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EFFECT OF *DE NOVO* PURINE SYNTHESIS INHIBITORS ON 5-FLUOROURACIL METABOLISM AND CYTOTOXICITY*

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Abstract—Methotrexate pretreatment of L1210 cells had been shown previously by us to cause an enhancement of the intracellular accumulation of 5-fluorouracil and of the formation of 5-fluorouracil nucleotides which was correlated with synergistic cytotoxicity. This effect of methotrexate was associated with increases in 5-phosphoribosyl-1-pyrophosphate, the cofactor required for the conversion of 5-fluorouracil to 5-fluorouridine-5′-monophosphate (FUMP). Because these influences on 5-fluorouracil metabolism were most likely mediated by the activity of methotrexate as an inhibitor of purine synthesis, the effects of other agents that inhibit purine synthesis were examined. An inhibitor of amidophosphoribosyltransferase, 6-methylmercaptopurine ribonucleoside, the glutamine antagonists, azaserine and 6-diazo-5-oxo-L-norleucine (DON), and the L-aspartate analogue inhibitor of adenylsuccinate synthetase, L-alanosine, all reduced the incorporation of [1-^{14}C]glycine into adenine and guanine bases isolated from nucleic acids. Each drug also resulted in intracellular elevations of 5-phosphoribosyl-1-pyrophosphate that were 15- to 25-fold greater than control levels. These alterations in *de novo* purine nucleotide synthesis were associated with enhanced intracellular 5-fluorouracil accumulation and synergistic cytotoxicity.

The interaction of methotrexate (MTX)‡ and 5-fluorouracil (FUra) has been an interest to many investigators [1–5]. The interaction of these two drugs on the generation of thymidylate (dTMP) has been shown to be antagonistic regardless of the drug sequence [1, 2]. We observed that, when suspension cultures of L1210 cells were pretreated with MTX, there was an enhancement of the intracellular accumulation of FUra. This FUra accumulation was 5-fold greater in L1210 cells after 6 hr of 100 μM MTX than in control cells. The FUra nucleotide derivatives, FUMP, FUDP, FUTP and FdUMP, were also increased 5-fold in the MTX-treated cells. Although the inhibition of [^{3}H]dUrd incorporation into the acid precipitable cell fraction was not greater following sequential treatment with MTX and FUra, there was a 5-fold greater incorporation of FUra into the RNA fraction. This sequence produced synergistic cell killing that could not be explained by the interaction of these drugs on dTMP synthesis [6, 7].

In cells treated with MTX there was an 8-fold increase in intracellular 5-phosphoribosyl-1-pyrophosphate (PRPP). The increase in either FUra or PRPP accumulation could be prevented by the addition of leucovorin (N^5-formyltetrahydrofolate) at concentrations that protected cells from the growth inhibitory effects of MTX. Moreover, there was no enhancement of FUra accumulation if the PRPP concentrations were reduced following MTX by adding hypoxanthine to the cell cultures which utilized PRPP for ribosylphosphorylation to inosine monophosphate (IMP).

This enhanced intracellular FUra accumulation was thought to be the consequence of the antipurine effect of MTX. The elevated PRPP concentrations were presumed to be the result of a reduction in the normal feedback control that the end products of *de novo* purine synthesis have on PRPP synthesis and utilization. The resultant increased PRPP concentration following this inhibition of *de novo* purine synthesis was now available for utilization in the ribosylphosphorylation of FUra to FUMP by orotate phosphoribosyltransferase (EC 2.4.2.10), the rate-limiting step in FUra intracellular metabolism [8].

Since our hypothesis is that MTX affects FUra metabolism indirectly by inhibiting purine synthesis, other inhibitors of purine synthesis should produce similar results.

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‡ Abbreviations: MTX, methotrexate; FUra, 5-fluorouracil; FUMP, 5-fluorouridine-5′-monophosphate; FUDP, 5-fluorouracil-5′-diphosphate; FUTP, 5-fluorouracil-5′-triphosphate; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; FUrd, 5-fluorouridine; FdUrd, 5-fluoro-2′-deoxyuridine; PRPP, 5-phosphoribosyl-1-pyrophosphate; MMPR, 6-methylmercaptopurine ribonucleoside; AZA, azaserine; DON, 6-diazo-5-oxo-L-norleucine; L-Ala, L-alanosine; and Tbc, tubercidin.

