1982

Uptake, metabolism, and toxicity of cytosine arabinoside in human leukemia cells following sequenced 3-deazauridine and pyrazofurin pretreatment

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A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

1982

ABSTRACT

The effect of pretreatment with 3-deazauridine (DAU) and pyrazofurin(PF), alone or in sequence, on the uptake, metabolism, and *in vitro* cytotoxicity of cytosine arabinoside(Ara-C) was studied in murine(L1210) and human (HL-60) leukemic cells. PF and DAU block synthesis of CTP at different steps of the pyrimidine biosynthetic pathway. Pretreatment with either agent alone for three hours at 5μM better than halved the expected cell survival following one hour of 5μM Ara-C in L1210. In HL-60 such pretreatment resulted in only marginally better than additive cytotoxicity with 3μM Ara-C(1 hour), and PF pretreatment was as effective as DAU pretreatment at reducing cell survival despite the fact that the latter significantly increased Ara-C uptake and Ara-CTP formation while the former had no effect on total Ara-C uptake and actually depressed Ara-CTP levels.

Pretreatment with both PF and DAU, in either sequence, resulted in almost total cell kill in L1210 cells exposed to 2μM or 5μM Ara-C for one hour. When the Ara-C exposure precedes PF and DAU, synergy is still noted in these cells, but it is an order of magnitude smaller. In HL-60, pretreatment with both drugs results in marked synergy with Ara-C cytoxicity(3μM Ara-C for 1 hour). Greater cytotoxic synergy was evident when PF exposure preceded DAU exposure than when DAU was administered first, even though the latter resulted in a significantly higher rate of Ara-C uptake. In addition, HL-60 cells pretreated with both PF and DAU, in either order, had Ara-CTP levels no higher than cells exposed to Ara-C without pretreatment. The key to understanding the synergy exhibited between the pretreatment regimes and Ara-C is the linear relationship between the Ara-CTP/CTP ratio and clone survival. At the concentrations and durations of exposure studied, PF depleted cellular CTP to a much greater degree than DAU, and the combination of the two, especially when PF exposure is first, further depleted CTP. Ara-CTP formation is apparently influenced by the level of UTP as well as the level of dCTP, and the fraction of Ara-C taken up that is converted to Ara-CTP may depend on the relative concentrations of the pyrimidine nucleotides. These findings may be helpful for the evaluation of other pretreatment regimes designed to enhance Ara-C cytoxicity. Further studies are necessary to define the possible utility of the PF/DAU/Ara-C combination.

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ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to my thesis advisor, Dr. Ed Cadman, for his guidance and support. I would also like to thank the following people in Dr. Cadman's lab for their technical assistance and suggestions: Douglas Armstrong, Chris Benz, Ted Boritzki, Lynn Danhauser, Robert Heimer, and Frank Rauscher III. Special thanks to Joan Gesmonde and Barbara Stanley.

for my darling Nancy-

who showed me that medical school
can be an experience in loving
as well as in learning

I. INTRODUCTION

Two relatively recent developments in the design of antineoplastic chemotherapy are the concept of malignant transformation-linked enzymic alterations and the use of deductive, as opposed to empiric, reasoning to construct drug combination regimes. The former arises from observations like those of Geoge Weber and his co-workers (111, 112,113,115) that malignant neoplasms of varying origins exhibit elevated activities of a number of key enzymes and their products and that the height of those elevations can, to a certain extent, be correlated with growth rates in a series of tumors. The recognition and control of such enzymes and their products may well be the key to treating neoplasia. The latter is exemplified by the use of an agent to deplete the cell of a vital molecule before administration of a drug whose toxic effect is exerted in competition with that compound. This project applies both approaches to the design and study of a novel combination of antineoplastic drugs and studies them in murine (L1210) and human (HL-60) leukemic cells.

One of the major targets for chemotherapeutic agents has been the synthesis of deoxyribonucleotides, based on the increased need of rapidly dividing cells, as opposed to normal tissue, for DNA precursors. Notable among the enzymes found elevated in a variety of tumors

are a large number of the enzymes involved in pyrimidine biosynthesis and salvage, and levels of pyrimidine ribo- and deoxyribonucleotides are elevated as well (111,113, 115). Pyrimidine catabolic pathways are, on the other hand, depressed in neoplasia.

Cytosine arabinoside(Ara-C, Cytarabine, 1-beta-D-arabinofuranosylcytosine) is a cytosine analog which at present is a major agent used in the treatment of acute myeloid leukemia in adults and being studied for use in combination with agents in a number of other tumors (94), but whose clinical utility is limited by rapid detoxification and emergence of resistant clones. It acts by inhibiting DNA synthesis, via direct incorporation into DNA and/or inhibition of DNA polymerase, in competition with the natural substrate dCTP. 3-Deazauridine(DAU, 1-beta-D-ribofuranosyl-2,4-pyridinedione) is a structural analog of uridine which competitively inhibits cytidine triphosphate synthetase, the enzyme which converts UTP to CTP. By depleting cellular CTP and, presumably, dCTP, it removes feedback inhibition of deoxycytidine kinase, the enzyme responsible for phosphorylating, and thus activating, Ara-C; it may also inhibit Ara-C deamination(detoxification) by feedback inhibition of cytidine deaminase. Thus, despite modest antitumor effect of its own, there is reason to expect that it could enhance the toxicity of Ara-C, and perhaps act on

Ara-C resistant mutants who relied entirely on de novo pyrimidine synthesis. However, DAU limits its own toxicity in two ways. First, build-up of UTP behind the competitive block could lead to break-through CTP synthesis. Second, UTP excess would inhibit uridine kinase, the enzyme resposible for transformation of DAU to its active form.

Pyrazofurin(PF,4-hydroxy-5-beta-D-ribofuranosyl-pyrazole-3-carboximide) is a C-nucleoside analog which strongly inhibits orotidylate decarboxylase, the enzyme responsible for UMP production from orotidylate 5'-monophosphate. This agent thus blocks de novo synthesis of pyrimidines, and the resultant UTP and CTP depletion could, as with DAU, be expected to enhance Ara-C toxicity. However, PF is incapable of blocking uridine re-utilization, a major source of pyrimidines in some cell lines and, presumably, in some human tumors, expecially those derived from cells which import much of their pyrimidines. Perhaps the combination of PF and DAU, blocking both de novo and salvage pathways, and preventing UTP build-up and subversion of DAU inhibition of cytidine triphosphate synthetase, could enhance Ara-C cytotoxicity more than either alone. This project investigates the effects of DAU and PF pretreatment on the uptake, metabolism, and toxicity of Ara-C in L1210 and HL-60 cells.

A. Pyrimidine Nucleotides: Biosynthesis, Interconversion, and Control Mechanisms

Before discussing the effects of the agents being studied, it is in order to review briefly pyrimidine biosynthesis, the interconversions by which uridine 5'-monophosphate(UMP) is transformed into the other pyrimidine nucleotides, and the mechanisms which control these processes. Excellent reviews of recent advances in our understanding of pyrimidine biosynthesis and metabolism are available (51,69,99), and the readers are referred to them for a more detailed discussion and bibliography.

1. Biosynthesis

The pathways by which UMP is synthesized and converted to cytidine and thymidine nucleotides as well as its own are illustrated in Figure 1. De novo biosynthesis of UMP begins with the formation of carbamyl phosphate by carbamyl phosphate synthetase-II(CPS-II). In contrast to mitochondrial carbamyl phosphate synthetase-I, which utilizes ammonia as a nitrogen source for the urea-synthetic pathway, cytoplasmic CPS-II utilizes glutamine as its normal nitrogen source as indicated by enzyme kinetics (reviewed in (69)). Carbamyl phosphate is combined with aspartate to form carbamyl aspartate, a reaction catalyzed by aspartate transcarbamylase (ATC), which is then converted

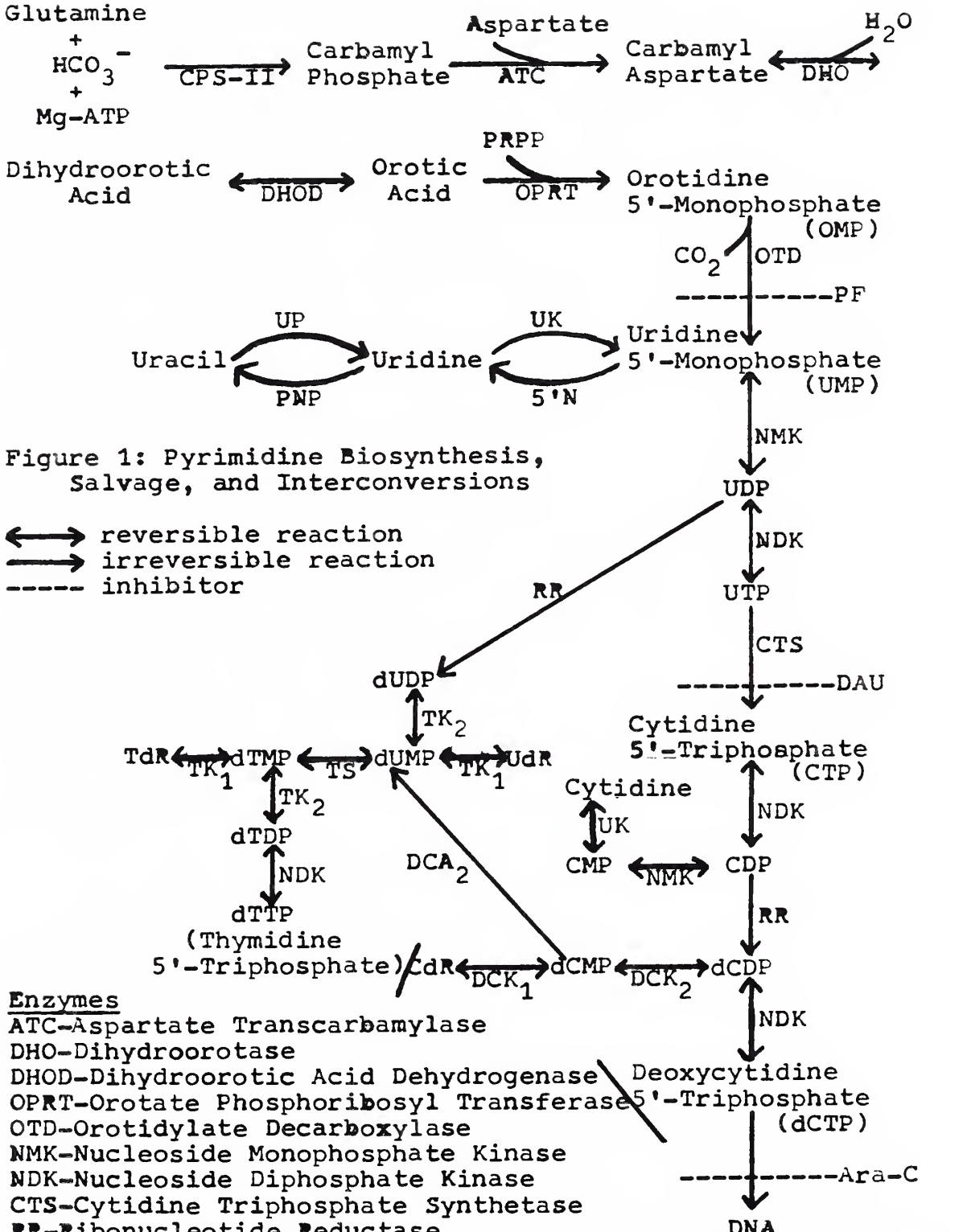


Figure 1: Pyrimidine Biosynthesis, Salvage, and Interconversions

⟷ reversible reaction
⟶ irreversible reaction
----- inhibitor

Enzymes

ATC-Aspartate Transcarbamylase
DHO-Dihydroorotase
DHOD-Dihydroorotic Acid Dehydrogenase
OPRT-Orotate Phosphoribosyl Transferase
OTD-Orotidylate Decarboxylase
NMK-Nucleoside Monophosphate Kinase
NDK-Nucleoside Diphosphate Kinase
CTS-Cytidine Triphosphate Synthetase
RR-Ribonucleotide Reductase
DCK_1-Deoxycytidine Kinase
DCK_2-Deoxycytidylate Kinase
TS-Thymidylate Synthetase
CPS-II-Carbamyl Phosphate Synthetase II
DCA_2-Deoxycytidylate Deaminase
PNP- Pyrimidine Nucleoside Phosphorylase
UK-Uridine Kinase
UP-Uridine Phoshorylase
TK_2-Thymidylate Kinase
5'N-5'-Nucleotidase
TK_1-Thymidine Kinase

to dihydoorotic acid by dihydroorotase(DHO). Since CPS-II, ATC, and DHO can be co-purified on column chromatography of tissue fractions, it has been suggested that these three enzymes exist in cells as a multifunctional complex (reviewed in (99)), perhaps providing a channeling of product to assist separation of mitochondrial and cytoplasmic carbamyl phosphate pools.

Dihydroorotic acid is oxidized to orotic acid by dihydroorotic acid dehydrogenase(DHOD), which, in contrast to the other enzymes of the de novo pathway, appears to be associated with cellular membranes (reviewed in (99)). The orotic acid is combined with 5-phosphoribosyl pyrophosphate(PRPP) by orotate phosphoribosyl transferase(OPRT) to form orotidine 5'-monophosphate(OMP). OPRT exists as a cytoplasmic complex with the final de novo pathway enzyme, orotidylate decarboxylase(OTD), which catalyzes the irreversible decarboxylation of OMP to produce UMP.

Regulation of the de novo pathway (see Table 1) is primarily directed at the initial step, the formation of carbamyl phosphate from glutamine, bicarbonate, and ATP by CPS-II. This is present at lower activity than the other enzymes in its cluster in a number of tissues studied, as presented by Moyer (80), and the presence of UTP, an ultimate product of the pathway, inhibits the enzyme, apparently acting in an allosteric fashion by decreasing its

affinity for ATP, the only substrate not present in excess, by as much as a factor of ten (69,80). PRPP, on the other hand, has been shown to increase the affinity of CPS-II for ATP, thus enhancing its activity (reviewed in (99)). Stimulated cells, such as phytohemaglutinin(PHA)-stimulated human lymphocytes and isoproterenol-stimulated salivary glands, have elevated CPS-II activities, and CPS-II activity has been correlated with the mitotic index in normal tissues and growth rates of a series of tumors (reviewed in (69)). To a lesser extent, PRPP may activate the activity of OPRT, and both UMP and cytidine 5'-monophosphate(CMP) inhibit OTD, though the usually low levels of the monophosphates and the relatively high inhibition constants for them make this unlikely to be an important regulatory mechanism (69).

2. Interconversion

Synthesis of cytidine and deoxycytidine nucleotides from UMP begins with the production of UTP by the action of nucleoside monophosphate kinase(NMK) and nucleotide diphosphate kinase(NDK), enzymes of high activity (49) that provide for rapid equilibrium of mono-, di-, and triphosphate nucleotide pools, with the triphosphates the predominant form unless cellular energetics are compromised (80). Cytidine triphosphate synthetase(CTS) then catalyzes the transformation of UTP to CTP, from which the other

cytidine ribonucleotides are derived. CDP is the substrate from which ribonucleotide reductase(RR) produces deoxycytidine 5'-diphosphate(dCDP), which NDK phosphorylates to produce dCTP, the form used in DNA synthesis. Removal of phosphate groups from dCDP, by deoxycytidylate kinase (DCK_2) and deoxycytidine kinase (DCK_1), results in the formation of dCMP and deoxycytidine(CdR), respectively.

