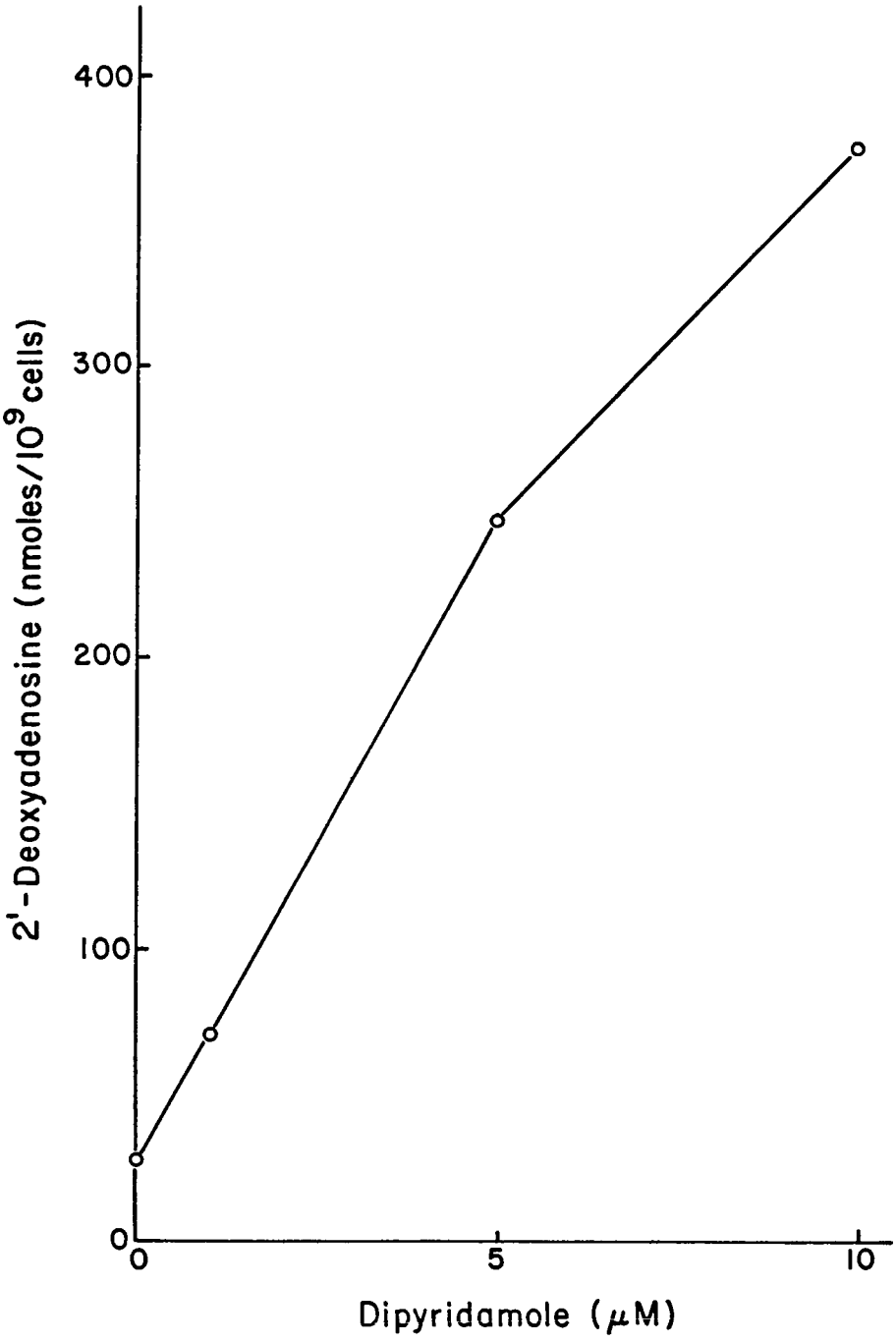

100
50
0
0
20
40
60
80
2'-Deoxyadenosine (nmoles/10^9 cells)
2'-Deoxyadenosine (μM)

FIGURE 22

EFFECTS OF DIPYRIDAMOLE ON dAdo LEVELS IN L1210 CELLS

Cells were incubated with dCF (1.1 μM) for 1 hour and then incubated with dAdo (40 μM) and different concentrations of dipyridamole for 3 hours.