METHODS

Drugs. [6-^{3}H]FUra (2 Ci/mmole) was purchased from Amersham (Arlington Heights, IL). [2-^{3}H]Adenine (24.5 Ci/mmole), which was used for the PRPP assay, and [1-^{14}C]glycine (20 mCi/mmole), which was used to evaluate purine synthesis, were obtained from the New England Nuclear Corp. (Boston, MA). MTX was provided by the National Cancer Institute (Bethesda, MD). FUra was purchased from the Sigma Chemical Co. (St. Louis, MO).

Cells. L1210 murine leukemia cells, with a doubling time of 10–12 hr were maintained as stationary suspension cultures in Fischer's medium plus 10% horse serum, transferred twice weekly, and incubated in a 5% CO_2 atmosphere at 37°. The cell density at which experiments were performed was 1–5 × 10^5 cells/ml. Mycoplasma contamination was not detected on routine monthly testing. A model ZBI Coulter Counter (Hialeah, FL) was used to enumerate cells.

Cloning. The biological antitumor effect of the various drugs was determined by cloning cells in soft agar by the technique we have previously reported [9, 10] that is a modification of the method initially described by Chu and Fischer [11]. Following the indicated single drug exposure to logarithmically growing cell cultures, the second drug was added for the specified time. The drug-containing medium was then removed after centrifuging at 1000 *g* for 5 min at 37°. The cell pellet was resuspended in drug-free medium and then recentrifuged as before. This washing procedure was repeated twice to remove any extracellular drug before cloning.

Fifty cells were pipetted into 10-ml culture tubes that contained 1 ml of liquified agar (37°) and 3 ml of drug-free Fischer's medium plus 15% horse serum. The tubes were capped and placed upright and incubated at 37° in a 5% CO_2 atmosphere. The amount of agar in the culture medium was 0.88 g/100 ml; the consistency of this mixture does not allow cell settling but does permit cell growth. Cells that have remained viable after the drug exposure, as defined by having the continuing ability to divide and produce progeny, will form individual cell colonies after 10 days of incubation. All clones were counted with an inverted microscope. Single cells, which can be visualized in suspension cultures, were not observed in the cloning medium after the 10-day incubation, indicating that all viable cells had developed into clones. The percent viability is the ratio of clones formed from drug-treated cultures to clones formed from untreated cultures multiplied by 100. The cloning efficiency of L1210 cells in this system was 85–90 per cent. All experiments were done in triplicate on three separate occasions. Mean values are shown; the maximum range was ± 5 per cent.

Total intracellular FUra accumulation. The total amount of intracellular [^{3}H]FUra that accumulated within the cells was determined by the microfuge method that we described previously in detail [12]. The pretreatment drugs were given for 3 hr at the indicated concentrations and then [^{3}H]FUra was added to the cell culture to achieve a concentration of 3 μM. Aliquots were removed over the course of 1 hr and the total [^{3}H]FUra accumulated was determined. The accumulation of FUra was linear to 1 hr and is reported as pmoles·min^{-1}·10^6 cells^{-1}.

Purine synthesis. The incorporation of [1-^{14}C]glycine into the purine bases of nucleic acid was used as a measure of *de novo* purine synthesis. Drugs were added to the cell cultures for 1–2 hr before adding the [1-^{14}C]glycine to 10 μM; 2 hr later the cells were centrifuged and the supernatant fraction was discarded. The cell pellet was precipitated in 1 N $HClO_4$ and the nucleic acid was depurinated by incubating at 100° for 1 hr. After neutralizing with 4 N KOH the supernatant fraction was analyzed by high pressure liquid chromatography on an ODS-2 column (Whatman Inc., Clifton, NJ), eluting with 0.1 M sodium acetate, pH 5.5, and acetonitrile at a gradient from 0 to 7.5% over 30 min. Nonradiolabeled adenine and guanine were used as markers. Absorbance was recorded at 254 and 280 nm, 0.5-ml fractions were collected, and the radioactivity was quantitated. Glycine that was not incorporated into bases appeared in the void volume.

PRPP assay. Intracellular PRPP was quantitated from the conversion of [^{3}H]adenine to [^{3}H]AMP by adenine phosphoribosyltransferase (EC 2.4.2.7). This reaction requires PRPP; appropriately prepared samples were assayed simultaneously with known quantities of PRPP as a standard. The technique used is a modification of that reported by Henderson and Khoo [13] and described in detail by us [7].