Thymidine nucleotides are produced by the action of thymidylate synthetase(TS) on deoxyuridine 5'-monophosphate(dUMP). dUMP can be formed by two pathways. dUDP formed by the action of RR on UDP is dephosphorylated by thymidylate kinase(TK_2) to dUMP. Alternately, dCMP can be directly converted to dUMP by deoxycytidylate deaminase(DCA_2). A recent study by Jackson (61) suggests that the latter pathway is the major source of dUMP in mammalian cells, the impact of which will be considered below. TK_2 converts dTMP to dTDP, then NDK catalyzes the formation of dTTP.

3. Salvage and Uptake

Pyrimidine nucleotides can also be generated by salvage of free nucleosides within the cell or in the extracellular space or from intracellular uracil, as illustrated in Figure 1. Uridine phosphorylase(UP) catalyzes the reaction between uracil and ribose-1-phosphate to form uridine. Cytidine and uridine are phosphorylated by uridine kinase(UK), also known as uridine-cytidine kinase,

to CMP and UMP. Deoxyuridine(UdR) and thymidine(TdR) are converted to dUMP and dTMP by thymidine kinase(TK_1), and CdR is converted to dCMP by DCK_1.

In a review of pyrimidine transport in animal cells, Berlin and Oliver(6) discuss transport mechanisms for nucleosides, which must be differentiated from uptake as used later in this paper. The latter term refers to total accumulation of a substance and its metabolites; in the case of nucleosides these would be the nucleotides formed rapidly under most metabolic conditions. Transport refers to intracellular accumulation of a substance in the absence of conversion which would maintain the diffusion gradient between extra- and intracellular compartments. For nucleosides measurement of transport rates requires cells deficient in the phosphorylating enzyme or cells depleted of ATP. Under such conditions, Berlin and Oliver discuss evidence that at low extracellular substrate concentrations transport of nucleosides is a saturable, non-accumulative, temperature-dependent process, suggesting carrier-mediated facilitated diffusion, while at high extracellular nucleoside concentrations uptake is proportional to concentration and only weakly temperature-dependent, suggesting simple diffusion (6). Both processes are inhibited by other nucleosides, but not by free bases, sugars, or amino acids. Nucleoside analogs with ionized, but not uncharged, substituents on the base moiety or the

replacement of ribose with sugars other than arabinose reduce affinity for the low concentration carrier (reviewed in (6)). Plagemann et al.(86) have recently shown that in Novikoff rat hepatoma cells transport of CdR and Ara-C is so rapid to equilibrate intra- and extracellular concentrations within less than one minute, as they had previously shown for adenosine, thymidine, and uridine (reviewed in (6)). The rates of transport were 1-2 orders of magnitude higher than the rates of phosphorylation, suggesting that the latter is the limiting step in uptake (86).

4. Regulation of Interconversion and Salvage

Regulation of the enzymes involved in pyrimidine nucleotide intercoversions and re-utilization of nucleosides described above is complex and incompletely understood. Effects of nucleotides themselves studied to date are presented in Table 1.

Table 1: Regulation of Pyrimidine Synthetic Enzymes

Enzyme	Urd	UMP	UTP	PRPP	CTP	dCTP	dTTP	ATP	dATP
CPS-II			-	+					
OPRT				+					
OTD		-			-				
RR						-	-	+	-
CD	-								
DCA_2		-				+	-		
DCK^1			+			-	+@		
CTS							-		
TK							-		
UK			-		-				

(+) Activated by increased levels
(-) Inhibited by increased levels
@ See Text

The best studied of these is dTTP. As reviewed by Harrap and Renshaw (51), dTTP restricts its own biosynthesis by allosteric inhibition of three enzymes. Salvage of thymidine and deoxyuridine is inhibited via inhibition of TK_1. By inhibiting RR, which catalyzes both the reduction of UDP to dUDP and CDP to dCDP, dTTP affects both pathways for dUMP production. This inhibition also explains the decline in dCTP levels and commensurate increase in CdR uptake noted by Grant et al.(46) after thymidine exposure. It also explains the phenomenon of CdR-reversible thymidine toxicity (61,70). The pathway to dUMP from CDP is also inhibited by dTTP via inhibition of DCA_2. It is interesting that TS, target for pyrimidine analogues, such as 5-fluorodeoxyuridylate, and antifolates, which limit dTMP production by depletion of the carbon group donor, is not subject to short-term regulation by thymidine nucleotides (61).

DCK_1, the best studied of the cytidine pathway enzymes by virtue of its role in Ara-C activation(see next section), is inhibited by dCTP and, to a lesser extent, dCMP, dCDP, UDP, CDP, and dTDP (60), while it is stimulated by dUTP and UTP. The action of dTTP on DCK_1 is complex. At low levels, dTTP stimulates the enzyme, reversing dCTP inhibition at relatively low levels of dCTP. At higher nucleotide concentrations, dTTP replaces the dCTP effect as the

major source of DCK_1 inhibition (60). dCTP also restricts its own synthesis via feedback inhibition of RR. Evidence for CTS inhibition by dTTP in E. coli has been presented (as referred to in (61)), but it is not known whether this control operates in mammalian cells. Further control of RR is exerted by ATP and dATP, higher ratios of which activate the enzyme (61). Finally, a recent study by Drake et al.(35) suggested that CD and DCA_2 are inhibited by their products, uridine and dUMP, respectively, and analogs of those products, especially DAU and DAU-MP.

A number of authors have shown that the activities of many enzymes involved in dNTP synthesis increase at the onset of S phase, notably TK_1, TK_2, DCK_1, and DCA_2 (reviewed in (51)). Mitotically-selected or isoleucine-synchronized Chinese hamster cells that had been transformed by a virus demonstrated an expansion of dNTP pools, usually very small relative to NTP pools, during S phase, with the level of dCTP corresponding most closely with DNA synthesis (see (51)). Transport rates for nucleosides also rise significantly from G_1 to S, then fall into G_2 (6). The concentration of maximal transport (K_m) remains the same with increases in V_m, the maximum rate of transport, most likely due to enhanced phosphorylation (as discussed in (6)).

PHA-stimulated human lymphocytes exhibit an increase in re-utilization pathway enzymes, uridine and thy-

midine kinases, followed by stimulation of de novo biosynthetic pathway enzymes (69). Finally, as noted above, Weber and co-workers (111,115) have studied pyrimidine pathway enzymes in normal liver and a variety of hepatomas with different growth rates. They have noted increases in most of the de novo and salvage pathway enzymes and corresponding decreases in pyrimidine catabolic enzymes. In particular, they have reported a correlation between the activities of certain enzymes, most notably TK_1, TK_2, DCA_2, and CTS, and the growth rates of the tumors studied. Although it is intuitively obvious that rapidly dividing cells require more deoxyribonucleotides, the pivotal roles of dCTP and dTTP, as discussed above, should be evident as we proceed to the discussion of Ara-C, DAU, and PF toxicity.

B. Cytosine Arabinoside(Ara-C)

Cytosine arabinoside(Ara-C) is a synthetic derivative of naturally occuring arabinonucleosides. These are isomers of the ribosyl derivatives but epimeric at the 2' position of the sugar moiety, which were first discovered in an unusual sponge in the early 1950's (see (24) for further details). First synthesized in 1959, it was soon demonstrated to be an effective inhibitor of murine leukemia (38). In the late 1960's, extensive clinical tests demonstrated Ara-C to be an effective agent in the treatment of acute leukemia in adults (8,37). At present, Ara-C is a first-line drug in a variety of combined drug programs for remission induction and maintainence in acute myeloid leukemia(AML) (87,94). Although it is also effective in a percentage of patients with acute lymphocytic leukemia(ALL), its value as first-line therapy is limited in comparison to the high percent of response obtained with other agents (94). However, a good percent of responses have been obtained in patients who are resistant to standard therapy(90,94). Ara-C is not effective against the chronic or blastic phases of chronic leukemias (116). Its role in the treatment of solid tumors has yet to be fully defined. A modest response to Ara-C has been reported for gastric, ovarian, and pancreatic cancer (1, 30,31,94), and its effectiveness in combination with other

chemotherapeutic agents is being studied.

The major and dose-limiting side effect produced by Ara-C in man is bone-marrow suppression, with resultant thrombocytopenia, anemia, and leukopenia with immunosuppression. Continuous infusion administration produces a greater degree of leukopenia for a longer period than does bolus administration (94). The other limiting factor to Ara-C treatment is the appearance of a clone of leukemic cells that are resistant to the drug. This will be discussed later.

The structure and metabolism of Ara-C are shown in Figure 2. Studies revealed that the antitumor effect of the drug requires uptake and phosphorylation by the cells (21,65). Transport of Ara-C corresponds to that of deoxycytidine (86), and no barrier to drug uptake has been identified in cells resistant to the effects of Ara-C (65), but drug resistance in murine leukemia cells, both *in vitro* (21) and *in vivo* (106), and in normal and leukemic human blood cells *in vitro*(64,65) has been associated with a decreased ability to phosphorylate both Ara-C and deoxycytidine.(98). The enzyme which catalyzes this phosphorylation has been identified as deoxycytidine kinase(DCK_1) (36,78). Kinetic studies with the enzyme revealed greater affinity for the natural substrate than the drug and, as noted in the previous section, significant inhibition by

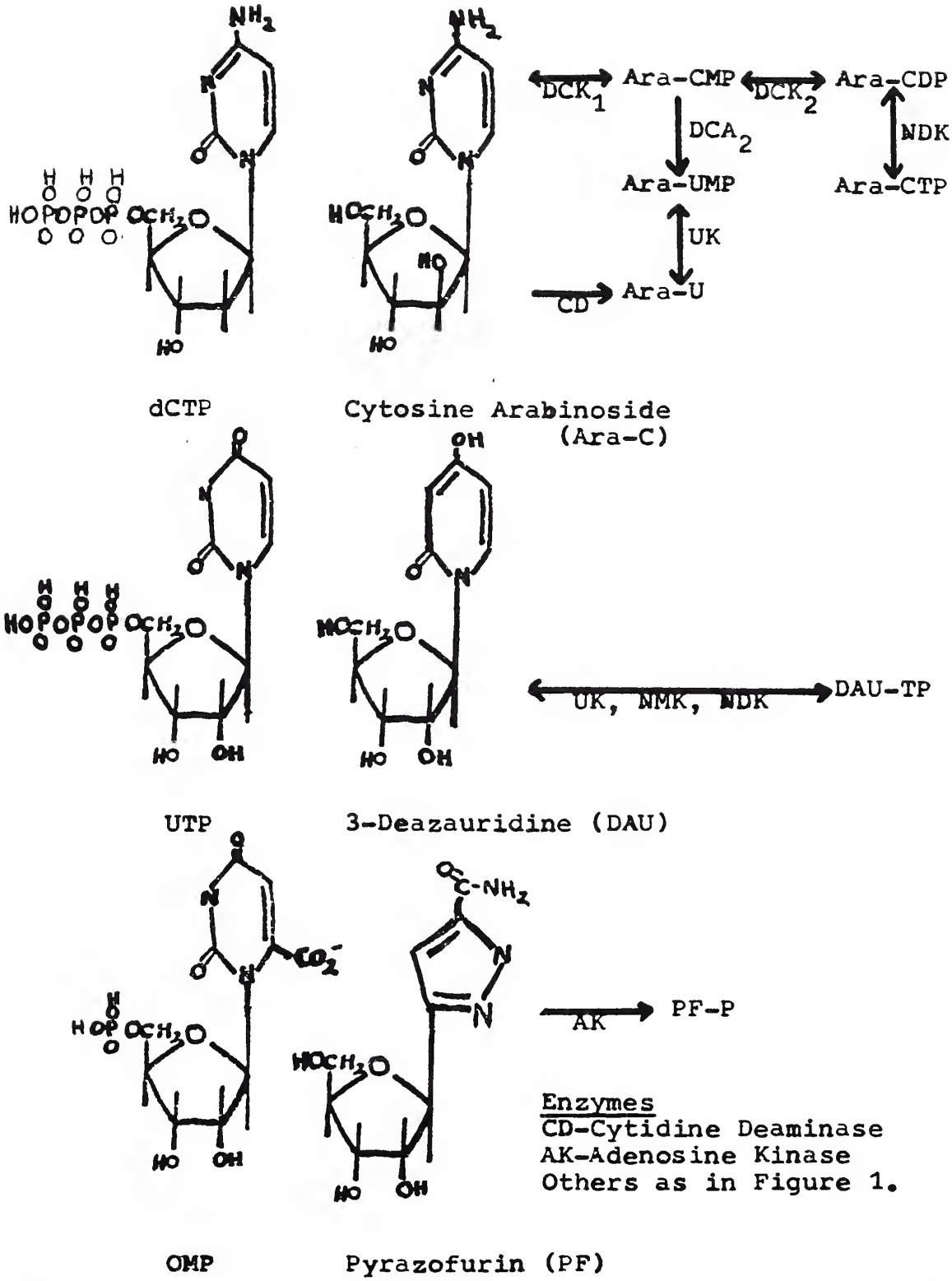


Figure 2: Structure and Metabolism of Ara-C, DAU, and PF, and Comparison to Natural Substrate for Enzyme Inhibited

cytidine deoxyribonucleotides (60,78). The production of Ara-CMP by DCK_1 represents the rate-limiting step in the production of Ara-CTP (49), the toxic form of the drug (20,65). The ability to form and retain Ara-CTP has been correlated with response to Ara-C treatment by Rustum and co-workers in both animal(95) and human(97) tumors, and and expansion of dCTP pools, with resultant DCK_1 inhibition, has been related to Ara-C resistance in Chinese hamster fibroblasts (32).

Ara-C is effective only when administered parenterally, and even after rapid intravenous administration the drug has an initial plasma half-life of only 3-15 minutes (52,59) with a second plasma decay phase of 2-2½ hours (59). The reason for the rapid drop of plasma levels is the action of cytidine deaminase(CD), an enzyme that is distributed throughout the body, especially in liver, kidney, and intestine (16,59). This enzyme converts Ara-C to the inactive form Ara-U (see Figure 2). In most species 90-95% of the Ara-C administered appears in the urine as Ara-U, humans included (24). Rats, which are low in CD, excrete 70% of administered Ara-C unchanged. Administration of a synthetic inhibitor of CD, tetrahydrouridine, decreased the rate of Ara-U production in both mice (57) and humans (19) and increased Ara-CTP levels in leukemias usually resistant to Ara-C (19). Clinical studies

with THU demonstrate significant myelosuppression at much lower Ara-C dosage than without the inhibitor (117), but have yet to show improvement in response rate. Ara-CMP is subject to deamination by DCA_2 to dUMP, but the significance of this reaction is unknown.

Steuart and Burke (100) reported elevated CD levels in the bone marrows of patients with leukemias resistant to Ara-C relative to CD levels in the marrows of patients with leukemias resposive to Ara-C. Tattersall et al.(105) found high levels of CD activity in tandem with low levels of DCK_1 activity in human AML cells resistant to Ara-C. DCA_2 activity was unchanged in this study. Ho (57) calculated ratios of DCK_1:CD in a number of normal and neoplastic tissues. He found low ratios in normal peripheral white cells and marrow, and in cells from patients with CML, while marrow and WBC from patients with AML and CLL had high DCK_1:CD ratios. A recent study using tetrahydrouridine to inhibit CD has questioned the validity of studies relating Ara-C resistance to increased CD activity (52), citing the use of lysed cells as a source of overestimating CD activity, but failed to settle the matter. Finally, CD has been shown to be inhibited by uridine and its analogs, most notably DAU (35), which may in part explain the ability of DAU to increase cellular Ara-CTP levels (35).

