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ENHANCEMENT OF 6-THIOGUANINE CYTOTOXIC ACTIVITY WITH METHOTREXATE[1]

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SUMMARY: Studies were completed to characterize the cytotoxic and biochemical interaction of methotrexate (MTX) and 6-thioguanine (6-TG). Pretreatment of L1210 leukemia cells for 3 hr with MTX substantially enhanced the cytotoxicity of 6-TG. The LD_{90} of 6-TG in cells pretreated with 1μM MTX was 0.9pM, compared to an LD_{90} of 800 pM when the two drugs were given concurrently and an LD_{90} of 30 pM resulted with 6-TG alone. HPLC analysis of intracellular metabolites demonstrated an increased conversion of 6-TG to 6-TG-nucleotides in cells pretreated with MTX. A marked enhancement of 6-TG incorporation into RNA also was noted (MTX→6-TG: 350 fmol/μg RNA vs 6-TG: 98 fmol/μg RNA). However, there was a suppression of 6-TG incorporation into DNA when cells were pretreated with MTX (MTX→6-TG: 170 fmol/μg DNA vs 6-TG: 690 fmol/μg DNA). These results suggest that: 1) an enhancement of 6-TG antileukemic activity can be obtained with MTX pretreatment, and 2) the enhancement of 6-TG cytotoxicity following MTX exposure is not associated with 6-TG incorporation into DNA, but rather with incorporation of 6-TG into RNA. This drug sequence may be beneficial in the clinical treatment of leukemia.

Methotrexate (MTX) and 6-thioguanine (6-TG) are antimetabolites used both singly and in combination for the treatment of various forms of leukemia and other cancers (1). The mechanism of action of MTX has been well characterized, and is mediated through the direct inhibition of dihydro-

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4 Abbreviations used: 6-TG, 6-thioguanine; MTX, methotrexate, FUra, 5-fluorouracil; PRPP, 5-phosphoribosylpyrophosphate.

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folate reductase, which leads to a depletion of the cellular folates required for de novo thymidylate and purine biosynthesis (1). This results in a suppression of DNA synthesis and cell death. The mechanism mediating 6-TG cytotoxicity is far less established. 6-TG is apparently inactive itself, and must be converted to a 6-TG-nucleotide for activity. This is dependent upon the action of hypoxanthine-guanine phosphoribosyltransferase which, using 5-phosphoribosyl-1-pyrophosphate (PRPP) as the cofactor, converts 6-TG to 6-TG-ribosemonophosphate (6-TGRP). 6-TGRP has been shown to have inhibitory actions on several enzymes involved in de novo purine biosynthesis (1). However, the primary mechanism mediating 6-TG cytotoxicity has been reported to result from its incorporation into DNA (1). The incorporation of 6-TG into RNA has been demonstrated, although any contribution of this to cytotoxicity has not been established (1).

A major biochemical effect of MTX is the suppression of purine biosynthesis which can result in the expansion of the PRPP pool. This increases the availability of PRPP for other PRPP-utilizing reactions. Previous studies (2,3) have demonstrated that pretreatment of cells with MTX resulted in PRPP pool expansion and the conversion of 5-fluorouracil (FUra) to FUra-ribosephosphate via PRPP-transferase was markedly enhanced, increasing the activation and the cytotoxicity of FUra. The dependence of 6-TG activity upon activation by PRPP-transferase suggests that a similar synergistic interaction between MTX and 6-TG may exist, and an increase in 6-TG anticancer activity may result.

The present study characterized the cytotoxic and biochemical interaction of MTX and 6-TG in L1210 mouse leukemia cells and demonstrates that methotrexate can markedly alter 6-TG activity.

MATERIALS AND METHODS

Chemicals. MTX was purchased from Lederle Laboratories, Wayne, N.J. 6-TG was purchased from Sigma Chemical Co., St. Louis, Mo. [^{14}C]6-TG was purchased from Moravek Biochemicals, City of Industry, Calif. All other biochemicals were purchased from Sigma Chemical Co. All tissue culture supplies were purchased from Gibco Laboratories, Grand Island, New York.

