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AUGMENTATION OF METHOTREXATE CYTOTOXICITY IN HUMAN COLON CANCER CELLS ACHIEVED THROUGH INHIBITION OF THYMIDINE SALVAGE BY DIPYRIDAMOLE*

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Abstract—In HCT 116 cells, a human colon cancer cell line, the levels of thymidine (0.6 μM) and hypoxanthine (9 μM) contributed to the tissue culture medium by the fetal bovine serum significantly reduced the growth inhibition and lethality produced by 0.1 μM methotrexate. Dipyridamole, an inhibitor of nucleoside transport, potentiated the growth inhibitory effects of methotrexate when the cells were grown in medium that was changed daily. However, when the medium was supplemented with dialyzed serum, methotrexate cytotoxicity was not increased by dipyridamole. Similarly, in cloning experiments, dipyridamole increased the cell killing produced by methotrexate. The potentiation of methotrexate toxicity produced by dipyridamole was mediated through inhibition of thymidine uptake. The uptake of 1 μM thymidine was inhibited 50% by 0.12 μM dipyridamole but neither hypoxanthine nor guanine uptake was decreased by dipyridamole (5 μM). As a result, the decrease in dTTP pools produced by methotrexate was augmented by dipyridamole. In contrast, dipyridamole did not influence the effect of methotrexate on ribonucleoside triphosphate pools. HCT 116 cells avidly salvaged low concentrations of thymidine, and methotrexate increased this capacity. Conversion of 0.11 μM thymidine to thymidine triphosphate was increased by 55%, from 16.6 to 25.7 pmoles/10^6 cells, following exposure to 1.0 μM methotrexate. Dipyridamole blocked this pool expansion. This study suggests that the salvage of physiological levels of thymidine may diminish the cytotoxic effects of methotrexate on human colon cancer cells. Inhibition of thymidine uptake by dipyridamole may be an effective strategy to increase the cytotoxicity of methotrexate.

Methotrexate, an antifolate, plays an important role in the treatment of numerous neoplastic disorders [1]. Methotrexate exerts its antineoplastic effect by inhibiting dihydrofolate reductase and limiting the synthesis of reduced folates which serve as cofactors in several critical enzymatic reactions. This leads to inhibition of dTMP and purine synthesis and, consequently, the cytotoxicity of methotrexate [1]. Unfortunately, resistance to methotrexate may develop and thereby limit its usefulness. Such alterations include a decrease in the uptake of methotrexate, a decrease in the affinity of dihydrofolate reductase, an increase in the intracellular levels of dihydrofolate reductase, a decrease in the polyglutamation of methotrexate, or a decrease in the activity of the enzyme thymidylate synthetase [1].

The salvage of nucleotide precursors may be another mechanism by which cells circumvent methotrexate toxicity. *In vitro* and *in vivo* studies have shown that the toxicity of methotrexate can be reduced markedly by the addition of thymidine and a purine source [2–11]. In addition, as seen in human colon carcinomas [12, 13], the murine 3924A hepatoma [14] and in a rat sarcoma [15], the activities of salvage enzymes are often elevated in tumor as compared to normal tissues. These findings raise several questions regarding the use of methotrexate. Is salvage a potentially important pathway for the development of resistance to methotrexate in human tumor cells? Are physiological levels of nucleic acid precursors sufficient to reduce methotrexate toxicity? Can an inhibitor of nucleoside transport restore methotrexate cytotoxicity by preventing precursor utilization?

Dipyridamole, an inhibitor of nucleoside transport [16–18], can increase the toxicity of inhibitors of *de novo* nucleotide synthesis by decreasing nucleoside salvage. For example, in combination with acivicin [14, 19, 20], DON** [21], or methotrexate [10, 21], dipyridamole can antagonize the protection afforded by the salvage pathways. Similarly, NBMPR, also an inhibitor of nucleoside transport, has been used

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** Abbreviations: DON, 6-diazo-5-opo-L-norleucine; NBMPR, 6-[(4-nitrobenzyl)thiol-9-β-D-ribofuranosylpurine; Mtx, methotrexate; Dp, dipyridamole; PALA, *N*-(phosphonacetyl)-L-aspartate; and PBS, phosphate-buffered saline (pH 7.4).

to potentiate methotrexate toxicity in the presence of nucleotide precursors [22].