A number of agents have been shown to increase Ara-C uptake and toxicity. As noted above, cells pretreated with thymidine have depleted dCTP pools and exhibit increased uptake of CdR from the extracellular space (46). Thymidine-depletion of dCTP also results in enhanced Ara-C uptake and formation of Ara-CTP (46,86), and increased Ara-C incorporation into DNA. This effect was maximized at thymidine concentrations of 0.1mM; higher concentrations resulted in suboptimal enhancement (46). Harkrader et al.(50) demonstrated similar effects with deoxyadenosine(AdR) and deoxyguanosine(GdR). Both depleted dCTP and dTTP pools to below 50% of control, and AdR depleted CTP and UTP pools as well(GdR was not studied). Both enhanced incorporation of Ara-C into DNA, but their effects differed in that AdR increased cellular Ara-CTP while GdR-treated cells had Ara-CTP levels equal to that of control cells. In spite of that, GdR enhanced incorporation of Ara-C into DNA to three times normal while AdR increased incorporation to twice normal, and GdR displayed greater synergy of cytotoxic effect than did AdR (50).

Methotrexate(MTX) has been shown to synergistically enhance Ara-C cytotoxicity in L1210 cells (13), but only when the concentration of MTX used was high enough to result in free, that is, not bound to dihydrofolate reductase, intracellular MTX. Under these conditions, pretreat-

ment with MTX decreased intracellular dCTP levels substantially and resulted in enhanced uptake and phosphorylation of Ara-C, presumably via reduction of feedback inhibition on DCK_1 by dCTP.

A recent paper by Rauscher and Cadman (89) investigated the effects of hydroxyurea(HU) pretreatment on Ara-C uptake, metabolism, and toxicity, reasoning that HU inhibition of ribonucleotide reductase would result in depletion of deoxyribonucleotides, including dCTP. They demonstrated a time and dose mediated HU enhancement of Ara-C uptake and Ara-CTP production for both L1210 murine leukemia and HL-60 cells, and remarkable synergy of cytotoxicty in both cell lines, related to dCTP depletion (89). Workers in the same lab, working with the L1210 cell line, have also recently correlated enhanced Ara-C lethality following fluoropyrimidine(5-fluorouracil, 5-fluorouridine, and 5-fluoro-2'-deoxyuridine, but not 5'-deoxy-5-fluorouridine) with dCTP depletion, increased Ara-C uptake and increased Ara-CTP formation (45). Thus a number of agents have been identified which enhance Ara-C toxicity presumably by lowering dCTP levels. Studies with DAU and PF will be discussed in the following sections.

Ara-C toxicity is thus a well studied phenomenon, but the mechanism by which Ara-CTP kills cells is still a matter of controversy. Although early studies suggested

that Ara-C might act by inhibiting ribonucleotide reductase (21), or by incorporation into RNA (22), evidence soon accumulated that although labelled Ara-C could be found in both RNA and DNA (22), the drug acted primarily by inhibiting DNA synthesis (24,41,88,94). Thus, its action is cell-cycle dependent - it exerts its major toxic effect during S phase (41). However, it also tends to block the progression of cells from G_1 to S, thus to a certain extent limiting its own toxicity (41,74). Vadlamudi and Goldin (107) reported enhanced Ara-C toxicity in vitro and in vivo after cell-cycle synchronization with demecolcine(Colcemid) or vinblastine, timing the Ara-C administration to correspond to the entry of the majority of the synchronized cells into S phase for maximum lethal effect.

Ara-CTP acts in competition with dCTP, and cells treated with Ara-C can, up to a certain point, be rescued with CdR (22). The dispute is over whether Ara-C cytotoxicity is the result of inhibition of DNA polymerase or incorporation into DNA and subsequent disturbance of DNA replication. Favoring the former hypothesis are kinetic studies with viral (74), bacterial (88), and mammalian (39,74) DNA polymerases demonstrating interference with dCTP utilization without affecting transcription of a poly-dAT template (reviewed in (24)), and the discovery of altered DNA poly-

merase sensitivity to Ara-C inhibition in bacteria (88) and human ALL blast cells (104) resistant to Ara-C. On the other hand, a number of studies have demonstrated disorders of DNA replication following Ara-C incorporation (3,33,118) and an early study correlated number of chromatid breaks with cell death following Ara-C exposure (63). More recently, a number of studies with Ara-C alone and in combination with drugs that enhance its lethality have correlated cytoxicity convincingly with Ara-C incorporation into DNA (45,46,66,89). Therefore, although the question of Ara-C toxicity has yet to be resolved, the weight of evidence appears to be shifting in favor of Ara-C incorporation into DNA, allowing for the development of a DNA polymerase that distinguishes between Ara-CTP and dCTP as a mode of resistance to the drug.

C. 3-Deazauridine(DAU)

3-Deazauridine(DAU) is a uridine analog in which the N_3 of the pyrimidine ring is replaced by carbon (see Figure 2), first synthesized by Robins and Currie (92) in 1968. It was shown to inhibit the growth of E.Coli and L1210 cells *in vitro* (93) and to increase the lifespan of L1210 tumor-bearing mice (96). Uridine kinase phosphorylates the drug to DAU-MP (9), from which DAU-TP, the active form of the drug (76), is formed. The drug inhibits both DNA and RNA synthesis without significantly affecting protein synthesis (9). McPartland *et al.* demonstrated inhibition of the enzyme responsible for the deamination of UTP to CTP, cytidine triphosphate synthetase(CTS), by DAU-TP (76), and this was supported by findings that DAU toxicity could best be reversed by cytidine and to a lesser extent by deoxycytidine or uridine (9,76). Reversal of toxicity by uridine agrees with findings that DAU inhibition of CTS is competitive with respect to UTP (76). Deoxyuridine and thymidine did not reverse DAU inhibition of nucleic acid synthesis. Recently, DAU and DAU-MP have been shown to inhibit CD and DCA_2, respectively (15,35), although the importance of this in the intact organism is uncertain. On administration of the drug to humans relatively rapid excretion of unchanged drug in the urine is noted, with an initial plasma

half-life of 1-1½ hours (27), and a terminal half-life from 4 to 10 hours (27,28) after rapid administration. The drug has a relatively large volume of distribution (18.8 liter/kg), thus continuous infusion led to a higher terminal half-life, averaging 21.3 hours in a study by Benvenuto et al.(4). Dose-limiting toxicities include nausea, vomitting, and oral stomatitis; marrow suppression is rare (27). Clinical trials with DAU in acute leukemia (120) and solid tumors (101) have been disappointing, leading to suggestions that DAU may prove most useful in combination with other agents.

Early studies with DAU by Brockman et al.(9) and Rustum et al.(96) demonstrated that L1210 cells resistant to the effects of Ara-C were more susceptible to DAU than L1210 cells susceptible to Ara-C. Evidence for three possible mechanisms for this effect appeared. First, L1210 cells resistant to Ara-C(L1210/A) had lower baseline dCTP levels than L1210 cells sensitive to Ara-C(L1210/0) (9,96), likely due to deficient DCK_1 activity, thus making them more dependent on the CTS pathway for dCTP. Second, L1210/A cells formed more DAU-TP than L1210/0 cells (96). Finally, despite equal UTP pools, CTP production in L1210/A cells from labelled UTP was lower than CTP production in L1210/0 cells (96). Thus the evidence suggests DAU in combination with Ara-C would help guard against the emergence

of an Ara-C resistant clone. In addition, CTP depletion following DAU exposure suggested its use to enhance Ara-C action in Ara-C sensitive cells as well (77).

Studies combining DAU and Ara-C have noted marked increases in Ara-C uptake (86), formation of Ara-C phosphates, especially Ara-CTP (77), and doubled incorporation of Ara-C into DNA (86), the likely source of the potentiated anti-proliferative effect noted in some (77) but not all (62) cell lines. DAU inhibition of cytidine deaminase is another possible source of enhancement (15,35). Lauzon et al.(68) noted arrest at the early S phase of cells exposed to 10μM DAU for 16 hrs, with enhanced Ara-C toxicity when exposed after transfer to medium containing no DAU. Unfortunately, enhanced toxicity towards tumor cells in culture may also signal more severe toxic side-effects in the whole animal, and Paterson et al.(84) noted drug-sequence dependent severe small bowel mucosal injury and death in mice treated with relatively small doses of DAU then Ara-C.

In a single clinical study on the effects of DAU and Ara-C for patients with relapsed acute leukemia (2), Barlogie et al. noted lower DAU-TP levels for either drug sequence than for DAU alone. Moreover, DAU-caused CTP depletion was not consistently associated with elevated Ara-CTP levels, and little clinical response was obtained,

though it should be noted that this was a group of patients who had already been exposed to Ara-C.

In combination with 5-azacytidine, a cytidine analog incorporated into RNA and presumed to exert its lethal effect by inhibiting protein synthesis (reviewed in (44)), DAU exerts a synergistic lethal effect, likely via depletion of CTP, the feedback inhibitor of UK, the enzyme required to activate 5-azacytidine. In combination with D-galactosamine, which depletes hepatic UTP, DAU-mediated depletion of CTP in rat hepatomas was very pronounced (up to 86%) (71), and in hepatomas this combination was strongly synergistic, as opposed to only additive effects in non-hepatic cell lines (62).

D. Pyrazofurin(PF)

Pyrazofurin(PF) is a C-nucleoside antibiotic (see Figure 2) isolated from the fermentation broth of a strain of Streptomyces candidus (103) demonstrated to inhibit growth of many, though far from all, tumors in vitro (11,47,103) and to prolong survival in rats inoculated with sensitive tumors (103). Most encouraging were the responses of solid tumors usually resistant to chemotherapy. Drug toxicity results from the formation of PF-MP from the action of adenosine kinase(AK) on PF (see Figure 2) (34); di- and triphosphate derivatives are formed but are inactive (34). This is corroborated by inhibition of PF phosphorylation by adenosine, but not other nucleosides (34), and the development of drug-resistant sublines demonstrated to be freely permeable to PF but lacking AK activity (34).

Dix et al.(34) demonstrated rapid competitive inhibition of orotidylate decarboxylase(OTD), the enzyme responsible for conversion of OMP to UMP (see Figure 1), as suggested by earlier studies which noted blockade of orotate metabolism (11) and reversal of PF toxicity in the presence of uridine or cytidine, but not purine nucleosides (11,83,85). Resistance in some cases may be due to enhanced ability to re-utilize uridine (11), and has been associated with elevated OTD activity (34,102).

The major toxic effect of PF exposure is inhibition of DNA synthesis, as indicated by profound suppression of deoxyadenosine incorporation in treated cells, and reversal of PF toxicity with deoxycytidine and thymidine in experiments by Plagemann and Behrens (85). PF enhances uridine uptake and eventual incorporation into DNA in cell lines capable of sufficient salvage (11,85).

OTD activity, as well as pyrimidine nucleotide (ribo- and deoxyribonucleotides) levels, were elevated in a variety of tumors studied by Weber et al.(114). PF severely depletes animal tumor cells of these nucleotides (10,113,114) in culture. Grown in ascitic fluid, UTP levels remained low in both sensitive and resistant cell lines, but CTP levels, initially depressed in both, returned to normal levels within 10 hours in a resistant cell line (11). Evidence for PF inhibition of purine synthesis, except via competitive inhibition of adenosine reutilization (85), is scant, though Worzalla and Sweeney (119) have reported inhibition of purine biosynthesis and PF-treated cells have exhibited ATP and GTP depletion as well as UTP and CTP depletion (10,113).

Cell cycle specificity of PF action has been studied by Hill and Whelan (55) and Olah et al.(83). The former pair related cell kill more to duration of exposure than concentration, finding cell kill predominantly

occurring in G_2 at low drug concentrations, but S phase toxicity at higher concentrations, with accumulation of cells in S phase or at the G_1/S boundary. The latter group agreed with the dominance of S phase killing at high concentrations(300μM), but found equal toxicity to early G_1 and early S phase cells for lower (10-25μM) concentrations. The importance of S phase killing is underscored by the limitation of PF toxicity to cells in logarithmic growth-plateau phase cells were unaffected (83).

Studies of PF in combination with Ara-C have suprising results. Greater than additive effects inhibiting cell growth and clone formation have been reported (11,55), but the mechanism of this synergy is unclear. While CTP (and presumably dCTP) depletion would remove feedback inhibition of DCK_1, leading, in theory, to increased Ara-C uptake and phosphorylation, PF pretreatment has not been found to increase Ara-C uptake or acid-soluble Ara-CTP (11,86). However, simultaneous PF and Ara-C markedly stimulated Ara-C uptake (86), and Ara-C incorporation into DNA was 15X control over the first 60 minutes (86).

PF markedly enhanced 5-azacytidine toxicity against murine leukemia cells <u>in vitro</u> (12) by increasing 5-azacytidine 5'-triphosphate formation, presumably by reducing UTP/CTP inhibition of uridine kinase. PF and D-galactosamine in combination synergistically depressed

UTP and CTP pools and exhibited more than additive cytotoxic effect (114).

In response to data from *in vitro* and animal studies demonstrating significant antitumor activity with PF alone (11) or in combination with 5-azacytidine (12,18), extensive clinical trials were undertaken. After some initial encouraging results (81), studies have indicated no significant antitumor effect of PF alone in a large variety of tumors (14,17,26,30,43,67,109,110) or improvement of 5-azacytidine effect in acute leukemia (73,108). Significant toxicity in the form of dermatitis and stomatitis was universally noted, and myelosuppression was noted after prolonged treatment, though decreasing dose frequency to weekly seemed to attenuate this side effect (30). Unless PF can be shown to enhance the antitumor effect of other agents, its usefulness as a chemotherapeutic agent will likely be limited.

II. MATERIALS AND METHODS

A. Materials

Ara-C, Ara-CTP, ATP, CTP, GTP, and UTP were purchased from Sigma Chemical Company(St. Louis, MO). PF was a gift from Ely Lilly & Company(Indianapolis, Ind.). DAU was obtained from the Drug Development Branch, National Cancer Institute, Bethesda, Md. 5,6-^{3}H-Ara-C (18 Ci/mmol) was purchased from Moravek Biochemicals, Inc.(City of Industry, CA). These were stored as powders in a dessicator tray at -5°C. Solutions of Ara-C, PF, and DAU were prepared by adding the drug to sterile water and stored at -5°C between experiments. 10^{-4}M ^{3}H-Ara-C was prepared by adding 0.1 ml labelled Ara-C to 1.0 ml 1.044×10^{-4}M 'cold' Ara-C. Ara-CTP, ATP, CTP, GTP, and UTP standards for the HPLC were prepared using carefully weighed ammounts of the pure compounds in 5.0 ml 75% 0.5M $NH_4H_2PO_4$(pH 4.85):25% 5mM $NH_4H_2PO_4$(pH 2.8), the buffer mixture at which the nucleoside triphosphates were eluted from the column.

Liquiscint was purchased from National Diagnostics(Somerville, NJ). All media referred to in the next section were purchased from Grand Island Biologicals Company(Gibco; Grand Island, NY).

B. Cells

Murine leukemia L1210 cells were maintained as a stationary suspension culture in Fischer's medium with 10% horse serum added at 37°C in a 5% CO_2 atmosphere and transferred twice weekly. Cultures done every two months for mycoplasma contamination were negative. Cells were inoculated at 5 X 10^4 cells/ml and used in logarithmic growth phase, between 1 and 1.5 X 10^5 cells/ml.