400
300
200
100
0
0
5
10
2'-Deoxyadenosine (nmoles/10^9 cells)
Dipyridamole (μM)

dipyridamole.

4. Dependence of dAdo on Dipyridamole Concentration

dAdo accumulation in the cell treated with dCF (1.1 μM) and dAdo (40 μM) increased with increasing concentration of dipyridamole. The highest amount of dAdo was about 375 nmoles/10^9 cells, when dipyridamole was used at 10 μM (Figure 22).

5. dAdo Accumulation with Time (with Dipyridamole)

dAdo accumulation in cells treated with dCF (1.1 μM), dAdo (40 μM), and dipyridamole (10 μM) increased with incubation time. In contrast to the results on dATP accumulation which showed a maximum concentration at 7 hours, dAdo concentration increased continously with time studied. The results imply that a possible inhibition of deoxyadenosine kinase is caused by the deoxyadenosine accumulating in L1210 cells where adenosine deaminase is inhibited (Figure 23).

6. Dependence of dAdo Accumulation on dCF Concentration

dAdo accumulation in cells treated with dAdo (40 μM) and dipyridamole (1 μM) with different concentrations of dCF (0.4 μM - 3.7 μM) did not increase with increasing concentration of dCF up to 3.7 μM (Figure 24).

FIGURE 23

dAdo ACCUMULATION WITH TIME IN L1210 CELLS IN THE PRESENCE OF DIPYRIDAMOLE

L1210 cells were treated with dCF (1.1 μM) for 1 hour and then incubated with dAdo (40 μM) and dipyridamole (10 μM) for various times.