The results of this study attest to the effective salvage capacity of human colon cancer cells and support the idea that physiological levels of thymidine are sufficient to antagonize the cytotoxic effects of methotrexate against a human colon cancer cell line. Although methotrexate augments thymidine salvage, dipyridamole can inhibit the utilization of thymidine, potentiate the reduction of intracellular dTTP pools, and augment methotrexate cytotoxicity.

MATERIALS AND METHODS

Materials

Methotrexate, dipyridamole, nucleobases, nucleosides, nucleotides, and poly d(AT) were purchased from either the Sigma Chemical Co. (St. Louis, MO) or P-L Biochemicals (Milwaukee, WI). Moravek Biochemicals, Inc. (Brea, CA) supplied [5-^{3}H]uridine (20 Ci/mmole) and [2-^{3}H]hypoxanthine (18 Ci/mmole). [8-^{3}H]Deoxyadenosine 5′-triphosphate, ammonium salt (24 Ci/mmole), [8-^{14}C]guanine sulfate (51 μCi/mmole) and [*methyl*-^{3}H]thymidine (25 Ci/mmole) were obtained from the Amersham Corp. (Arlington Heights, IL). Alamine (tri-*n*-octylamine) and freon (1,1,2-trichlorotrifluoroethane) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). DNA polymerase I was obtained from Boehringer-Mannheim (Indianapolis, IN). The thin-layer chromatographic sheets, MN PEI Cel UV, were obtained from Brinkmann Instruments (Westbury, NY).

Cell culture

HCT 116 cells, a human colorectal carcinoma cell line, were obtained from Dr. M. Brattain at the Bristol-Baylor Laboratory (Houston, TX) and have been characterized previously [23]. These cells were grown in Eagle's Minimum Essential Medium (Grand Island Biological Co., Grand Island, NY) supplemented with non-essential amino acids (Grand Island Biological Co.), 2 mM L-glutamine, 50 μg/ml gentamicin (Schering Co., Kenilworth, NJ), 10 μg/ml insulin, 0.2 nM triiodothyroxine, 5 nM sodium selenite, 2 μg/ml transferrin, and 8% fetal bovine serum (Hyclone Lot No. 100389, Logan, UT). The final concentrations of thymidine and hypoxanthine in the medium which were contributed by the serum were 0.6 and 9 μM respectively. Stock cultures were passed weekly when approximately 90% confluent. These cells were found to be free from contamination by mycoplasma (Hoechst stain kit for detection of mycoplasma in cell culture, Flow Laboratories, Inc., McLean, VA). The fetal bovine serum was dialyzed against a saline solution to give a 1:6433 dilution (Union Carbide Corp., Chicago, IL).

Cytotoxicity

Inhibition of cell growth. Approximately 4×10^4 cells were plated in 35 mm culture dishes and maintained in a humidified 5% CO_2 atmosphere. The cells were allowed to grow for 24 hr before the drugs were added. Incubation was continued for an additional 72 hr before the cells were counted. The use of dialyzed or undialyzed serum is specified for each experiment in Results. The cells were enumerated using a Coulter Counter (model ZBI, Coulter Electronics, Inc., Hialeah, FL) after removal from the dish with 0.1% trypsin. The population doubling time for the HCT 116 cells was 24 hr.