HL-60 is a human promyelocyte leukemia cell line derived from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia. As described by Gallagher et al (40), the cell line has been identified as a neutrophilic promyelocyte with prominent nuclear/cytoplasmic asynchrony. HL-60 cells were passed twice weekly in RPMI 1640 medium supplemented with 1% 10mM Sodium Pyruvate, 1% 100mM Nonessential Amino Acids (NEAA), and 10% heat-inactivated, dialyzed fetal bovine serum(DFBS) and maintained at 37°C in a 5% CO_2 atmosphere.

Double-strength McCoy's medium(2X McCoys) used for HL-60 cloning experiments was prepared by dissolving a 1 liter package of powdered Gibco McCoy's 5a Medium in 450 ml sterile water and adding

2.2 grams Sodium Bicarbonate
6.0 ml Gibco $NaHCO_3$ solution(pH 7.57)
10.0 ml Gibco 10mM Sodium Pyruvate
8.0 ml Gibco MEM Amino Acids

4.0 ml Gibco NEAA
4.0 ml Gibco MEM Vitamins
2.5 ml Gibco Penicillin/Streptomycin
15.0 ml Gln-Ser-Asn solution,

the last of which was prepared by adding 160 mg L-asparginine and 84 mg L-serine to 100 ml sterile water, to which is then added 40 ml Gibco L-glutamine solution.

Cells were counted in 0.9 M NaCl on a Coulter Model ZBI Counter (Hialeah, FL).

C. Soft Agar Cloning Assay

The soft agar cloning assay used to determine survival of L1210 cells has been described by Cadman et al.(12) as a modification of a technique initially reported by Chu and Fischer (22). Log phase L1210 cells at 1 - 1.5 X 10^5 cells/ml were used for the cloning studies. 10 ml of cells were exposed to DAU, PF, or Ara-C as per the experimental design. For sequential drug exposures, at the end of the first drug exposure the cell suspension was centrifuged at 1000 rpm for 8 minutes. The drug-containing supernatant was discarded and the cell pellet resuspended in fresh medium. This process was repeated to remove any extracellular drug before the addition of the second drug. An identical washing sequence was used at the end of the drug exposures.

A 1:10 dilution of the resuspended cells was

counted, and a final cell suspension with a concentration of 50 cells/ml was achieved with further dilution. 2.0 ml of this suspension was added to 3.0 ml of a 0.2% Difco Bacto-Agar - Fischer's medium with 15% horse serum mixture in 10 ml culture tubes and gently rocked to mix the contents without introducing air bubbles. The tube was then set upright in ice for 3 minutes to hasten setting of the soft agar, then incubated at 37°C in a 5% CO_2 atmosphere.

The consistency of the soft agar allowed continued cell growth and division without cell settling or random motion. Within 10 days, cells that had remained viable and retained the ability to divide after the drug exposure(s) had produced single cell colonies (clones). The effect of drug treatment can therefore be expressed as the percentage of clones formed relative to untreated cells, which had a cloning efficiency of 70%. All experiments were done in quadruplicate, and values represent mean values.

D. Bilayer Cloning Assay

The method used for determining the effect of cytotoxic drug exposures on the viability of HL-60 cells was a modification of the bilayer cloning assay described by Hamburger et al.(48). The bilayer consists of a basal

nutrient layer onto which the layer containing the cells being studied is applied. The consistency of the cell layer does not allow cell settling or random cell motion, but does permit continued growth. Cells that remain viable after drug exposure, as defined by the capability to continue to divide, form individual cell colonies (clones) that are visible at 40 X on a phase-contrast microscope within 15 days.

The nutrient layer consists of a 2:1:2 mixture of 2X McCoys:DFBS:1.2% Difco Bact-Agar, the last of which had been melted and allowed to cool to approximately 40°C before adding it to the other ingredients. Under sterile conditions 1.0 ml of this mixture was dispensed into the base of four of six 35mm diameter, 14mm deep wells, being careful to avoid introducing bubbles that would hinder counting. The other two wells were filled with 2 ml sterile water to prevent the agar from drying out. The bottom layer was allowed to harden at least 2 hours at 37°C.

Log phase HL-60 cells at 1.5 - 2 X 10^5cells/ml were counted, then diluted to 50,000 cells/ml.with fresh medium. 2.0 ml of this cell suspension were exposed to DAU, PF, or Ara-C as per the experimental design. For sequential drug exposures, at the end of the first drug exposure the cell suspension was centrifuged at 1200 rpm for 10 minutes, the supernatant poured off, and the cells

resuspended in 2.0 ml fresh medium before the second drug was added.

At the end of the drug exposures the cell suspension was centrifuged at 1200 rpm for 10 minutes, the supernatant poured off, the cell pellet washed in fresh medium and recentrifuged as before to remove extracellular drug. The cell pellet was resuspended in 2.5 ml 1X McCoys, of which 1.0 ml was used to establish an accurate cell count. A final concentration of 2500 cells/ml(5000 for those drug treatments presumed highly toxic) was achieved by adding to 3.0 ml 1X McCoys an ammount of the cell suspension determined by the formula

$$\text{Ammount(in ml)} = \begin{cases} 7500/(X-2500) & \text{for 2500 cells/ml} \\ 15000/(X-5000) & \text{for 5000 cells/ml} \end{cases}$$

where X equals the cell count per ml of the initial solution.

The diluted cell suspension was mixed well, then 0.5 ml was added to a mixture of 0.75 ml 2X McCoys and 0.5 ml DFBS. To this mixture was added 0.75 ml 1.0% Bacto-Agar that had been melted then allowed to cool to approximately 40°C. Before this mixture began to gel, 1.0 ml was dispensed into each of two wells onto the now-solid basal nutrient layer. Thus 500(or 1000) cells were seeded per well, and incubated at 37 C with 5% CO_2.

Clones were counted at 15 days using a phase-contrast microscope to scan the entire well at 40 X.

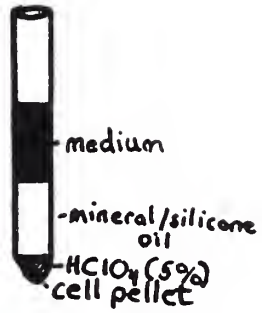
Growing clones were easily distinguished from debris or small cell clumps. The effect of the drug treatments can be expressed as the percentage of clones formed after drug treatment versus clones formed by untreated cells. The cloning efficiency of control cells was 67%.

E. Ara-C Uptake

The procedure used to quantitate Ara-C uptake was essentially as described by Grant et al.(46). 50 ml of logarithmically growing cells, between 1.5 and 2 X 10^5cells/ml, were exposed to DAU and/or PF as per the experimental design, then centrifuged at 1200 rpm for 10 minutes. The drug containing medium was removed and the cell pellet was resuspended in 2.1 ml fresh medium. After 0.1 ml was removed for a determination of the cell concentration, the 2.0 ml solution (at 4 - 7 X 10^6cells/ml) was placed in a 10 ml stoppered flask and allowed to equilibrate in a 37°C shaker water bath. ^{3}H-Ara-C was added (0.06 ml of a 10^{-4}M solution) to achieve a final concentration of 3μM with a specific activity of 1718 mCi/mmol.

At 5, 10, 15, 20, 30, and 60 minutes 0.1 ml aliquots of the agitated cell suspension were added to 0.5 ml plastic microfuge tubes which had previously been layered with 0.04 ml 5% perchloric acid and 0.1 ml of an 84:16 mineral oil:silicone oil mixture. The aliquots were

immediately centrifuged at 10,000 rpm for 15 seconds. The oil layer permitted only the cells, not the medium, to pass to the perchlorate-containing tip (see diagram at right), where the cells lysed. The tubes were frozen in a 70% ethanol/dry ice bath and cut in the oil layer, which, according to Grant et al.(46), retains no radioactive material, to separate intracellular from extracellular label. Each fraction was immersed in 4 ml Liquiscint in a 7 ml counting vial. After vigorous agitation to dislodge the cell pellet and distribute the radiolabel, each fraction was counted for three minutes by a Packard Model 3255 Tri-Carb Liquid Scintillation Spectrometer.



Two samples were taken for each time point and the Ara-C content of the cell fractions was determined using the following equation:

$$\frac{^3\text{H-Ara-C}}{\text{ml}} \times \frac{\text{ml}}{\text{cells}(\times 10^6)} \times \frac{\text{CPM(Cell Fx)}}{\text{CPM(Cell Fx)} + \text{CPM(Medium)}} = \frac{\text{Ara-C uptake}}{10^6\text{cells}}$$

Values from repeated experiments were averaged and used to generate lines whose slopes can be expressed as ^{3}H-Ara-C uptake/10^6cells perminute.

F. Ribonucleotide Triphosphate and Ara-CTP Analysis

Ribonucleotide triphosphate levels were deter-

mined using a modification of procedures described by Harkrader et al.(50) and Lui et al.(71). After drug exposures, cell concentration was measured, then approximately 10^7 cells were harvested by centrifugation at 1500 rpm for 3 minutes. The supernatant was meticulously removed to avoid contamination of the cell extracts by substances in the medium; this included wiping down the walls of the centrifuge tube with a paper towel. The cell pellet was extracted in 0.4 ml of ice-cold 0.7 N perchloric acid, vigorously agitated, then centrifuged at 1500 rpm to remove precipitated protein and nucleic acids. To the supernatant was added potassium bicarbonate until the pH was >7, followed by centrifugation to remove precipitated potassium perchlorate. 0.3 ml of this supernatant was added to 0.06 ml 1 N HCl, bringing the pH approximately to 5.

Aliquots of 0.25 ml were analyzed using an Altex Model 334 High Pressure Liquid Chromatograph. A 4.0 mm X 25 cm column of Partisil-10 SAX(Whatman, Clifton, NJ) was eluted with a starting buffer of 5mM ammonium phosphate, pH 2.8. At 4 minutes 0.5M ammonium phosphate, pH 4.85, was added to the starting buffer. Figure 3 illustrates the relation between column time and percent high pH buffer. Total flow rate was 2.0 ml/min. and column pressure was approximately 1500 psi. Peaks were detected by ultraviolet absorbance at 280 nm, the longer wavelength

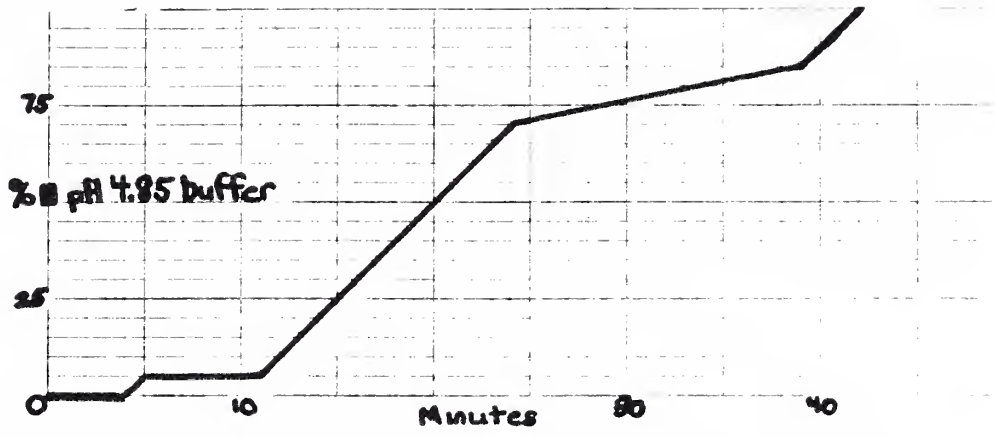
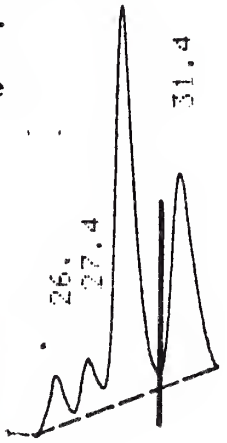


Figure 3: Percent High pH Buffer Vs. Column Time

substituted for the standard 254 nm to enhance CTP detection, and displayed as a continuous function of absorbance vs. time (see Figure 14 for an example).

Peaks were translated into NTP levels using the cut-and-weigh procedure. To correct for baseline drift due to the buffer system's intrinsic increased absorbance as the percent of the more concentrated high pH buffer increased, the baseline was interpolated under the closely-spaced peaks in the triphosphate region. The dotted line in the diagram at right is an example of this. Overlap areas were divided by dropping a perpendicular from the nadir between the peaks(the solid line in the diagram).



The chromatograms were copied onto xerox paper of measured average density ($7.133 \times 10^{-2} mg/mm^2$) and carefully cut out and weighed. Location of the NTP peaks and the NTP concentrations were determined by comparison

with chromatograms produced by known ammounts of pure compounds. Division of the number generated by this method by the cell count determined to be in the 0.25 ml aliquot applied to the column yielded the result in picomoles/10^6 cells.

A pure sample of Ara-CTP yielded a single peak in the NTP region of the chromatogram (see also Figure 12). ^{3}H-Ara-CTP was quantitated by collecting the eluent for the NTP region (between 24 and 34 minutes column time) as 1 minute fractions. These fractions were combined with 3 ml Liquiscint in a 7 ml counting vial and the decay of the tritium label was averaged over three minutes by the Packard Liquid Scintillation Spectrometer. Investigation revealed, however, that the gel formed by the combination of the aqueous buffer solution with the Liquiscint resulted in a 44% counting efficiency relative to the same ammount of radioactive material in the same volume of pure Liquiscint. Consequentially, values obtained by this method were multiplied by 2.29 to relate them to values obtained in the drug uptake studies.

G. Statistical Analysis

Synergy of cytotoxic drug combinations was determined simply by dividing the observed clone survival by the expected clone survival, where the expected survival

is the product of observed survival rates for the individual drugs. For triple drug exposures a second 'expected' value was obtained by multiplying the observed survival rate for the first drug alone by the observed survival rate for the combination of the other two, when that combination had been tested. If Obs/Exp < 1.0, the drug combination was considered to have synergistic cytotoxic effects. When Obs/Exp > 1.0, the drug combination was considered antagonistic. When Obs/Exp $\cong 1.0$, the effects of the drugs were considered additive.

For Ara-C uptake studies, lines of best fit were determined using the method of least squares. The correlation coefficient r was calculated as

$$r = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\Sigma(x-\bar{x})\Sigma(y-\bar{y})} \qquad R^2 = (r)^2$$

and the significance of r was determined using the Student-t test with

$$t_{n-2} = r(n-2)^{\frac{1}{2}}/(1-r^2)^{\frac{1}{2}}$$

For the experiments summarized in Figure 10 and Table 4 $p<0.001$ in all cases. R^2 values were calculated for the lines obtained from mean uptake values and one-tailed t tests were performed to determine significant differences in ^{3}H-Ara-C uptake.

III. RESULTS

A. Cloning Experiments

1. L1210

The results of the cloning experiments with L1210 are presented in Table 2. By themselves, DAU and Ara-C exhibited substantial toxicity towards L1210 cells in culture. The toxic effect of PF at the concentrations/duration studied was limited. Greater than additive effect on clone survival was noted for both DAU and PF when combined with Ara-C. In both cases the synergy was greater when Ara-C was preceded by the CTP-depleting drug than when Ara-C exposure was first, with a more noted difference for DAU than PF. Triple drug exposures resulted in remarkable synergy when DAU and PF, in either order, preceded Ara-C. Ara-C followed by PF and DAU exhibited synergy of the same order as the double drug exposures.