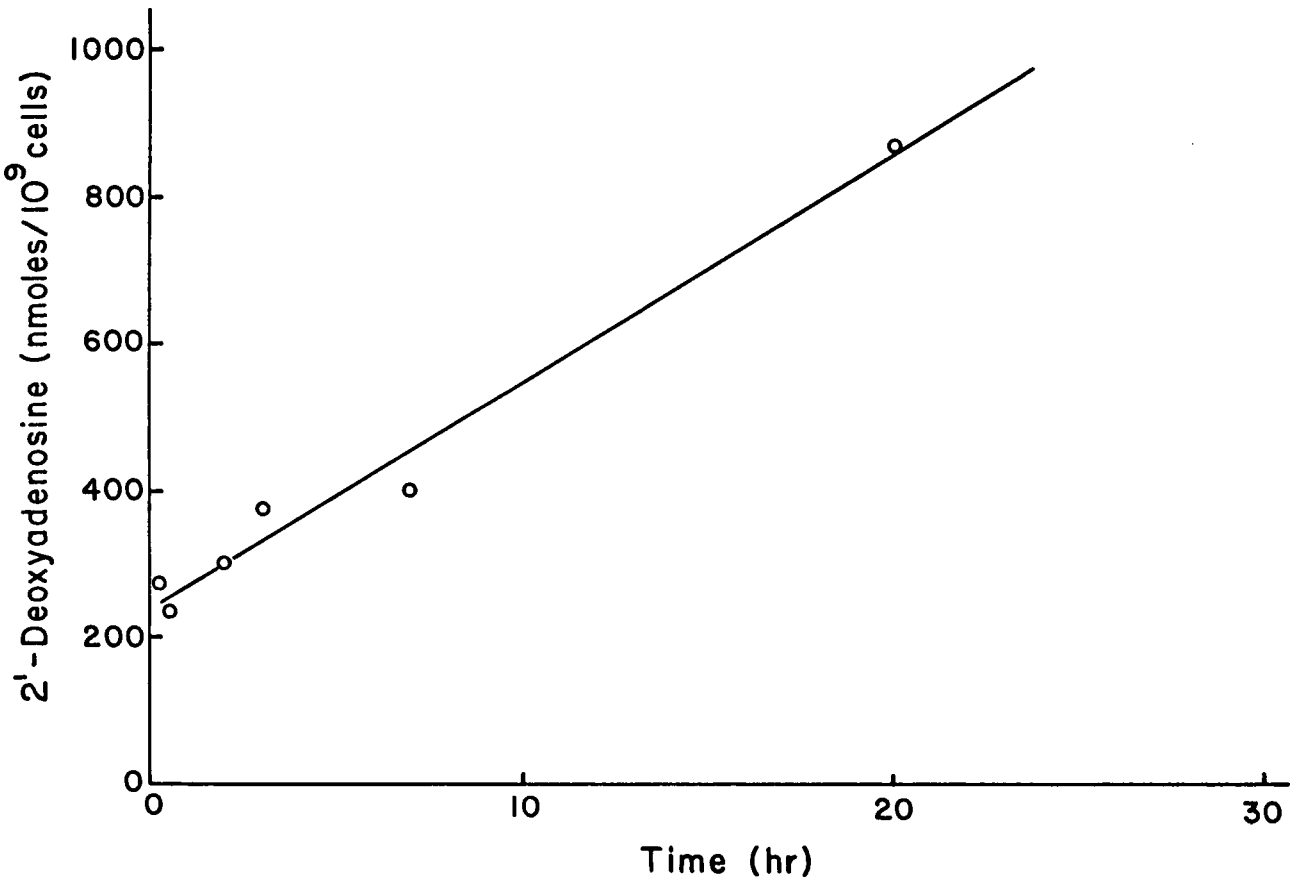

2'-Deoxyadenosine (nmoles/10^9 cells)
1000
800
600
400
200
0
0
10
20
30
Time (hr)

FIGURE 24

EFFECTS OF dCF ON dAdo LEVELS IN L1210 CELLS

Cells were incubated with various concentrations of dCF for 1 hour and then were incubated with dAdo (40 μM) alone or combination of dAdo (40 μM) and dipyridamole for 3 hours.

O Without dipyridamole

X With dipyridamole (1 μM)

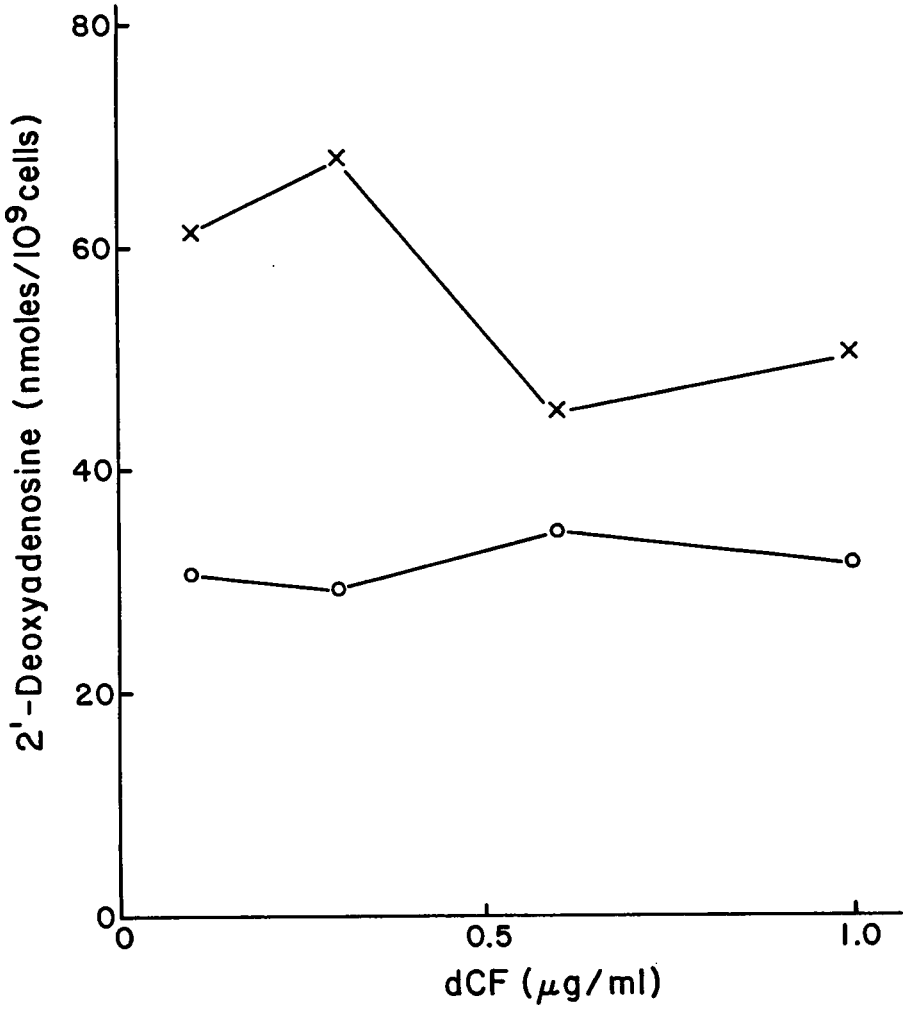

80
60
40
20
0
0
0.5
1.0
2'-Deoxyadenosine (nmoles/10^9cells)
dCF (μg/ml)