Inhibition of colony formation. In these experiments and those involving measurement of ribonucleoside triphosphate and dTTP pools, approximately 5×10^5 cells were plated in 100 mm culture dishes and maintained at 37° in a humidified 5% CO_2 atmosphere for 24 hr. At that time the drugs were added and the exposure was continued for 24 hr. The cells were then counted, diluted to 300–2000 cells per 3 ml in medium containing non-dialyzed serum, and replated into 35 mm plates. These cells were then allowed to grow for 7 days at which time they were stained with a 0.1% methylene blue in methanol solution. Colonies, which were defined as 50 or more cells, were counted.

Measurement of ribonucleoside triphosphate pools

The nucleoside triphosphates were extracted with 2–3 ml of iced 0.5 N $HClO_4$. The extract was then neutralized with 2 vol. of 0.5 M alamine in freon [24]. Neutralized samples were stored at −20° until analyzed by high pressure liquid chromatography. A Spectra-Physics 8700 solvent delivery system, a Kratos Spectroflow 773 UV spectrophotometer monitoring 254 nm, a LDC/Milton Roy CI-10 integrator, and a Whatman Partisil 10/25 SAX anion exchange column equipped with a guard column were used. Peaks were identified by retention times and peak areas were linear over the range of 0.1 to 2.3 μmoles. Standards were run on each day of assay. The ribonucleotides were eluted isocratically with 0.3 M NaH_2PO_4, pH 5.0, using a flow rate of 2 ml/min. Typical retention times (min) for the ribonucleotides were as follows: UTP 6.3, CTP 8.7, ATP 11.2 and GTP 15.8. In this assay the coefficient of variation for samples run on different days was less than 10% for each of the nucleotides.

Measurement of dTTP pools

The enzymatic assay used for the measurement of dTTP was a modification of the method of Solter and Handschumacher [25] as described by Hunting and Henderson [26]. The neutralized extracts were lyophilized and reconstituted with 100 μl of double-distilled water. Ten microliters of Poly d(AT) (2 absorbance units/ml) was used as the template for the dTTP assay in a total volume of 170 μl. Ten and twenty microliters of cell extract were incubated with 10 μl of DNA polymerase I (0.75 units/ml) for 45 min at 45°. Twenty microliters was then spotted onto Whatman DE81 filter paper strips. After drying, the strips were washed three times in 25 ml of 5% trichloroacetate–1% sodium pyrophosphate and twice in 95% ethanol. The assay was linear from 5 to 100 pmoles dTTP. The addition of known amounts of dTTP to the extracts yielded the expected results.

Nucleoside uptake

Approximately 2×10^5 cells were plated in 60 mm culture dishes and incubated for 48 hr at which time

they were approximately 70% confluent. Dipyridamole, radiolabeled nucleosides, and bases were added, and the cells were incubated for 1 hr. The cells were then washed three times in PBS and extracted with 1.5 ml of ice-cold 0.5 N $HClO_4$. One milliliter of the extract was counted in a liquid scintillation spectrometer. The pattern of incorporation of [^{3}H]thymidine into dTMP, dTDP and dTTP was determined by thin-layer chromatography using polyethyleneimine sheets and 1.0 M LiCl as the solvent. In these experiments, neutralized acid extracts were lyophilized and resuspended in 40 μl of water prior to spotting the samples. The chromatograms were cut into 1 cm strips, and the radioactivity was eluted with 1 ml of buffer (0.7 M $MgCl_2$ and 0.02 M Tris–HCl, pH 7.5) and quantified.

RESULTS AND DISCUSSION

Cytotoxicity

An important question addressed in this study was whether the HCT 116 cells could salvage physiological levels of nucleotide precursors sufficiently well to diminish the cytotoxicity exerted by methotrexate. Nucleoside salvage was perturbed either by growing the cells in medium supplemented with dialyzed serum, which depletes the medium of nucleic acid precursors, or by adding an inhibitor of nucleoside transport, dipyridamole, to the medium. Initially, growth studies were performed using medium containing undialyzed serum in which both the medium and the drugs were changed daily. This was done to reduce depletion of nucleotide precursors from the medium. Thymidine, for example, was utilized rapidly by the HCT 116 cells. At 5.3×10^4 cells/dish, the half-life of thymidine at a concentration of 1.8 μM was about 24 hr (data not shown). Similarly, L1210 cells can deplete the medium of uridine within 24 hr in the presence of PALA [27].