2. HL-60

The results of the cloning experiments with HL-60 are presented in Table 3. By itself, DAU exposure, up to 10μM for 3 hours, had no effect on the ability of HL-60 cells to produce a clone. PF exhibited modest toxicity, and Ara-C exhibited a linear log-dose X response relationship between concentrations of 0.1 to 20μM (R^2=0.96, slope = -23.35% clone survival/$\log_{10}$concentration). Pretreatment with 3μM DAU did not enhance Ara-C toxicity, but 5μM DAU

Table 2 : Results of L1210 Cloning Experiments

Treatment*	% Clone Survival (Control=100.0) Observed	Expected (Additive)@	Obs/Exp
PF 2μM	100.0	----	----
PF 5μM(1 hr)	88.9	----	----
PF 5μM	79.4	----	----
DAU 1μM	83.0	----	----
DAU 2μM	70.5	----	----
DAU 5μM	46.7	----	----
Ara-C 1μM	96.1	----	----
Ara-C 2μM	62.1	----	----
Ara-C 5μM	46.0	----	----
Ara-C 7.5μM	33.4	----	----
Ara-C 10μM	22.5	----	----
PF 2μM, Ara-C 2μM	25.4	62.1	.409
PF 2μM, Ara-C 5μM	22.2	46.0	.483
PF 5μM, Ara-C 2μM	26.2	49.3	.531
PF 5μM, Ara-C 5μM	16.7	36.5	.458
Ara-C 2μM, PF 2μM	34.1	62.1	.549
Ara-C 2μM, PF 5μM	29.4	49.3	.596
DAU 1μM, Ara-C 1μM	31.0	79.8	.388
DAU 1μM, Ara-C 3μM	16.3		
DAU 1μM, Ara-C 5μM	10.9	38.2	.285
DAU 2μM, Ara-C 1μM	23.3	67.8	.344
DAU 2μM, Ara-C 3μM	12.4		
DAU 2μM, Ara-C 5μM	11.6	32.4	.358
Ara-C 1μM, DAU 5μM	31.0	44.9	.690
Ara-C 5μM, DAU 2μM	23.3	32.4	.719
Ara-C 5μM, DAU 5μM	16.3	21.5	.758
PF 2, DAU 2, Ara-C 2	0.4	43.8	.009
PF 2, DAU 2, Ara-C 5	0.8	32.4(11.6)	.025(.069)
PF 5, DAU 2, Ara-C 2	2.0	34.8	.057
PF 5, DAU 2, Ara-C 5	2.0	25.7(9.2)	.078(.217)
DAU 2, PF 5, Ara-C 2	1.2	34.8(18.5)	.034(.065)
Ara-C 2, PF 5, DAU 2	13.5	34.8	.385
Ara-C 5, PF 5, DAU 2	9.9	25.7	.385

*-PF,DAU treatments lasted 3 hrs, Ara-C treatments 1 hour.

@-Determined by multiplying single drug values. Values in parentheses were determined by multiplying the value for the first drug alone by the value obtained for the combination of the second and the third drugs.

Table 3: Results of HL-60 Cloning Experiments

Treatment	% Clone Survival(Control=100.0) Observed	Expected (Additive)	Obs/Exp
DAU 3μM	100.0	----	----
DAU 5μM	100.0	----	----
DAU 10μM	100.0	----	----
PF 3μM	100.0	----	----
PF 5μM	92.5	----	----
PF 10μM	83.1	----	----
Ara-C 0.1μM	100.0	----	----
Ara-C 0.3μM	92.0	----	----
Ara-C 1μM	85.0	----	----
Ara-C 3μM	73.8	----	----
Ara-C 5μM	66.4	----	----
Ara-C 10μM	52.5	----	----
Ara-C 20μM	47.5	----	----
DAU 3μM, Ara-C 1μM	89.7	85.0	1.06
DAU 3μM, Ara-C 3μM	84.1	73.8	1.14
DAU 3μM, Ara-C 5μM	77.3	66.4	1.16
DAU 5μM, Ara-C 3μM	56.4	73.8	0.76
DAU 5μM, Ara-C 5μM	29.3	66.4	0.44
Ara-C 3μM, DAU 5μM	61.4	73.8	0.83
PF 3μM, Ara-C 1μM	100.0	85.0	1.18
PF 3μM, Ara-C 3μM	73.6	73.8	1.00
PF 3μM, Ara-C 5μM	70.2	66.4	1.06
PF 5μM, Ara-C 3μM	59.5	68.3	0.87
PF 5μM, Ara-C 5μM	54.2	61.4	0.88
Ara-C 3μM, PF 5μM	43.2	68.3	0.63
DAU 5μM, PF 5μM	91.7	92.5	0.99
PF 5μM, DAU 5μM	81.2	92.5	0.88
D 2μM, P 2μM, A 1μM	68.2	85.0	0.80
D 2μM, P 2μM, A 3μM	40.1	73.8	0.54
D 2μM, P 5μM, A 1μM	63.2	78.6	0.80
D 2μM, P 5μM, A 3μM	56.9	68.3(59.5)	0.83(0.96)
D 5μM, P 2μM, A 1μM	45.4	85.0	0.53
D 5μM, P 2μM, A 3μM	37.4	73.8	0.51
D 5μM, P 5μM, A 1μM	62.9	78.6	0.80
D 5μM, P 5μM, A 3μM	34.1	68.3(59.5)	0.50(0.57)

Table 3: Results of HL-60 Cloning Experiments

Treatment	% Clone Survival(Control=100.0) Observed	Expected (Additive)	Obs/Exp
P 2μM, D 5μM, A 1μM	21.1	85.0	0.25
P 2μM, D 5μM, A 3μM	19.6	73.8(56.4)	0.27(0.35)
P 5μM, D 5μM, A 3μM	18.4	68.3(52.2)	0.27(0.35)
P 5μM + D 5μM*, A 3μM	40.0	66.4	0.60
*simultaneous exposure to DAU and PF			
A 3μM, P 5μM, D 5μM	41.5	68.3(59.9)	0.61(0.69)
A 3μM, D 5μM, P 5μM	48.4	68.3(67.7)	0.71(0.71)
D 5μM, A 3μM, P 5μM	48.3	68.3(43.2)	0.71(1.12)
P 5μM, A 3μM, D 5μM	17.0	68.3(56.8)	0.25(0.30)

PF and DAU treatments lasted 3 hours, Ara-C treatments 1 hr.

was associated with observed/expected clone survival less than 1. Experiments with the sequence reversed had similar results. PF pretreatment (2 or 5μM for 3 hours) of Ara-C exposed cells resulted in additive effects on clone survival, with the reverse sequence suprisingly more effective at reducing clone survival. PF did not appreciably enhance DAU toxicity, though PF preceding DAU was marginally more toxic than PF following DAU.

DAU followed by PF prior to Ara-C exposure resulted in greater than additive cytotoxicity, cutting survival in half, vs. expected values, for 3μM Ara-C. The most toxic regimen was PF followed by DAU then Ara-C, with which clone survival was cut to 20% of controls, about one-fourth of expected survival. A similar result was noted when PF and

Ara-C were followed by DAU, but not when DAU and Ara-C precede PF. Treating with Ara-C first, followed with DAU and PF in either order, was less effective than when Ara-C was the final drug to which the cells were exposed. PF and DAU simultaneous exposure was as effective as DAU followed by PF in sensitizing the cells to Ara-C, but not as effective as the PF followed by DAU sequence.

B. Ara-C Uptake

Presenting the results of the Ara-C uptake studies requires two brief explanations. First, as discussed above, the transport of nucleosides, including Ara-C, into cells is a very rapid, first-order process (6,86), but the uptake of a nucleoside is dependent upon its rate of phosphorylation (86) and, in the case of Ara-C, its conversion to other forms (Ara-U, Ara-UMP) which establish their own concentration gradients across the cell membrane. In these experiments, beyond approximately 20 minutes exposure to Ara-C, the rate of uptake declined, and total uptake began to level off. For statistical purposes, results were plotted as total uptake vs. time and lines, whose slopes are expressed in uptake per minute were drawn using values from the first four time-points (5, 10, 15, and 20 minutes) only. Figures 6 and 7 contrast this method of extrapolation with actual uptake values, and the 60 minute values are identified on all figures. Justification for treating the data in this fashion comes from the observation that in the intact organism Ara-C is rapidly cleared and metabolized (52,59), thus the initial rate of uptake becomes the most vital determinant of total uptake.

Second, statistical comparisons between drug regimes were performed on rates of Ara-C uptake instead of

absolute values of total uptake. Though in most cases significant differences in rate were accompanied by significant differences in total uptake (by 15 minutes exposure), rates of uptake varied less than total uptake in repeated experiments - compare Figure 10 with Table 4.

1. L1210

In a single experiment performed with L1210 cells, pretreatment with DAU enhanced intracellular Ara-C accumulation (Figure 4), increasing the rate of

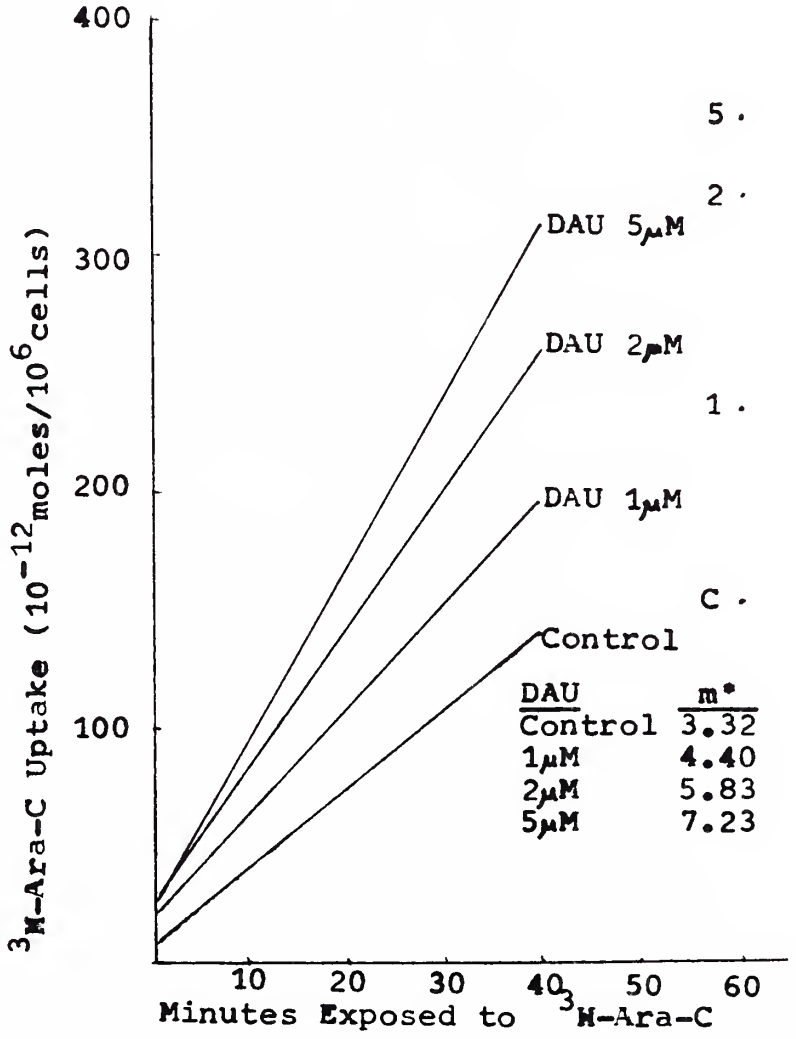


Figure 4:

Effect of Concentration of DAU Pretreatment on ^{3}H-Ara-C (5μM) Uptake in L1210 Cells

—— extrapolated from 5 and 20 minute timepoints

---- actual uptake values

*m=rate of ^{3}H-Ara-C Uptake as $\frac{10^{-12}\text{moles}/10^6\text{cells}}{\text{minute}}$

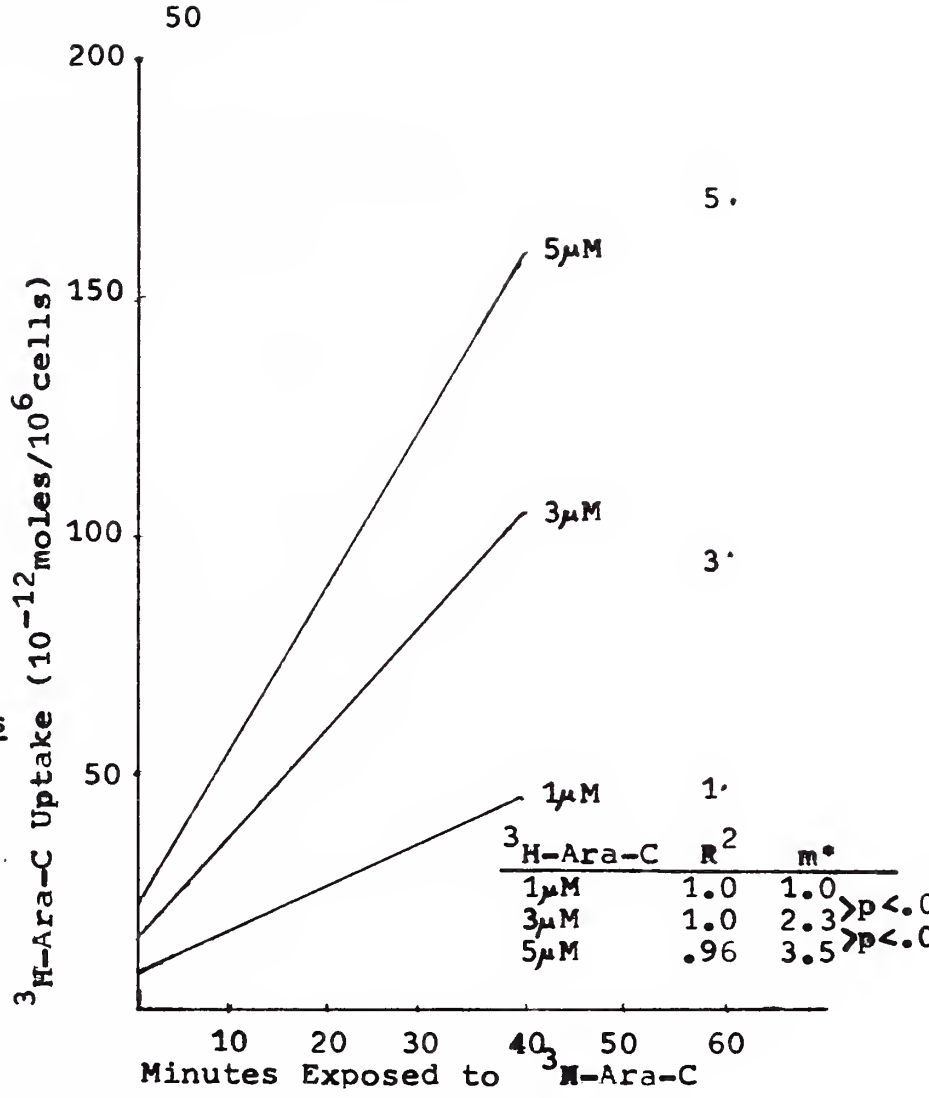


Figure 5 :

Effect of ^{3}H-Ara-C concentration on ^{3}H-Ara-C Uptake

*m=rate of ^{3}H-Ara-C Uptake as $\frac{10^{-12}\text{moles}/10^6\text{cells}}{\text{minute}}$

——extrapolated from 5 and 20 min. timepoints
----actual uptake values

uptake from 3.32 X 10^{-12}moles/10^6cells minute^{-1} for untreated cells to 4.40, 5.83, and 7.23 X 10^{-12}moles/10^6cells minute^{-1} for cells pretreated for 3 hours with 1μM, 2μM, and 5μM, respectively.