FIGURE 25

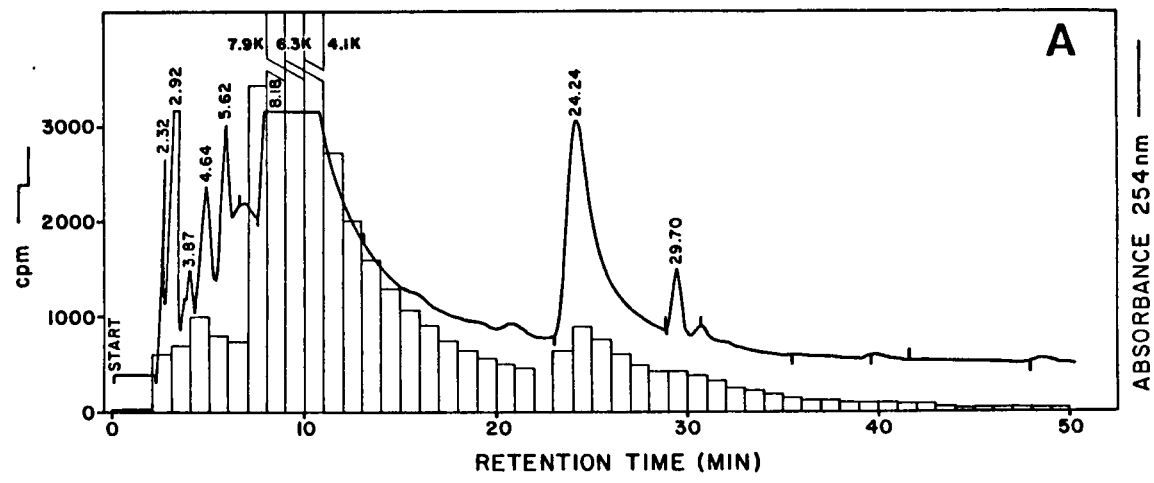
HPLC ANALYSIS OF ACID SOLUBLE EXTRACTS FROM L1210 CELLS TREATED WITH [^{3}H]dAdo (NUCLEOSIDES)

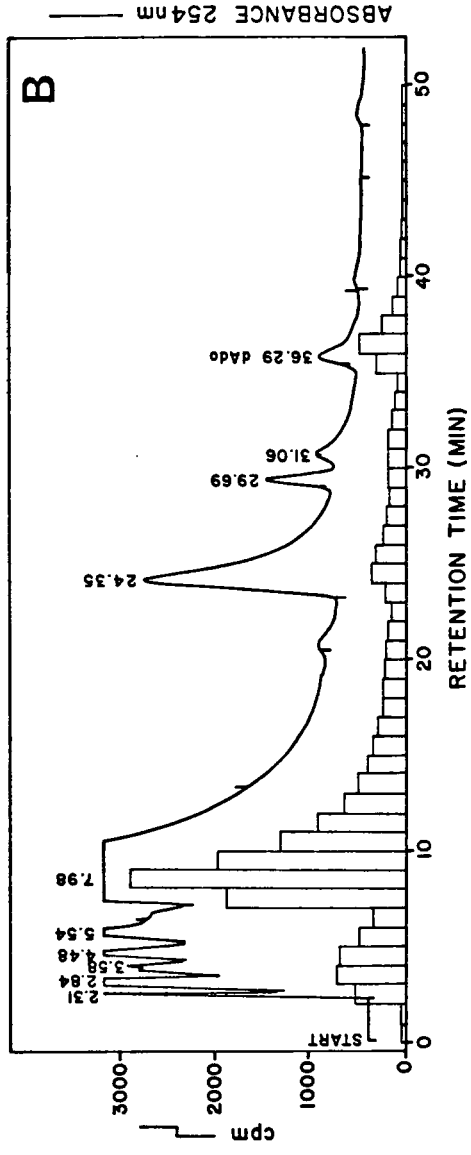
[^{3}H]dAdo (0.4 µCi/ml) specific activity: 1 µCi/0.07 nmol) was used.