The results of these growth inhibition experiments, which are summarized in Fig. 1, demonstrate that the salvage of low levels of nucleic acid precursors can diminish methotrexate cytotoxicity. If salvage was reduced, either by the use of medium supplemented with dialyzed serum or by the addition of dipyridamole, the cytotoxicity of methotrexate was augmented. When HCT 116 cells were exposed to 0.1 μM methotrexate and grown in medium containing 8% fetal bovine serum which was changed daily, cell growth decreased to 33% of control. The addition of 5 μM dipyridamole further reduced cell growth to 7% of control. Dipyridamole also reversed the protective effect conferred by the addition of thymidine (1 μM) and hypoxanthine (50 μM). When the cells were exposed to methotrexate in medium supplemented with dialyzed serum, cell growth was substantially less (10% control). Dipyridamole did not inhibit cell growth nor did it augment the toxicity of methotrexate when the cells were grown in dialyzed serum.

To determine if dipyridamole also augmented the lethality of methotrexate, clonogenic assays were undertaken. The addition of 5 μM dipyridamole reduced the viability of cells exposed to 0.1 μM methotexate from 29 to 17% of control (Fig. 2).

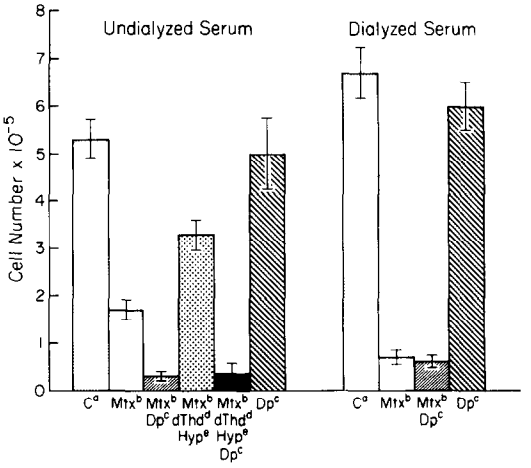

Fig. 1. Effects of dipyridamole on methotrexate cytotoxicity in HCT 116 cells. Key: C^a = vehicle control; Mtx^b = methotrexate, 0.1 μM; Dp^c = dipyridamole, 5 μM; $dThd^d$ = thymidine, 1 μM; and Hyp^e = hypoxanthine, 50 μM. Cells were plated and then incubated for 24 hr prior to the addition of drugs. Incubation was continued for 72 hr and then the cells were counted. Data are presented as the mean ± standard error, N = 4–6, for the control, Mtx, Mtx plus Dp, and Dp conditions in the first group of experiments. Mtx and Mtx plus Dp differed from each other at $P < 0.005$ (Student's *t*-test). The means and ranges are indicated for the other experimental conditions (N = 2).

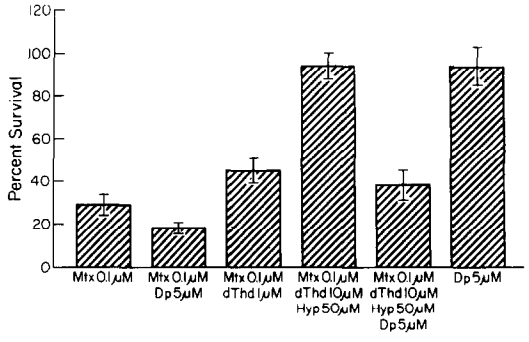

Fig. 2. Effects of dipyridamole on the lethality of methotrexate in HCT 116 cells. Approximately 5×10^5 HCT 116 cells were plated per 100 mm culture dish and then incubated for 24 hr. The cells were exposed to the drugs for 24 hr and then cloned into medium supplemented with non-dialyzed serum. The cells were allowed to grow for 7 days and were then stained, and the colonies containing 50 or more cells were enumerated. Data are presented as the mean ± standard error, N = 3, for Mtx, Mtx plus Dp, and Dp. Mtx and Mtx plus Dp differed from each other at $P < 0.05$ (Student's *t*-test). The other conditions are presented as the means and ranges (N = 2).