2. HL-60

Figure 5 demonstrates that increasing the initial concentration of ^{3}H-Ara-C from 1μM to 3μM to 5μM can significantly ($p<.05$) increase the rate of ^{3}H-Ara-C uptake by HL-60 cells. Pretreating cells for 3 hours with con-

centrations of PF ranging from 1 to 10 M had no significant effect on ^{3}H-Ara-C uptake (Figure 6) as previously noted by Cadman et al.(11) and others (86).

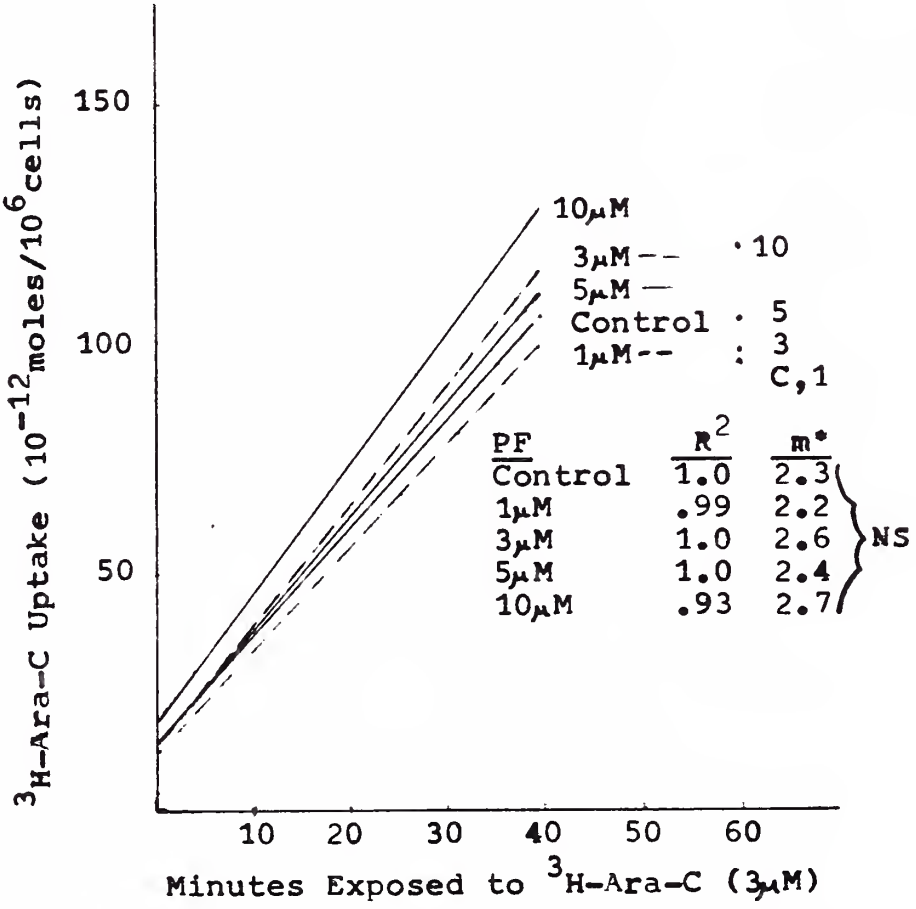


Figure 6: Effect of Concentration of PF Pretreatment on ^{3}H-Ara-C Uptake
*m=rate of ^{3}H-Ara-C uptake as $\frac{10^{-12}\text{moles}/10^{6}\text{cells}}{\text{minute}}$

In contrast, DAU pretreatment markedly increased uptake of the labelled drug. As illustrated in Figure 7, the baseline uptake of ^{3}H-Ara-C, expressed as picomoles per 10^{6}cells per minute, of 2.3 exhibited a modest increase in response to 3-hour pretreatment with 1μM(rate=2.5) or 3μM(2.6) DAU. Pretreatment with 5μM DAU resulted in significantly higher Ara-C uptake(rate=3.4, $p<0.01$) than control.

Pretreatment with 10μM DAU was no more effective at increasing ^{3}H-Ara-C uptake than 5μM.

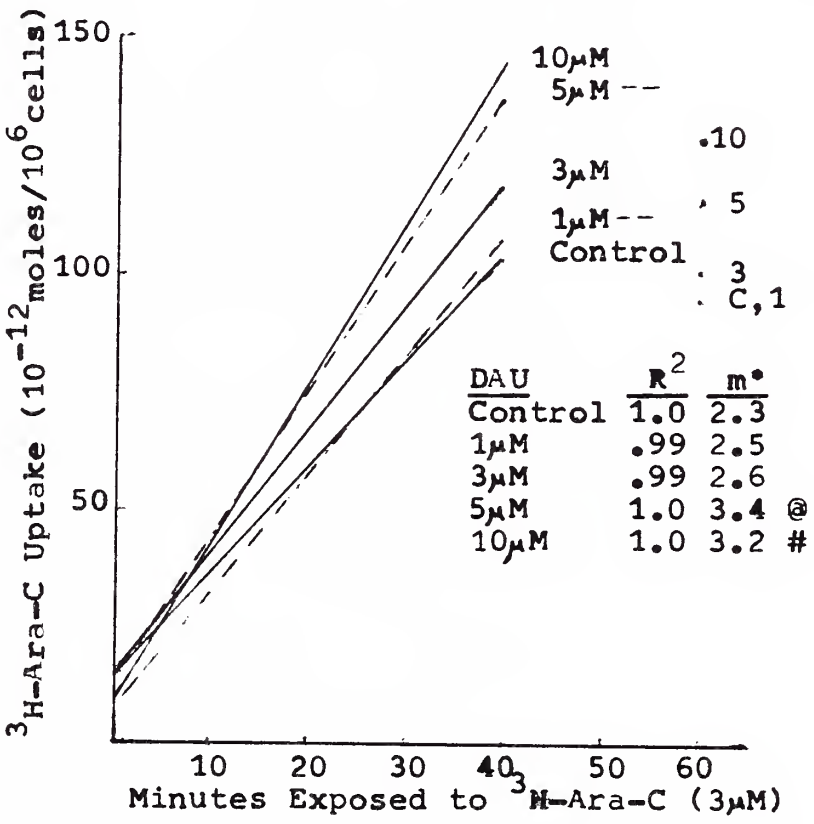


Figure 7: Effect of Concentration of DAU Pretreatment on ^{3}H-Ara-C Uptake
*m=rate of ^{3}H-Ara-C Uptake as $\frac{10^{-12}\text{moles}/10^{6}\text{cells}}{\text{minute}}$
@ p<0.01
# p<0.05

Figures 8 and 9 illustrate the effect of following DAU pretreatment with PF before exposing the cells to ^{3}H-Ara-C(3μM). In Figure 8, increasing concentrations of PF result in increases in the rate of ^{3}H-Ara-C uptake, from a control (DAU(5μM-3 hrs) alone) value of 3.4 to 4.4 for 5μM PF(p 0.01) and 4.6 for 10μM PF(p=0.05). The effect

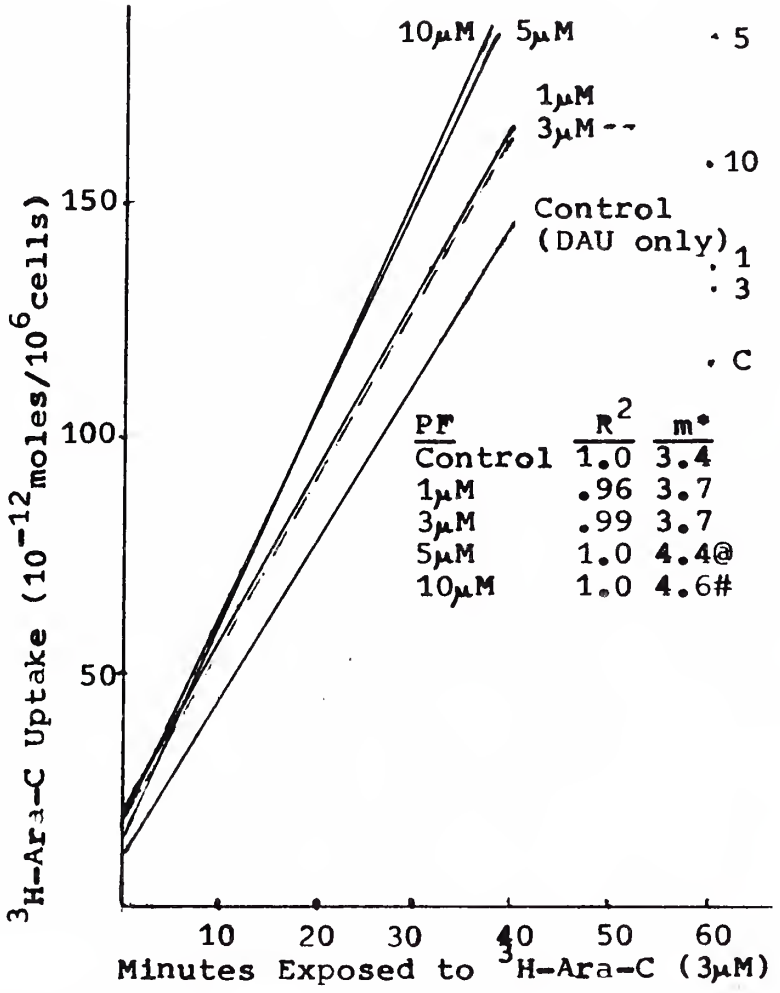


Figure 8: Effect of Concentration of PF Pretreatment Following DAU (5μM-3 hrs) on Ara-C Uptake
*m=rate of Ara-C Uptake as(10^{-12}moles/10^6cells)/minute
@p<0.01
#p=0.05

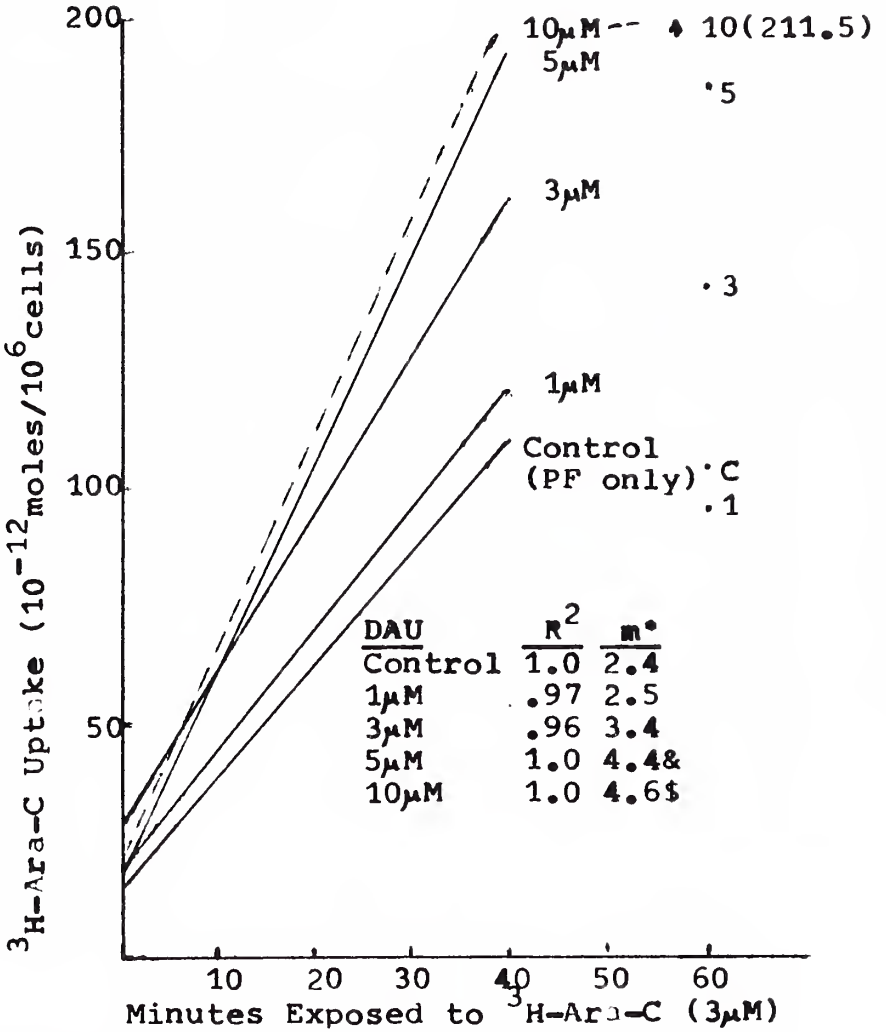


Figure 9: Effect of Concentration of DAU Pretreatment Followed by PF (5μM-3 hrs) on Ara-C Uptake
&p<0.001
$p<0.025

of DAU concentration on the marked enhancement of ^{3}H-Ara-C uptake exhibited by DAU and PF sequenced pretreatment is shown in Figure 9. Slight increases in rate for 1μM and 3μM DAU (2.5 and 3.4, respectively, vs. a control (PF(5μM-3 hrs) alone) value of 2.4) became significant increases in the rate of ^{3}H-Ara-C accumulation at DAU concentrations of 5μM (rate=4.4, $p<0.001$) and 10μM (rate=4.6, $p<0.025$).

Reversing the order of pretreatment markedly alters the effect of PF and DAU pretreatment on ^{3}H-Ara-C uptake, as presented in Figure 10. Intracellular accumulation is significantly higher when DAU precedes PF than when PF precedes DAU ($p<0.01$), with the latter resulting in a rate of uptake similar to that of DAU pretreatment alone.

The results of the uptake experiments are summarized in Figure 10 and Table 4.