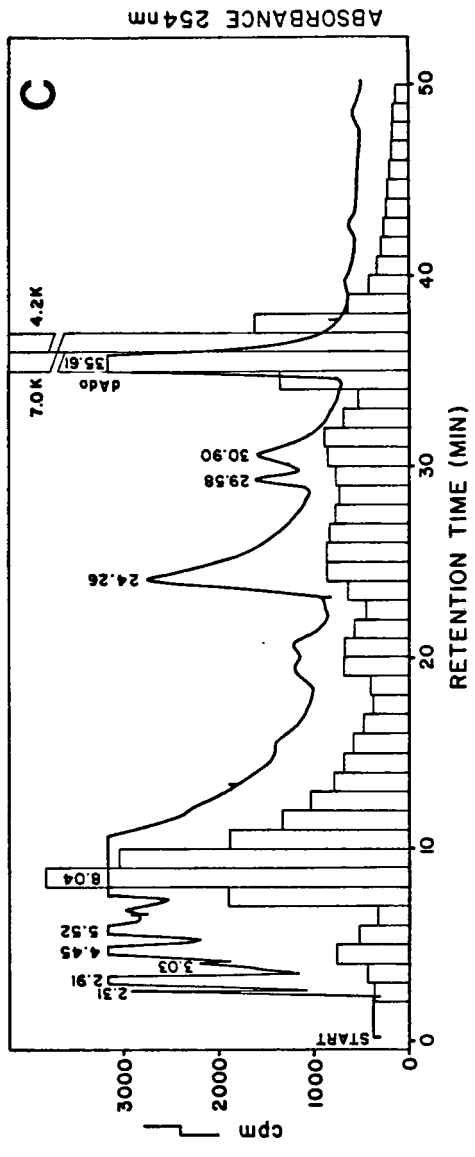
A) Cells (0.8 x 10^6/ml, 500 ml) were treated with dAdo (40 µM) for 3 hours.

B) Cells (0.6 x 10^6/ml, 500 ml) were treated with dCF (1.1 µM) for 1 hour and then incubated with dAdo (40 µM) for 3 hours.

C) Cells (0.85 x 10^6/ml, 500 ml) were treated with dCF (1.1 µM) for 1 hour and then incubated with dAdo (40 µM) and dipyridamole (10 µM) for 3 hours.


A
7.9K
6.3K
4.1K
2.32
2.92
3.87
4.64
5.62
8.18
24.24
29.70
START
cpm
3000
2000
1000
0
0
10
20
30
40
50
RETENTION TIME (MIN)
ABSORBANCE 254nm


B
ABSORBANCE 254nm
cpm
3000
2000
1000
0
START
2.31
2.84
3.58
4.48
5.54
7.98
24.35
29.69
31.06
36.29 dado
0
10
20
30
40
50
RETENTION TIME (MIN)


ABSORBANCE 254nm
C
4.2K
7.0K
35.61
dAdo
30.90
29.58
24.26
8.04
5.52
4.45
3.03
2.91
2.31
START
cpm
3000
2000
1000
0
0
10
20
30
40
50
RETENTION TIME (MIN)

7. The HPLC Analysis of Intracellular dAdo Using [^{3}H]dAdo

The HPLC profiles of [^{3}H]dAdo in acid soluble extracts from L1210 cells that were exposed to drugs are shown using an ODS column. The HPLC pattern shown in Figure 25(A) was obtained using extracts from cells that were treated with dAdo alone. The dAdo peak was too small to be quantitated and the radioactivity of the dAdo peak was negligible. The HPLC pattern in Figure 25(B) was obtained from cells incubated with dAdo (40 μM) in combination with dCF (1.1 μM). Incubation with this combination resulted in a increase in the dAdo peak which coincided with the radioactivity. The HPLC pattern in Figure 25(C) was obtained with cells incubated with the triple combination [dCF (1.1 μM), dAdo (40 μM), and dipyridamole (10 μM)]. This analysis showed more than a ten-fold increase in dAdo compared with the double combination [dCF (1.1 μM) and dAdo (40 μM)].