Cells. L1210 murine leukemia cells were maintained as a suspension culture in Fischer's medium supplemented with 10% heat-inactivated horse serum at 37° in a 5% CO_2 atmosphere. Drug toxicity was determined using a soft-agar cloning methodology as described previously (2). Viability was defined as the ability of a cell to produce progeny, which are visible as distinct individual clones or cell colonies. Percent viability or clonal growth was determined by the ratio of clonal growth of the treated cells compared to the clonal growth of untreated cells x 100. Drug concentrations were measured by spectrophotometric analysis, and solutions were prepared the same day of use.

6-TG Metabolism. Cells were exposed to [^{14}C]6-TG (56μCi/mmol) for time periods from 1 to 180 min. At the specified time, iced saline containing 50μM dipyridamole was added, the cell pellet immediately isolated, and then disrupted by the addition of 0.2N $HClO_4$. The acid-soluble fraction was isolated for HPLC analysis of intracellular 6-TG nucleotides. The RNA and DNA were isolated from the acid-precipitated as follows. The acid precipitate was washed twice with iced 0.2N $HClO_4$, followed by the addition of 3 ml of 0.3N NaOH. The precipitate was then digested for 1 hour at 37°C. After cooling on ice, DNA and protein were precipitated by addition of 300μl of 5.2 N $HClO_4$. 500μl of the isolated supernatant (containing the RNA fraction) was measured for radioactivity. This process was repeated twice to insure complete removal of all RNA from the remaining precipitate. Following removal of the RNA-containing supernatant, the precipitate was washed twice with iced 0.2N $HClO_4$ and then incubated at 70° for 30 min in 3ml of 0.5N HClO, to solubilize the DNA fraction. After cooling on ice, the sample was centrifuged and 500μl of the supernatant (containing the DNA) was measured for radioactivity. The precipitate was then washed twice with 0.5 $HClO_4$ and digested for 1 hour at 70° in 3 ml of 0.5N KOH. 500μl of the supernatnat (representing the protein fraction) was neuralized with 1N HCl, and measured for radioactivity. RNA was quantitated by the orcinal reaction, and DNA by a standard diphenylamine procedure (4). HPLC analysis was carried out on a SAX anion-exchange column using a 60 min linear gradient from 0.01M potassium phosphate (pH 5.7) to 0.75M potassium phosphate (pH 4.7).

RESULTS

Cytotoxicity. The cytotoxic interaction between MTX and 6-TG was characterized and the results are illustrated in figure 1. This graph depicts the clonal growth of L1210 cells following either a 3 hr exposure to 6-TG, a 3 hr exposure simultaneously with 1μM MTX and 6-TG, or a 3 hr exposure to 6-TG following a 3 hr pretreatment with 1μM MTX. A 3 hr exposure of cells to 1μM MTX alone resulted in a 52% inhibition of clonal growth, and is illustrated on figure 1 by the dotted line. It is apparent that the cytotoxic activity of 6-TG has markedly enhanced by preexposure of cells to MTX, as the LD_{90} for 6-TG is decreased from 0.8μM to 0.9pM when cells were pretreated with MTX. Furthermore, this modulation was absent when 6-TG and MTX were administered concurrently. In fact, MTX was antagonistic to higher concentrations of 6-TG under these conditions.