RESULTS AND DISCUSSION

The influence of MTX, tubercidin, 6-methylmercaptopurine ribonucleoside, azaserine, 6-diazo-5-oxo-L-norleucine, and L-alanosine on the intracellular accumulation of FUra and PRPP levels is shown in Table 1. The effect of MTX, which has been reported by us previously [6, 7], is thought to be the result of purine inhibition. Tbc, which is phosphorylated by adenosine kinase (EC 2.7.1.20), inhibits PRPP synthetase [14] and does not result in an increase in PRPP levels. In cells treated with this

Table 1. Effects of purine synthesis inhibitors on FUra accumulation and PRPP levels*

Drug	Concn.	FUra accumulation (Treated/Control)	PRPP
MTX	10	4.32	8
MMPR	1	4.35	15.7
MMPR	10	4.40	25.0
DON	10	3.44	ND†
AZA	10	3.00	15.0
L-Ala	10	1.8	25.0
Tbc	10	UD‡	0.1

* In control cells FUra accumulation was 0.025 pmole·min^{-1}·10^6 cells^{-1} and PRPP was 7 ± 1.2 ng/10^6 cells. Cell cultures were exposed to the drugs for 3 hr before evaluating FUra accumulation and PRPP levels. Abbreviations: MTX, methotrexate; MMPR, 6-methylmercaptopurine ribonucleoside; DON, 6-diazo-5-oxo-L-norleucine; AZA, azaserine; L-Ala, L-alanosine; and Tbc, tubercidin.

† Not done. ‡ Undetected.

drug, intracellular FUra was undetectable In addition, when MTX-treated cells were also given Tbc, there was no PRPP elevation or augmentation of intracellular FUra accumulation [7]. This is consistent with our hypothesis that the inhibition of *de novo* purine synthesis that results in an elevation of PRPP concentration is the important modulating effect of MTX.

MMPR, like tubercidin, is phosphorylated by adenosine kinase, and as the nucleoside triphosphate it inhibits amidophosphoribosyltransferase (EC 2.4.2.14) [15]. As expected, PRPP levels were increased following this drug and the FUra intracellular acccumulation was also enhanced. The higher PRPP levels following MMPR did not result in any greater intracellular FUra accumulation compared to those cells treated with MTX. The addition of both MTX and MMPR together did not result in a greater increase in PRPP levels, nor was the intracellular FUra accumulation enhanced further. This may have been a reflection of a concentration of PRPP in these cells that had exceeded by several-fold the K_m of orotate phosphoribosyltransferase, the enzyme which transfers the 5-phosphoribose moiety from PRPP to FUra forming FUMP.

AZA and DON are glutamine antagonists which can inhibit *de novo* purine synthesis at the two steps that require glutamine for the amide transfer [16]. This probably accounts for the increased PRPP levels and enhanced intracellular FUra accumulation seen in cells pretreated with these drugs. The magnitude of the enhanced intracellular FUra accumulation was not as great as that observed following MTX or MMPR. This could be explained partially by the possible inhibitory effects of these drugs on CTP synthetase and on other metabolic conversions that require glutamine for amination. This effect on pyrimidine synthesis could possibly lead to increased orotate levels which would compete with FUra for PRPP. Our current investigations with these cells have documented a K_m for Fura of approximately 600 μM, while that for orotate was near 8 μM (unpublished results). Therefore, any slight elevation in the orotate concentration could have a dramatic inhibitory effect on the ribosylphosphorylation of FUra.

L-Ala is an antibiotic that competitively inhibits the intracellular transport of L-aspartic acid. The major antitumor activity may be the result of the inhibition of several enzymes involved in L-aspartic acid metabolism, however, including adenylosuccinate synthetase (EC 6.3.4.4), 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide synthetase (EC 6.3.2.6), L-aspartyl-tRNA synthetase, L-aspartate transcarbamylase (EC 2.1.3.2), and L-aspartate animotransferases [17, 18]. In addition, this drug also inhibits L-glutamine synthetase (EC 6.3.1.2) and L-glutamate decarboxylase [18]. Since several of the enzymes inhibited by this drug are involved in *de novo* purine synthesis, it was not surprising to observe an elevation in PRPP levels in cell cultures treated with this drug. The modest enhancement of FUra intracellular accumulation that followed treatment with L-Ala may represent a modifying effect on cellular metabolism by the other activities of this drug.

Table 2. [1-^{14}C]Glycine incorporation into purine bases*

Drug	Exposure time (hr)	Concn. (μM)	[^{14}C] into adenine and guanine·10^6 cells·hr^{-1} (% control ± 10%)
MTX	3	10	16
MMPR	3	1	12
MMPR	3	10	7
AZA	3	10	14
DON	3	10	4
Tbc	1.5	10	17
L-Ala	4	10	60

* The control value was 10.1 ± 1 pmoles [^{14}C]glycine into adenine and guanine·10^6 cells^{-1}·hr^{-1}. See Table 1 for abbreviations.