C. Survival Studies Using dCF and dAdo Combination Therapy on L1210 Cells

A series of experiments were carried out to determine the effectiveness of dCF and dAdo either alone or in combination on BDFI female mice, bearing i.c. implants of 10^5 L1210 cells. Le Page *et al.* (1976) have previously shown that one dose of dCF (0.25 to 1.0 mg/kg) almost completely inhibited the deamination of ara-A in L1210 cells and which recovered to only about 15% of control by 24 hr. In this experiment, when dCF (0.5 mg/kg/dose) was given once on days 1, 4, and

7 alone or in combination with dAdo given every 3 hours for 8 doses on day 1, 4, and 7 at 25 or 50 mg/kg/dose, no increase in life spans of drug treated mice occurred. Either the dose of dCF or dAdo or both were insufficient. However, when dCF (0.5 mg/kg/dose) was given every 12 hours for 2 doses along with dAdo at 50 or 100 mg/kg/dose, then the % ILS increased and ranged from 72% (T/c = 172) to 167%, (T/c = 267). Several long term survivors were also obtained in these survival experiments. The 125 mg/kg and 150 mg/kg doses of dAdo in combination with dCF were considered toxic. Also an interesting observation was made regarding the median survival times (MST) of dying animals only where the animals had received the dose at 100 mg/kg plus dCF which was considered relatively nontoxic and therapeutic. The MST of dying animals only at this dosage was 13 days while the % ILS was 167% (based on 30 days) and the maximum weight loss of treated animals was only 13% occurring on day 10. (Table 3)

TABLE 3

EFFECTS OF dCF AND dAdo ON THE SURVIVAL OF BDF1 MICE BEARING i.p. L1210 CELLS

L1210 cells (10^5) were implanted i.p., and therapy was begun 24 hours later.

a. q3hr, every 3 hours; q12hr, every 12 hours.

b. $\frac{\text{(MLS treated - MLS controls)}}{\text{MLS controls}} \times 100$. The median life span (MLS) of the 36 untreated controls was 9 days. Mice surviving longer than 30 days were treated in the calculations of % ILS as surviving only 30 days.

EFFECTS OF dCF AND dAdo ON THE SURVIVAL OF BDF1 MICE BEARING i.p. L1210 CELLS

Drug	Dosage (mg/kg/dose)	Schedule	Max. % Wt. loss (Day)	% ILS[b]	MST Dying Animals (Range)	50 Day Survivors
dCF	0.5	once daily, days 1,4,7	-	0	9 (9-10)	0/6
dCF	0.5	q12hr[a] x 2, days 1,4,7	-	-11	8 (8-9)	0/6
dAdo	25	q3hr x 8, days 1,4,7	-	-11	8 (8-9)	0/6
dAdo	50	q3hr x 8, days 1,4,7	-	-11	8 (8-9)	0/6
dAdo	100	q3hr x 8, days 1,4,7	-	-22	7 (7-10)	0/12
dAdo	150	q3hr x 8, days, 1,4,7	-	0	8 (8-11)	0/12
dCF + dAdo	0.5 + 25	dCF, once daily, days 1,4,7 dAdo, q3hr x 8, days 1,4,7	-	11	10 (10-11)	0/6
dCF + dAdo	0.5 + 50	dCF, once daily, days 1,4,7 dAdo, q3hr x 8, days 1,4,7	-	11	10 (10-11)	0/6
dCF + dAdo	0.5 + 50	dCF, q12hr x 2, days 1,4,7 dAdo, q3hr x 8, days 1,4,7	9 (13)	72	15 (12-16)	2/6
dCF + dAdo	0.5 + 100	dCF, q12hr x 2, days 1,4,7 dAdo, q3hr x 8, days 1,4,7	13 (10)	167	13 (11-37)	11/24
dCF + dAdo	0.5 + 125	dCF, q12hr x 2, days 1,4,7 dAdo, q3hr x 8, days 1,4,7	17 (9)	toxic	14 (6-14)	(4/6)
dCF + dAdo	0.5 + 150	dCF, q12hr x 2, days 1,4,7 dAdo, q3hr x 8, days 1,4,7	24 (10)	toxic	12 (7-16)	(4/12)

CHAPTER IV

DISCUSSION

The biochemical metabolism of the naturally occurring purine deoxynucleoside, deoxyadenosine(dAdo) has been studied because dAdo analogs are important as anticancer and antiviral drugs, and because one form of the human severe combined immunodeficiency disease syndrome is due to deficiency of ADA activity. In the inherited deficiency of adenosine deaminase activity associated with immune dysfunction, patients have markedly elevated dAdo levels in serum and urine, and dATP levels in erythrocytes, marrow cells, and lymphocytes (Donfri et al., 1978; Giblett et al., 1972; Simmonds et al., 1978).