Thymidine and hypoxanthine reduced the lethality of methotrexate and dipyridamole antagonized that protection. The viability of HCT 116 cells was not affected by 5 μM dipyridamole.

Warnick *et al.*, using NBMPR [22], and Marz *et al.*, using dipyridamole [10], demonstrated that these inhibitors of nucleoside transport could prevent the reversal of methotrexate cytotoxicity seen with the addition of dThd and a purine. Their studies demonstrated that growth rate can be dependent on the

membrane transport of precursors. Recently, Nelson and Drake [21] reported that dipyridamole can enhance methotrexate toxicity in mice. The key point in the cytotoxicity experiments in this study is that low, physiological concentrations of precursors are of considerable importance in human cancer cells. The augmentation of toxicity by dipyridamole indicates that the amounts of dThd (0.6 μM) and hypoxanthine (9 μM) contributed by the 8% fetal bovine serum in this study were sufficient to partially reverse methotrexate toxicity. Since the reported values for most nucleosides in human plasma range from 0.1 to 1 μM (2, 8, 28–32] and those for hypoxanthine in cancer patients range from 0.2 to 64 μM [2, 8], salvage of these physiological amounts of nucleic acid precursors may be an important mechanism for methotrexate resistance *in vivo*.

Effects on nucleic acid precursor uptake and nucleoside triphosphate pools

Dipyridamole can inhibit the transport and, therefore, the uptake of both nucleosides and bases in various cell types [16–21]. To assess the mechanism by which dipyridamole augments methotrexate toxicity, uptake studies were undertaken. The effects of dipyridamole on incorporation of precursors into the acid-insoluble and acid-soluble fractions were similar. Dipyridamole inhibited the uptake of 1 μM uridine by 50% at 70 nM and had a similar effect on the uptake of 1 μM thymidine at 120 nM (data not shown). These data are consistent with the literature [14, 33, 34]. The uptake of the nucleobases, guanine and hypoxanthine, was not, however, inhibited by concentrations of dipyridamole as high as 5 μM. This is in contrast to the results we obtained in another human colon cancer line, VACO 5, in which dipyridamole effectively inhibited guanine uptake [19]. The uptake of the nucleobases adenine, guanine and hypoxanthine is inhibited by dipyridamole in cultured Novikoff hepatoma cells [35] and that of hypoxanthine in Chinese hamster lung fibroblasts [36]. Other investigators have found little or no effect by dipyridamole on adenine uptake in murine tumor cells [37], guinea pig heart cells [38], human platelets [39], and chick fibroblast cells [40]. These findings emphasize the heterogeneity in nucleoside and base transport in different cell types. This heterogeneity could be an advantage if precursor transport in key normal cells, such as the bone marrow, was less sensitive than tumor cells to inhibition by dipyridamole. On the other hand, if a significant percentage of tumor cell populations is relatively insensitive to dipyridamole, then this approach will be of little value.

Since the uptake of hypoxanthine was not affected by dipyridamole in HCT 116 cells, the enhancement of methotrexate toxicity appeared to result from inhibition of thymidine uptake. Thus, restoration of the depleted dTTP pools by salvage of thymidine should be prevented in those cells treated with the combination of methotrexate and dipyridamole. This was, in fact, the case (Fig. 3). When HCT 116 cells were treated with 0.1 μM methotrexate for 24 hr, dTTP pools fell to 29% of control. However, in cells additionally exposed to 5 μM dipyridamole, the dTTP pools were further decreased to 8% of control.