The effects of these drugs on the [1-^{14}C]glycine incorporation into purine bases are shown in Table 2. Each drug had an appreciable inhibitory effect on *de novo* purine synthesis. The reduction by L-Ala of [1-^{14}C]glycine incorporation was only 60 per cent of control values, compared to a much greater reduction seen with the other agents studied. There was, however, a 40 per cent reduction of labeled glycine incorporated into adenine bases, whereas no inhibition was observed on the incorporation of labeled glycine into guanine. This reflects the effect of this drug on the conversion of IMP to AMP by adenylosuccinate synthetase. All the other agents affected purine synthesis before the IMP branch point and, therefore, the total reduction of [1-^{14}C]glycine into purine bases was paralleled by identical reductions in both adenine and guanine.

The effects of increasing concentrations of MTX on the inhibition of *de novo* purine synthetase and their relation with PRPP elevations are represented in Table 3. These effects following MTX were directly related to the greatest FUra accumulation, which also occurred with the higher concentrations of MTX [6].

The cytotoxicities resulting from sequential treatment with these antipurine drugs before adding FUra in a clonogenic assay of L1210 cells as determined by soft agar cloning are shown in Table 4. The pretreatment of cell cultures with drug concentrations that resulted in increased FUra intracellular accumulation produced synergistic cell killing. The concept that an inhibitor of purine synthesis which is associated with increased PRPP levels could result in enhanced cytotoxicity when given before a second drug that requires PRPP for activation is not new.

Table 3. Effect of methotrexate concentration on inhibition of *de novo* purine synthesis*

MTX μM	% Control [1-^{14}]glycine into adenine and guanine	ng PRPP / 10^6 cells
0	100	1.0
0.01	88	
0.1	60	2.5
1.0	22	41.5
10.0	16	52.2
100.0	9	52.2

* The S.D. of PRPP for this assay was ± 0.1 ng/10^6 cells.

Table 4. Viability of L1210 cells*

Drug(1)	Drug(2)	μM(1)	μM(2)	hr(1)		hr(2)	Viability
Control	0	0	0	0		0	100
MTX		10		4			60
	FUra		10			1	100
MTX	FUra	10	10	3	→	1	10
MMPR		1		4			100
MMPR	FUra	1	10	3	→	1	6
AZA		6		4			77
AZA	FUra	6	10	3	→	1	10
L-Ala		10		4			106
L-Ala	FUra	10	3	3	→	1	41
DON		10		4		1	45
DON	FUra	10	10	3	→	1	25

* The arrow (→) indicates that drug 1 was given for the indicated time before adding drug 2. Total drug exposure was 4 hr before cloning the cells in soft agar. See Table 1 for abbreviations.

Nelson and Parks [19] reported that S-180 cells pretreated with MMPR would accumulate more metabolites of 6-thioguanine (6-TG). This laboratory also made a similar observation when MMPR preceded 6-mercaptopurine (6-MP) in the same cell lines [20]. Paterson and Wang [21] and Paterson and Moriwaki [22] had also reported on the synergistic interaction between MMPR and 6-MP.

We have examined the nucleotide derivatives of FUra in L1210 cells pretreated with MTX [6, 7] and MMPR (unpublished results). The ribonucleotides FUMP, FUDP and FUTP and the deoxyribonucleotide, FdUMP were all increased above control values by the same percentage as the increase observed in total intracellular FUra accumulation. Approximately 1 per cent of the intracellular drug was the base, [^{3}H]FUra, in both treated and control cells. These findings indicate that drugs which inhibit *de novo* purine synthesis can also modulate the metabolism of FUra. This, however, would most likely occur only with cells in which the metabolism of FUra to FUMP is by orotate phosphoribosyltransferase and requires the cofactor PRPP. FUra can be converted to nucleotides by two other pathways, neither of which would be expected to be enhanced from increased PRPP levels. FUra can react with ribose-1-phosphate in the presence of uridine phosphorylase (EC 2.4.2.3) to form FUrd [23], which is then phosphorylated to FUMP by uridine-cytidine kinase (EC 2.7.1.48) [24]. FUra can also be converted to FdUrd by thymidine phosphorylase (EC 2.4.2.4) [25]. This nucleoside then can be phosphorylated to FdUMP by thymidine kinase (EC 2.7.1.2) [26].

Our studies do not address the mechanisms responsible for the synergistic cell kill observed with these drug treatment sequences. Both FdUMP and FUTP would be expected to be increased as was observed in our previous evaluations with MTX. Further studies are required to delineate the relative importance of these two active nucleotide derivatives in producing the enhanced cytotoxicity when the various inhibitors of *de novo* purine synthesis precede FUra.

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