The data presented in this dissertation showed an enhancement of dAdo toxicity in L1210 cells produced by dCF which is a potent inhibitor of adenosine deaminase. dCF alone was not inhibitory for cell growth and viability of L1210 cells. dAdo alone was not inhibitory for cell growth up to 40 μM concentration, but showed slight inhibition in the early period of the cell culture and this might be due to the time for the deamination of dAdo to deoxyinosine. L1210 cells incubated with dAdo (40 μM) alone could accumulate up to 15 nmol dATP/10^9 cells in 3 hours but dATP was not detectable after a 24 hour incubation due to the decrease in concentration of dAdo produced by adenosine deaminase and by the catabolism of dATP.

Dipyridamole, a smooth muscle relaxant used as a coronary vasodilator, is known to be a competitive inhibitor of nucleoside

transport (Plagemann and Erbe, 1974). Dipyridamole, in the range of 25-100 μM, inhibited the inward transport of adenosine and deoxycytidine in L1210 cells. At a concentration of 100 μM, dipyridamole inhibited the outward transport of uridine and cytidine in L1210 cells which could not metabolize deoxycytidine (Kessel and Dodd, 1972; Kessel and Hall, 1970). Our data shows that dipyridamole in the range of 5-10 μM inhibited adenosine (1 μM) accumulation in the presence of 40 μM dAdo and dCF (0.3 μg/ml) but did not inhibit dAdo (40 μM) accumulation in the presence of dCF. The dipyridamole block on the uptake of [^{3}H]dAdo could be overcome by the addition of nonlabelled dAdo (10-40 μM) or by different nucleosides that are phosphorylated by the same kinase in cultured chicken fibroblasts (Scholtissek, 1968). Paterson et al. (1977) have shown that differences occur in the nucleoside transport systems between species and cell types. Dipyridamole (10 μM) has also been demonstrated to inhibit the transport of dAdo into L1210 cells without added dCF by 88% in short-term kinetic studies carried out for 18 seconds at 20°C (Kessel, 1978). Our results here are not at variance, since long-term exposure of L1210 cells to dAdo, dCF, and dipyridamole (1-10 μM) would allow for a considerable uptake of dAdo even at 88% inhibition.

The dramatic increase in dATP amount produced by adding dipyridamole to dCF and dAdo combinations in L1210 cells was related to cell toxicity. dATP is well known to be a potent feed-back inhibitor of ribonucleotide reductase. The intracellular concentration of dATP

could be a critical factor in regulating DNA synthesis. The cytotoxicity of dAdo may be related to the accumulation of dATP to a level which inhibits the formation of other deoxynucleotides (Thelander and Reichard, 1979).

The concentration of dATP required to give 70% inhibition of ribonucleotide reductase activity in partially purified Ehrlich tumor cells was about 20-30 μM (Sato and Cory, 1981). Ribonucleotide reductase activity was inhibited by 32% when 10 μM dATP was added back to the Dowex 1-treated EHNA and dAdo extracts of HeLa cells after 16.5 hours of incubation (Lin and Elford, 1980). The dATP concentrations in dCF and dAdo treated L1210 cells and dCF, dAdo and dipyridamole-treated L1210 cells were 25-30 μM and 400 μM respectively. The dATP amount (25-30 μM) at 3 hours after the addition of dCF and dAdo gave a 60% inhibition of ribonucleotide reductase activity. However, the reduced amount of ribonucleotide reductase activity was almost the same as that of the cells treated with dAdo alone. Therefore, the correlations of dATP amounts and cell cytotoxicity in L1210 cells treated with dCF and dAdo are not compatible with inhibitions of ribonucleotide reductase activity. One possible explanation for this discrepancy may be that the CDP reduction was not as susceptible to inhibition by dATP as the reduction of other three ribonucleotides. The radioactive label provided in ribo-CDP was not diluted by added dCTP during its incorporation into DNA showing that ribo-CDP does not pass through a dCTP pool in lysolecithin-permeabilized Chinese hamster