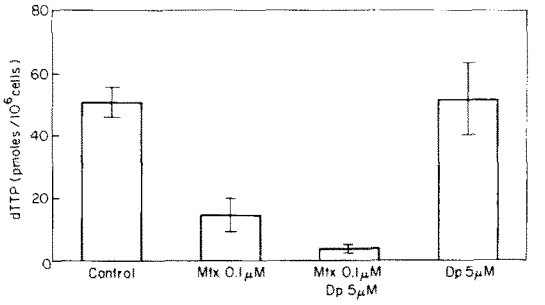

Fig. 3. Effects of dipyridamole and methotrexate on dTTP pools. Exponentially growing HCT 116 cells were incubated for 24 hr and then were exposed to the drugs for an additional 24 hr. Data are expressed as pmoles/10^6 cells and represent the mean ± standard error (N = 3).

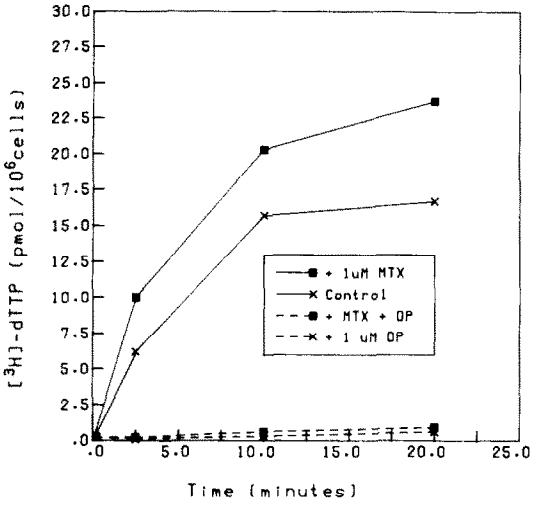

Fig. 4. Effect of methotrexate on the salvage of thymidine by HCT 116 cells. Cells were plated and then incubated for 24 hr. Either methotrexate or PBS was added to the dishes and the cells were incubated for an additional 2 hr. Tritiated dThd (0.11 μM) and either 1 μM dipyridamole or PBS were then added to the dishes. The cells were processed as described for nucleoside uptake after 2.5, 10 and 20 min of incubation. Approximately 72% of the label in the cellular extracts was [^{3}H]dTTP, and the data were calculated accordingly and are presented as [^{3}H]dTTP (pmoles/10^6 cells).

Dipyridamole alone did not alter dTTP pools. These data strongly suggest that dipyridamole can increase methotrexate toxicity by preventing the salvage of thymidine and are the first demonstration that the dipyridamole effect is accompanied by a drop in dTTP pools. The decrease in dTTP pools produced by methotrexate alone were similar to those reported by other investigators [41–43].

Since dThd kinase is subject to feedback inhibition by dTTP, a methotrexate-induced reduction in dTTP pools could enhance dThd salvage. Therefore, the effect of pretreating HCT 116 cells with methotrexate on their capacity to convert low levels of [^{3}H]dThd to [^{3}H]dTTP was determined. As shown in Fig. 4, 16.6 pmoles of [^{3}H]dTTP accumulated per 10^6 control cells within 20 min after the addition of 0.11 μM [^{3}H]dThd. If the cells were exposed to 1 μM methotrexate for 2 hr prior to adding the [^{3}H]dThd, salvage

Table 1. Effect of dipyridamole (Dp) and methotrexate (Mtx) on ribonucleotide triphosphate pools

Condition	Pool size (nmoles/10^6 cells) ATP	GTP	UTP	CTP
Control	3.2	0.8	1.0	0.4
Mtx (0.1 μM)	1.6	0.8	0.8	0.4
Mtx (0.1 μM) + Dp (5.0 μM)	2.0	1.0	0.8	0.5