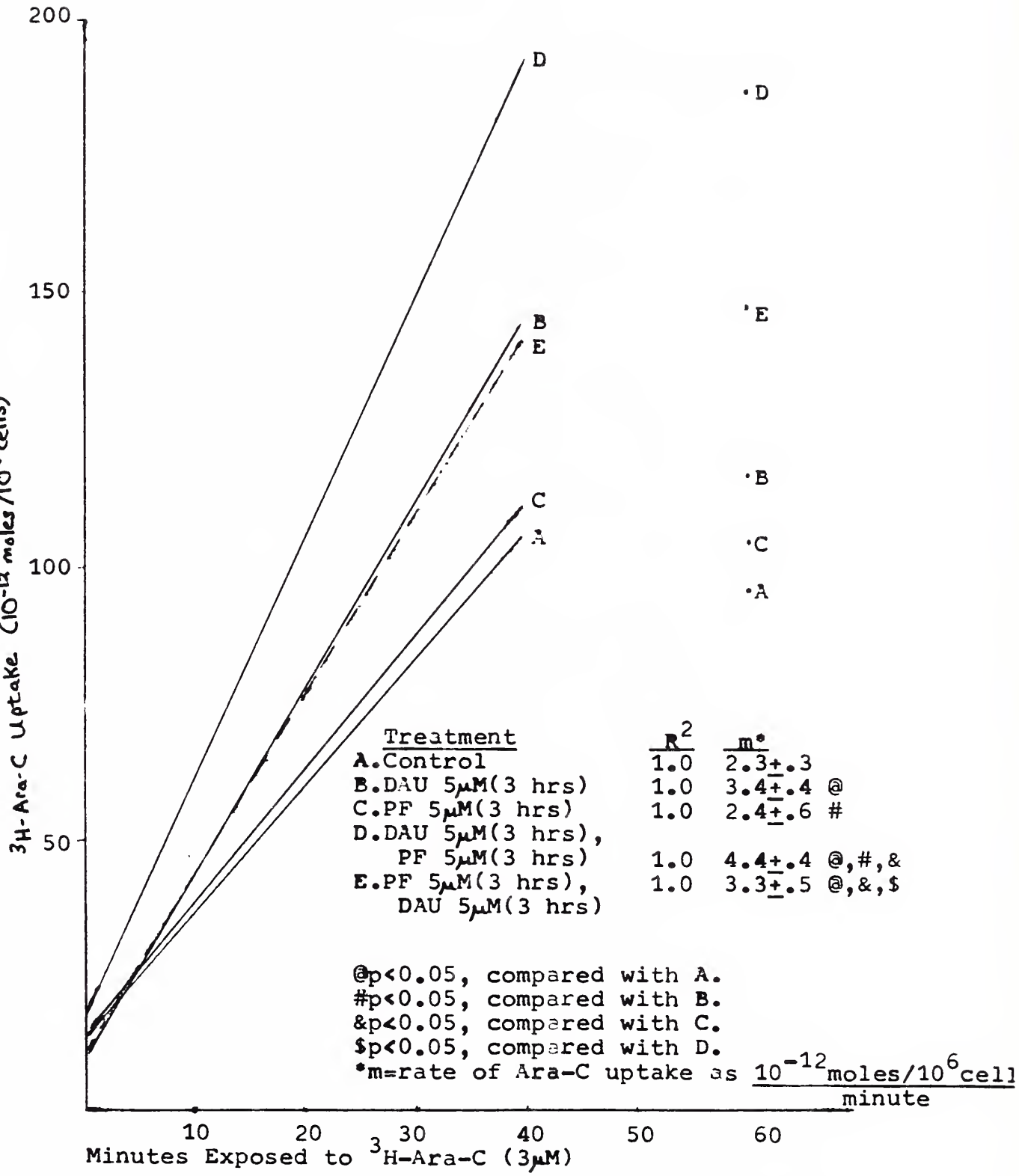
Table 4: ^{3}H-Ara-C Uptake

Pretreatment	5 min.*	10 min.	15 min.	20 min.	60 min.
A. Control(Ara-C 3μM)	24.7 ±8.8	37.3 ±6.0	47.0 ±9.1	59.7 ±7.8	95.3 ±7.4
B. DAU 5μM (3 hrs)	25.8 ±3.3	43.6 ±3.9	60.3 ±5.8^{A}	76.7 ±7.8^{A}	116.1 ±20.9
C. PF 5μM (3 hrs)	25.3 ±8.5	39.1 ±9.5	51.3 ±11.6	61.6 ±14.7	104.3 ±29.5
D. DAU 5μM (3 hrs), PF 5μM (3 hrs)	39.7 ±5.7$^{@}$	60.4 ±8.4$^{@}$	82.8 ±10.2$^{@}$	105.3 ±9.3$^{@}$	185.7 ±48.8$^{@}$
E. PF 5μM (3 hrs), DAU 5μM (3 hrs)	26.9 ±3.3	42.9 ±3.8	60.8 ±6.3^{A}	75.7 ±9.5^{A}	146.6 ±41.7

*-in moles X $10^{-12}/10^{6}$cells, ± 1 Standard Deviation

$^{@}$-$p<.05$, compared to other treatments

^{A}-$p<.05$, compared to Control



Treatment	R^2	m*
A.Control	1.0	2.3±.3
B.DAU 5μM(3 hrs)	1.0	3.4±.4 @
C.PF 5μM(3 hrs)	1.0	2.4±.6 #
D.DAU 5μM(3 hrs), PF 5μM(3 hrs)	1.0	4.4±.4 @,#,&
E.PF 5μM(3 hrs), DAU 5μM(3 hrs)	1.0	3.3±.5 @,&,$

@p<0.05, compared with A.
#p<0.05, compared with B.
&p<0.05, compared with C.
$p<0.05, compared with D.
*m=rate of Ara-C uptake as $\frac{10^{-12}\text{moles}/10^6\text{cell}}{\text{minute}}$

Figure 10: Summary of Uptake Experiments with HL-60

C. Ribonucleotide Triphosphate Levels in HL-60 cells

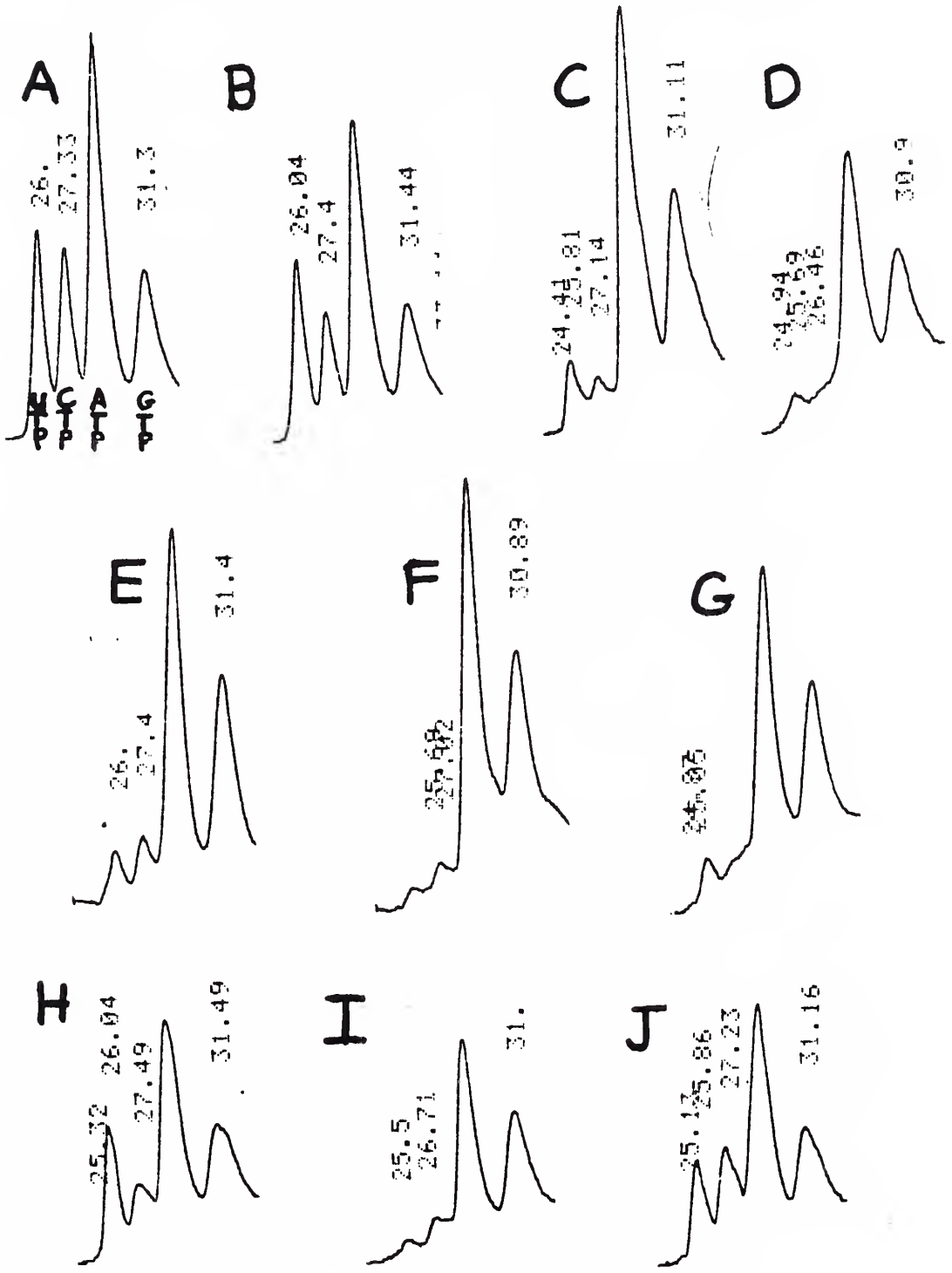
Table 5 and Figure 11 illustrate the changes in acid-soluble ribonucleotide TP pools in HL-60 cells subjected to DAU(5μM-3 hrs), PF(5μM-3 hrs), and/or ^{3}H-Ara-C(3μM-1 hr). Control values were as follows: ATP-3080.4pmol/10^{6}cells, GTP-875.8, UTP-1328.8, CTP-555.9. DAU treatment reduced CTP to 57.8% of control; UTP and ATP were reduced to 73.4 and 70.4% of control, respectively. PF following DAU further reduced CTP to 20.6% of control while reducing UTP to 28.7% of control in the face of ATP levels equal to control. PF alone reduced CTP to 28.6% and UTP to 20.7% of control, and subsequent DAU exposure further reduced CTP and UTP to 12.9 and 8.0 percent of control, respectively, with ATP levels about 80% of control in both cases. Ara-C alone reduced UTP and CTP levels to 60.9% and 78.9% of control, respectively, with modest reductions in ATP and GTP levels as well. This same effect was noted in cells pretreated with DAU then PF or PF alone. Cells treated with DAU alone had little change in NTP levels in response to Ara-C, and cells treated with PF then DAU responded with a modest increase in UTP and CTP (from 8.0 to 15.0 and 12.9 to 16.1, respectively), with slight reductions in ATP and GTP. (Note: GTP measurements may be less accurate than those of the other NTPs because of

Table 5: Ribonucleotide Triphosphate Levels

Sample	UTP*(%A)	CTP (%A)	ATP (%A)	GTP (%A)
A. Control	1328.8(100.0)	555.9(100.0)	3080.4(100.0)	875.8(100.0)
B. DAU 5μM (3 hrs)	975.0 (73.4)	321.1 (57.8)	2168.1 (70.4)	545.6 (62.3)
C. DAU 5μM (3 hrs), PF 5μM (3 hrs)	381.6 (28.7)	114.7 (20.6)	3096.9(100.5)	1138.7(130.0)
D. DAU 5μM (3 hrs), PF 5μM (3 hrs), Ara-C 3μM (1 hr)	196.0 (14.8)	68.2 (12.3)	2279.6 (74.0)	810.8 (92.6)
E. PF 5μM (3 hrs)	274.9 (20.7)	159.2 (28.6)	2484.5 (80.7)	1050.7(120.0)
F. PF 5μM (3 hrs), DAU 5μM (3 hrs)	106.8 (8.0)	71.9 (12.9)	2650.8 (86.1)	968.0(110.5)
G. PF 5μM (3 hrs), DAU 5μM (3 hrs), Ara-C 3μM (1 hr)	199.4 (15.0)	89.3 (16.1)	2011.7 (65.3)	802.5 (91.6)
H. DAU 5μM (3 hrs), Ara-C 3μM (1 hr)	967.8 (72.8)	285.7 (51.4)	2399.5 (77.9)	821.2 (93.8)
I. PF 5μM (3 hrs), Ara-C 3μM (1 hr)	134.7 (10.1)	121.3 (21.8)	2016.9 (65.5)	841.8 (96.1)
J. Ara-C 3μM (1 hr)	808.6 (60.9)	438.6 (78.9)	2860.0 (92.8)	750.2 (85.7)

*- picomoles/10^6 cells

Figure 11: Nucleotide Triphosphate Peaks at 280 nm
(see Table 5 for identification)



the difficulty of determining the end of the GTP peak)

Table 6: Ribonucleotide Triphosphate Ratios
(see Table 5 for identification)

Sample	CTP/UTP[@]	CTP/ATP	UTP/ATP
A.	.42(1.0)	.33(1.0)	.43(1.0)
B.	.33(.79)	.15(.82)	.45(1.0)
C.	.30(.72)	.04(.21)	.12(.29)
D.	.35(.83)	.03(.17)	.09(.20)
E.	.58(1.4)	.06(.36)	.11(.26)
F.	.67(1.6)	.03(.15)	.04(.09)
G.	.45(1.1)	.04(.24)	.10(.23)
H.	.30(.71)	.12(.66)	.40(.94)
I.	.90(2.2)	.06(.33)	.07(.16)
J.	.54(1.2)	.15(.85)	.28(.66)

[@]- Using values from Table 5. Figures in parentheses express ratios in terms of ratios of control cells.

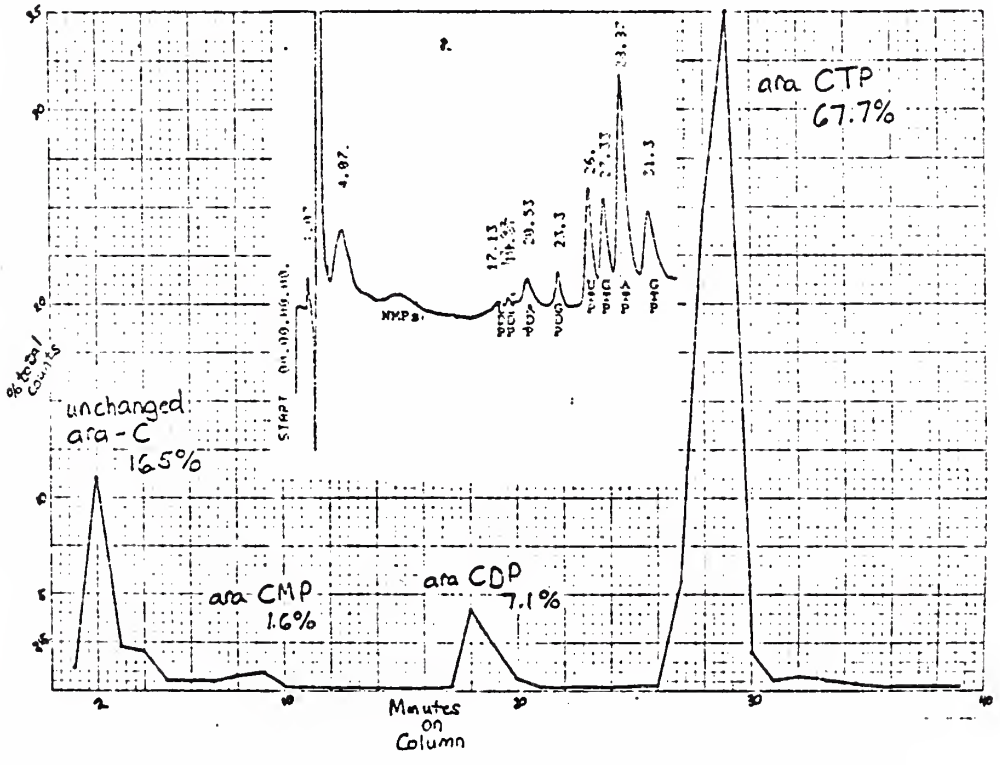
Relating the concentrations of CTP and UTP to each other and to ATP may shed some light on the effects of the drugs on HL-60 cells. As presented in Table 6, the CTP/UTP ratio yields the most new information. As expected, treatment with DAU alone lowered the CTP/UTP ratio (from .42 to .33, or 79% of control). Exposing these cells to PF, Ara-C, or both agents caused relatively little change in this ratio (compare B. to C., H., and D.). PF alone, on the other hand, increased the CTP/UTP ratio to .58 (140% of the control value), and subsequent DAU exposure did not reverse this effect. Treatment with Ara-C, noted above

to decrease UTP to a greater degree than CTP, enhanced this effect. Ara-C alone led to a CTP/UTP ratio of .54; in combination with PF the ratio rose to .90(220% of control value). The possible effects of these changes in CTP/UTP ratios will be discussed below.