embryo fibroblast cells in culture (Reddy and Pardee, 1982). If the metabolic intermediates of the ribonucleotide reductase pathway do not mix with the free dNTPs, but rather are "channeled" directly to DNA (Reddy and Pardee, 1982), the dATP produced by the addition of the dCF and dAdo in combination or the dCF, dAdo and dipyridamole in triple combination may not be effectively incorporated into DNA. This will result in the depletion of dATP for DNA synthesis since dATP is a known allosteric inhibitor of ADP reduction. The triple combination of dAdo, dCF, and dipyridamole when used at 40 μM, 0.3 μg/ml, and 10 μM concentrations, respectively, gave a dATP level in L1210 cells of 400 μM and ribonucleotide reductase activity was inhibited 80%. While this degree of enzyme inhibition may be significant, complete inhibition might have been expected since 400 μM dATP is approximately 40 times the concentration to give 50% inhibition in some purified systems (Moore and Hurlbert, 1966).

Another possible explanation is that when dAdo is used in the presence of dCF, dATP may be converted to dAdo, which can not be further catabolized in the presence of dCF. The dAdo accumulated in the cells may inactivate S-adenosylhomocysteine hydrolase, which results in the inhibition of transmethylation reactions mediated by S-adenosylmethionine (Hershfield, 1979; Cass et al., 1982). In this connection, when radiolabeled dAdo was used with dCF and dipyridamole, there was a large peak that eluted in the nucleoside region (solvent front) under conditions of HPLC separation of nucleotides from L1210

cell extracts. This peak was identified as dAdo. In contrast to the results with dATP accumulation which showed maximum concentration at 7 hours with the triple combination, the dAdo concentration increased up to 1 mM continuously during the time studied (20 hours). At this intracellular concentration of dAdo, phosphorylation of dAdo might be inhibited by the free dAdo accumulating in these cells as a consequence of the inhibition of ADA and by the action of dipyridamole. Carson et al. (1977) observed that the apparent Km value of a human thymus kinase for dAdo was about 400 μM, and Chang et al. (1982) observed that the apparent Km value of the purified deoxyadenosine kinase (L1210 cells) for dAdo was 1.25 mM in the presence of ATP. A 1 mM intracellular concentration of dAdo is insufficient to saturate deoxyadenosine kinase in L1210 cells, but might be sufficient to saturate adenosine kinase. Henderson et al. (1982) suggested that adenosine kinase is more important than deoxyadenosine kinase in the phosphorylation of dAdo because of its much greater total activity in cells.

Recently, a soluble deoxynucleotidase that preferentially dephosphorylates 2'-deoxynucleotides (Carson et al., 1981) was identified in mammalian cells. This enzyme activity correlates inversely with the sensitivity of the cells to dAdo toxicity when adenosine deaminase is inhibited. Since AMP deaminase has little capacity to deaminate dAMP, 5'-nucleotidase converts dAMP to dAdo. However, with the accumulation of dAdo caused by dCF, a substantial amount of dAMP,

dADP, and dATP are formed. It will be interesting to study the possible inhibition of 5'-nucleotidase activity by dipyridamole in L1210 cells and this mechanism may explain the different responses of T- and B-cells to dAdo toxicity.

The L1210 tumor was used here as a model of the non-T, non-B cell type (Halcenberg and Camitta, 1981) most commonly found in childhood ALL. In the presence of dipyridamole and when adenosine deaminase was inhibited with dCF, the L1210 cell reacted to dAdo toxicity like a T-cell type. The large increase in dATP and dAdo levels in the presence of dipyridamole is consistent with a greater inhibition of efflux than influx. This finding may possibly have utility in the clinic since the inhibition of dAdo efflux might enhance the cytotoxicity of dAdo.

Future studies are required to establish the exact biochemical mechanism of the triple combination, such as (1) transport studies (influx and efflux) of dAdo in the presence of dCF and dipyridamole; (2) inhibition studies of S-adenosylhomocysteine hydrolase activity in L1210 cells with dCF, dAdo, and dipyridamole.

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