HCT 116 cells (5×10^5) were plated in 100 mm culture dishes and incubated for 24 hr. Methotrexate, dipyridamole or PBS was added to the dishes and incubation continued for 24 hr. The ribonucleoside triphosphate pools were then extracted from the cells and assayed as described in Materials and Methods. In a similar experiment, performed in duplicate, the ribonucleoside triphosphate pools (ATP = 5.1 ± 0.1; GTP = 1.6 ± 0.01; UTP = 1.84 ± 0.03; CTP = 0.76 ± 0.01; mean ± range) were not reduced by methotrexate.

was increased and 23.7 pmoles of [^{3}H]dTTP accumulated per 10^6 cells. In five different experiments, the accumulation of [^{3}H]dTTP in the methotrexate-treated cells averaged 154 ± 7% that of the controls (mean ± SE). Thus, the salvage of low, physiological levels of thymidine can be substantially augmented by methotrexate. The total intracellular dTTP pools were also determined, in duplicate, in two of these experiments. The dTTP pools were reduced from 79 to 15 pmoles/10^6 cells following a 2-hr exposure to methotrexate. Within 20 min after the addition of 0.11 μM dThd, the dTTP pools were increased to 25 pmoles/10^6 in the methotrexate-treated cells and to 87 pmoles/10^6 control cells. The addition of 1 μM dipyridamole entirely blocked this dTTP pool expansion. Salvage was dependent on the concentration of dThd, and at 1 μM [^{3}H]dThd approximately 72 pmoles [^{3}H]dTTP/10^6 cells accumulated within 20 min. Taken together, these data emphasize the remarkably active salvage capability of the HCT 116 cells and focus on the potential importance of low concentrations of thymidine. The reduction in dTTP pools produced by methotrexate decreased the feedback inhibition of thymidine kinase and further enhanced the salvage of thymidine in these cells.

In addition, if the augmentation of methotrexate cytotoxicity produced by dipyridamole is dependent on the inhibition of thymidine, but not of hypoxanthine uptake, then dipyridamole should not affect ribonucleotide pools. As shown in Table 1, the amounts of ATP, CTP, UTP and GTP for the controls were 3.2, 0.8, 1.0, and 0.4 nmoles/10^6 cells respectively. Although the addition of 0.1 μM methotrexate decreased ATP pools to 1.6 nmoles/10^6 cells, the combination of 5 μM dipyridamole and methotrexate did not result in further changes in the ATP, CTP, UTP, or GTP pools. These data, as well as the finding that dipyridamole did not enhance methotrexate toxicity in cells grown in medium supplemented with dialyzed serum (Fig. 1), support the critical importance of perturbing thymidine metabolism in the HCT 116 cells. In contrast, we have found that the ability of dipyridamole to increase the cytotoxicity of fluorouracil is not mediated by a blockade of thymidine salvage [44]. A new and potentially important effect of dipyridamole, inhibition of methotrexate efflux, has been reported to occur in mouse sarcoma 180 cells [45]. This effect does not appear to be critical in the HCT 116 cells since dipyridamole did not enhance methotrexate cytotoxicity when the cells were grown in medium supplemented with dialyzed serum.

This study illustrates that methotrexate can increase significantly the uptake of physiological levels of dThd and that this salvage can reduce the anticancer effect of the drug. The concomitant use of dipyridamole, which can block dThd uptake, restored the cytotoxicity of methotrexate and may be a useful therapeutic approach. The effects of dipyridamole on thymidine salvage and methotrexate cytotoxicity were correlated with changes in the dTTP pools. However, micromolar concentrations of thymidine have also been reported to reverse the effects of methotrexate on mouse and human bone marrow cells [8, 11, 29]. Thus, the toxicity, as well as the anticancer effect of methotrexate, could be augmented by dipyridamole [21]. A phase I evaluation of methotrexate used in combination with dipyridamole will be carried out in order to assess these possibilities.

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