D. Ara-CTP levels

After 1 hour exposure to 3 M ^{3}H-Ara-C, 67.7% of the label recovered from the HPLC column eluted in the triphosphate region (Figure 12), between CTP and ATP peaks. The chromatogram of pure Ara-CTP identified this peak as

Figure 12: Chromatogram of Ara-C Derivatives with Standard Chromatogram(inset)



Ara-CTP. Smaller peaks in the nucleotide di- and monophosphate regions are likely Ara-CDP(7.1%) and Ara-CMP(1.6%), respectively. In this buffer system, unchanged Ara-C, present as 16.5% of total counts recovered from the column, elutes near the origin. It was discovered that perchloric acid precipitation through the application of the sample to the column resulted in a loss of almost 50% of the label compared to values from the uptake experiments, but all values for Ara-CTP below were calculated taking into account the decreased counting efficiency described in the Methods section, but without correction for the loss of label mentioned just above (since the Ara-CTP fraction of that loss is incalculable).

Table 7: Ara-CTP levels and Ratio to CTP Level

Treatment	Ara-CTP*	%Total Ara-C	Ara-CTP/CTP@
Control#	31.5	35.6	5.67
DAU 5μM(3 hrs)	45.2	33.0	14.02
PF 5μM(3 hrs)	18.9	23.6	11.87
DAU 5μM(3 hrs), PF 5μM(3 hrs)	33.6	18.0	29.29
PF 5μM(3 hrs), DAU 5μM(3 hrs)	30.7	18.8	42.70

*- in moles X $10^{-12}/10^{6}$cells

@- expressed as percent of CTP level

#- all ^{3}H-Ara-C exposures were 3μM-1 hr

Table 7 presents the data on Ara-CTP levels for control and pretreated HL-60 cells. Whereas pretreatment with DAU markedly enhanced the formation of Ara-CTP, PF pretreatment interfered with Ara-CTP formation, with the decline in actual level paralleled by a drop in percent total intracellular Ara-C recovered as the triphosphate (from 35.6% in control to 23.6% in PF pretreated cells). This effect was even more marked in cells pretreated with DAU and PF. In either order, total Ara-CTP was no greater than in control cells and reflected a cut in half of the percent total intracellular Ara-C recovered as Ara-CTP.

The final column of Table 7 relates Ara-CTP concentrations to CTP concentrations at the beginning of Ara-C exposure. The Ara-CTP/CTP ratio is doubled with PF pretreatment and two-and-a-half times control following DAU pretreatment, but the combination of the two drugs has a more marked effect. DAU followed by PF results in an Ara-CTP/CTP ratio five times control and PF followed by DAU results in a ratio seven-and-a-half times control. Figure 13 relates this ratio to HL-60 clone survival data for controls and pretreated cells and reveals a consistent (R^2=0.99) relationship between higher Ara-CTP/CTP ratios and enhanced cytotoxic effect.

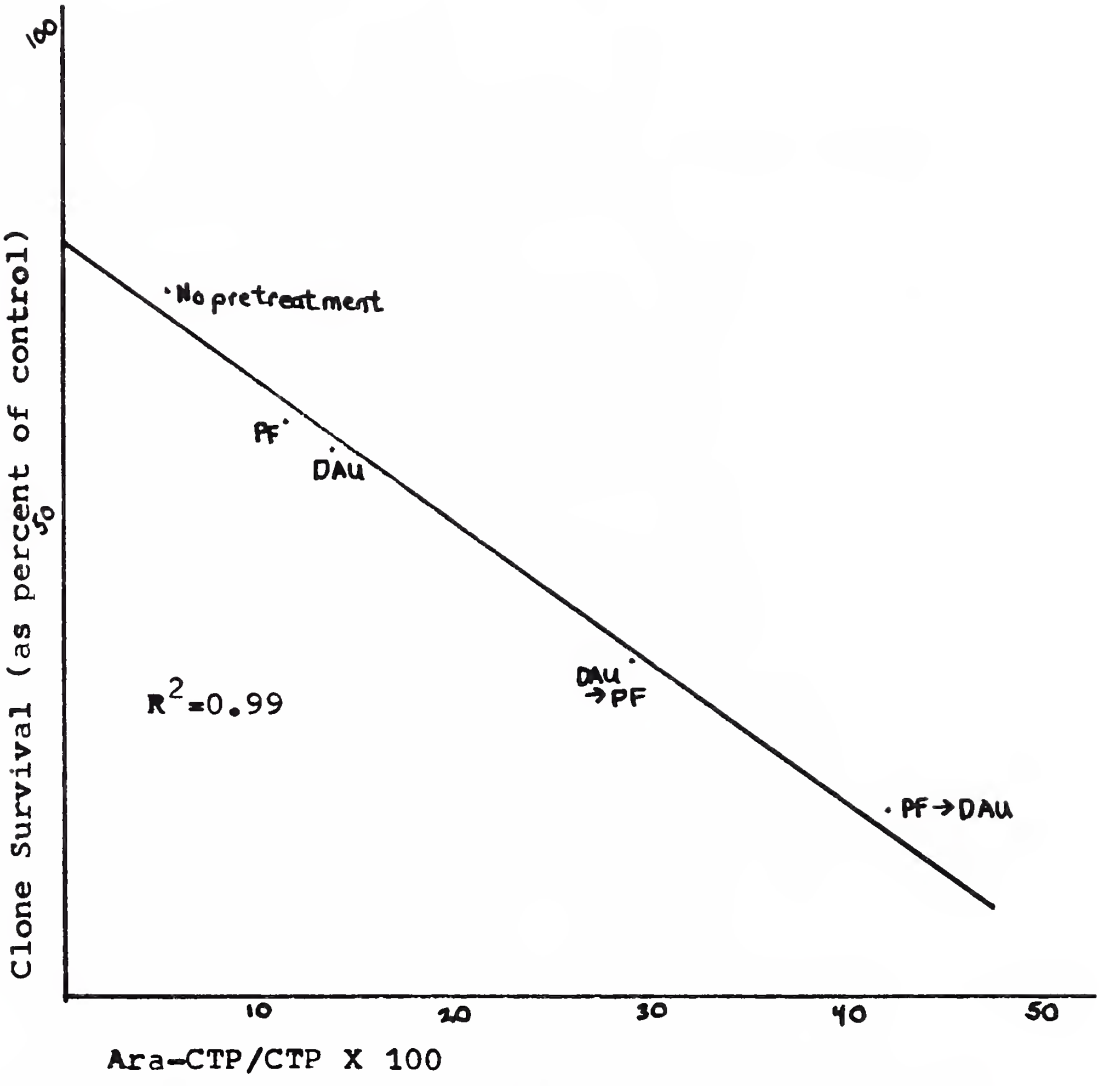


Figure 13: Clone Survival as a function of the Ara-CTP/CTP ratio

IV. DISCUSSION

These studies indicate that pretreatment of L1210 or HL-60 cells with PF then DAU sensitizes them to the cytotoxic effect of Ara-C, resulting in a synergistic decrease in the ability of the cells to form a clone. The mechanism of this synergy, in HL-60 cells, is apparently a marked increase in the Ara-CTP/CTP ratio compared to cells exposed to Ara-C without pretreatment. If, as suggested by earlier work, a reduction in CTP also depletes dCTP pools, these findings are consistent with a mechanism of Ara-CTP lethality in which Ara-CTP competes with dCTP. The reverse sequence, DAU then PF pretreatment, results in significantly higher uptake values, but because it is less effective at depleting CTP, the Ara-CTP/CTP ratio is lower, as is its ability to enhance Ara-C lethality.

At the outset, three remarks are in order. First, the experiment strongly suggests that measurement of Ara-C uptake is not sufficient for relating the effect of a pretreatment regimen to Ara-C toxicity. Because of different rates of phosphorylation and, even more important, the competitive nature of Ara-CTP toxicity, Ara-CTP levels and CTP or, better yet, dCTP levels must be measured. In addition, while much is made of dCTP inhibition of DCK_1, the enzyme responsible for initial phosphorylation of Ara-C, as indicated on Table 1 UTP and dTTP exert an activating influence

on this enzyme. PF alone or combined PF and DAU pretreatment in either sequence depletes cellular UTP and, likely, dTTP. This explains both the inhibition of total uptake and the decline of percent Ara-CTP of total intracellular Ara-C with those pretreatment regimes. A corollary to this finding is that an elevated CTP/UTP ratio, such as seen after PF or PF then DAU pretreatment, would inhibit DCK_1 to a greater degree than a depressed CTP/UTP ratio, such as seen after DAU then PF pretreatment.

Finally, when studying the effects of a cell-cycle specific agent like Ara-C, one must pay attention to the length of the cell cycle in the cells being studied. L1210 cells double approximately every 12 hours; HL-60 cells double approximately every 24 hours. Thus the likelihood of 'catching' a cell in S phase, either during the drug exposure or after the drug exposure at a time when Ara-CTP levels are still high enough to have a lethal effect is, disregarding different rates of Ara-CTP catabolism, roughly twice as great for L1210 than HL-60, a likely contribution to the higher order of cell kill for the mouse cells. The effect of cell cycle length on Ara-C toxicity is even more marked following pretreatment, especially if S phase or G_1/S boundary synchronization is considered a contributing factor to the enhancement of Ara-C toxicity, as has been postulated for DAU and PF. In these experiments, relatively

short drug exposures were used to simulate the *in vivo* administration of a drug bolus, after which all of the agents being studied are cleared relatively quickly, though drug effects may last for some time. Continuous infusion therapy would obviously allow for higher drug levels over a longer period, and thus synchronize a larger portion of the cells, but would likely also increase toxic side effects.

Ara-C uptake and Ara-CTP formation following Ara-C exposure without pretreatment in HL-60 cells, with Ara-CTP accounting for two-thirds of the intracellular Ara-C, are similar to those reported elsewhere (89). DAU pretreatment with 5μM for 3 hours modestly lowered CTP, relative to ATP, without affecting UTP. This, and perhaps inhibition of CD (35), enhanced Ara-C uptake and resulted in the highest levels of intracellular Ara-CTP. However, at this relatively low drug concentration for a much shorter drug exposure than used by earlier investigators (62,68,77), cytotoxic synergy was modest in HL-60 cells, though somewhat more impressive in L1210. The decline in CTP may not have been enough to significantly affect the dCTP pool, though the Ara-CTP/CTP ratio was two-and-a-half times control. If cell cycle arrest is a major determinant of cytotoxic enhancement for the DAU/Ara-C drug sequence, as suggested by Lauzon *et al.*(68), the shorter drug exposure would 'trap' fewer cells in the Ara-C-sensitive S phase. Finally, the self-limited nature of DAU toxicity discussed in the intro-

duction may also limit its ability to enhance Ara-C lethality

PF inhibits OTD much more effectively than DAU does CTS and, at 5μM for 3 hours, markedly depletes cellular UTP and CTP, with the former affected to a greater degree. This likely results in reduced dCTP as well, which, by itself, should enhance Ara-C uptake and phosphorylation. However, as noted by other investigators (11,86), Ara-C uptake is not increased, and in these experiments Ara-CTP was just 60% of control. The effect of UTP depletion on DCK_1 is a likely source of this discrepancy. The presence of marginally better than additive cytotoxicity in HL-60 cells treated with PF then Ara-C, and the synergy evident in L1210, is due to the doubling of the Ara-CTP/CTP ratio. This is likely the source for the marked enhancement of Ara-C incorporation into DNA following PF pretreatment reported by Plagemann et al.(86) as well. Also, if, as suggested by Hill and Whelan (55), PF arrests cells in the S phase or at the G_1/S boundary, the toxic effect of subsequent Ara-C exposure would be maximized.

Pretreating with both drugs leads to marked reductions of both CTP and UTP, with the greatest depletion noted when PF is the first drug administered. With the reduction of DCK_1 activation by UTP, while HL-60 cells exposed first to DAU had a significantly higher rate of Ara-C uptake than cells exposed first to PF, this occurred in

the absence of increased Ara-CTP formation, resulting in a lower Ara-CTP/CTP ratio for DAU then PF than PF then DAU. This paralleled the changes in HL-60 clone survival, as presented in Figure 13. This apparent intracellular accumulation of metabolites of Ara-C other than the triphosphate and/or the unchanged drug is of uncertain etiology, but it may well play a role in the levelling off of Ara-C uptake due to back-diffusion of substances such as Ara-U. At higher rates of uptake the enzymes which deaminate Ara-C and Ara-CMP to Ara-U and Ara-UMP may be activated, or accumulation of Ara-CMP and/or Ara-CDP may occur. Measuring Ara-U, Ara-UMP, Ara-CMP, and Ara-CDP levels could identify the Ara-C 'well.'

Figure 13 illustrates a fairly straightforward relationship between the Ara-CTP/CTP ratio and the effects of DAU and PF pretreatment on HL-60 clone survival, but enhancement of Ara-C toxicity may be more complex than suggested here. Recall the apparent inconsistency reported by Harkrader *et al.*(50), who found that while cells exposed to deoxyadenosine had a higher Ara-CTP level and a higher Ara-CTP/dCTP ratio than cells exposed to deoxyguanosine, whose Ara-CTP content was no higher than control cells, the latter had three times the Ara-C incorporation into DNA of controls while the former had only double the control value. Deoxyguanosine also resulted in more marked synergy with Ara-C than deoxyadenosine. To resolve these

questions in regards to the agents in this study, deoxyribonucleotide levels and Ara-C incorporation into DNA should be measured. Perhaps rapid incorporation of Ara-C into DNA, with or without concurrent inhibition of DNA polymerase, affects cellular expression of the metabolic alterations characteristic of S phase. These alterations, as noted in the introduction, include increased uptake and phosphorylation of nucleosides and their analogs, including Ara-C. The picture is that of a cell critically depleted of dCTP following PF and DAU pretreatment, activating transport and DCK_1 at the onset of S phase only to be greeted with a lethal relative concentration of Ara-CTP, shutting down the S phase processes. This would result in lower total Ara-CTP content, but might be revealed by plotting Ara-CTP/Total intracellular Ara-C against time exposed to Ara-C.

Because of more marked UTP, CTP, and, likely, dTTP depletion following PF pretreatment, cell cycle synchronization may be more marked than following DAU exposure. Thus, simply by lengthening the period of PF action from 3 to 6 hours by putting it first in the sequence, more cells may be 'caught' in the S phase, or released into the S phase, when Ara-C is administered.

A final brief hypothesis is required to explain synergy between Ara-C and PF and/or DAU even when Ara-C

is the first drug administered. Cells may be able to repair the damage done to their DNA under the influence of Ara-C, either through its incorporation into DNA or altered DNA synthesis secondary to inhibition of DNA polymerase. There is no evidence that Ara-C affects excision repair. However, the cell would require dCTP for normal repair, and if its synthesis is inhibited by PF or DAU, the cell will be unable to repair its DNA and may progress to cell death.

In conclusion, this study has demonstrated the efficacy of a drug regimen involving PF then DAU pretreatment of L1210 and HL-60 cells exposed to Ara-C. By depleting cellular UTP, PF enhances the ability of DAU to block CTP synthesis, resulting in increased Ara-C uptake via removal of feedback inhibition of DCK_1. A sevenfold elevation of the Ara-CTP/CTP ratio, perhaps accompanied by accumulation of cells in the S phase or at the G_1/S boundary, results in synergistic enhancement of Ara-C cytotoxicity. Though further studies are undoubtedly necessary, I would urge the eventual application of this rationally-designed drug combination regimen to patients with tumors otherwise unresponsive.

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