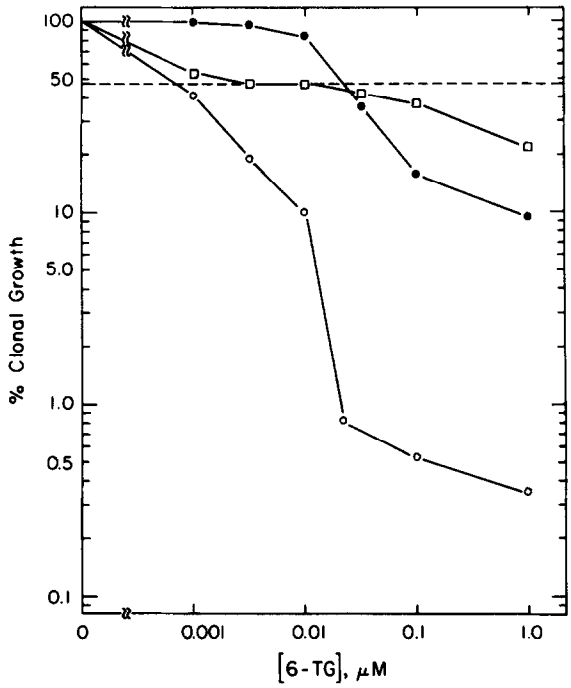

Figure 1. Cytotoxicity of 6-TG in L1210 cells. L1210 leukemia cells were exposed for 3 hr to 6-TG either alone (●), simultaneously with 1μM MTX (□) or following a 3 hr preexposure to 1μM MTX (○), and % clone growth determined. Clonal growth of cells exposed to 1μM MTX for 3 hr is illustrated by the dotted line. Results represent the mean of 4 separate experiments.

Metabolism of 6-TG. To determine the affect of MTX on the metabolism of 6-TG, cells were exposed to 0.5μM [^{14}C]6-TG for a total of 60 minutes and the intracellular metabolites of 6-TG were characterized. Simultaneous studies were completed in cells that had been preexposed to MTX for 3 hr. Table 1 list the levels of intracellular 6-TG and 6-TG-nucleotides measured in L1210 cells either with or without MTX pretreatment. This demonstrates that MTX pretreatment enhances the conversion of 6-TG to 6-TG-nucleotides.

Table 1

Metabolism of 6-TG to 6-TG-Nucleotides

L1210 cells both with and without preexposure to 1mM MTX were exposed to 0.5μM [^{14}C]6-TG for 60 min, and the cellular levels of 6-TG and 6-TG-nucleotides determined as described under "Materials and Methods".[1]

Drug	pmol/10^6 cells 6-TG	TGMP	TGDP	TGTP	Total Nucleotide
6-TG	3.43	0.90	0.28	0.37	1.55
MTX→6-TG	3.36	1.6	0.32	0.48	2.40

[1]Results are representative of 4 separate experiments.

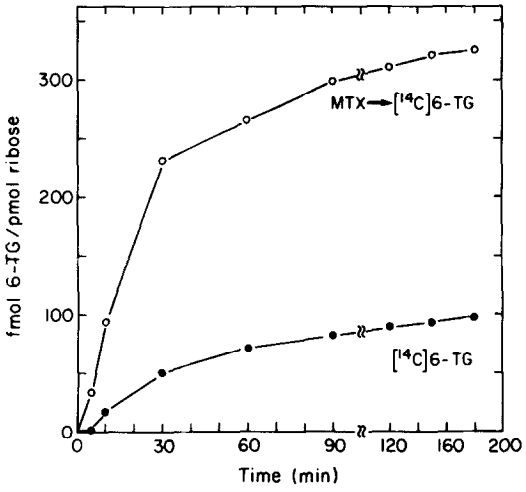

Figure 2. Incorporation of 6-TG into RNA. L1210 cells were exposed to [^{14}C] 6-TG for 5 to 180 minutes, the RNA isolated and radioactivity measured. Cells exposed to 6-TG alone (●), and cells pretreated with 1μM MTX, and then exposed to 6-TG (○). Results are representative of 4 separate experiments.

Incorporation of 6-TG into RNA and DNA. The incorporation of [^{14}C]6-TG into RNA and DNA was measured over a 3 hr period in cells both with and without a 3 hr preexposure to 1μM MTX. The incorporation of 6-TG into RNA is illustrated in figure 2. Reflecting the increased metabolism of 6-TG to 6-TG-nucleotides, a marked enhancement of the level and rate of 6-TG incorporation into RNA resulted in cells pretreated with MTX. However, the effect of MTX pretreatment on 6-TG incorporation into DNA, as illustrated in figure 3 is exactly the opposite. MTX pretreatment of cells resulted in a suppression of 6-TG incorporation into DNA compared to that occurring in cells exposed to 6-TG alone. Identical results were obtained using cesium-sulfate gradient separation of RNA and DNA (data not shown).

DISCUSSION

It is apparent from these results that MTX can modulate 6-TG cytotoxic activity, and the type of modulation is dependent upon their schedule of administration. Preexposure of cells with MTX resulted in a large increase in cytotoxic potency of 6-TG, whereas simultaneous exposure caused an antagonism of 6-TG cytotoxic activity. The preexposure of cells with MTX re-

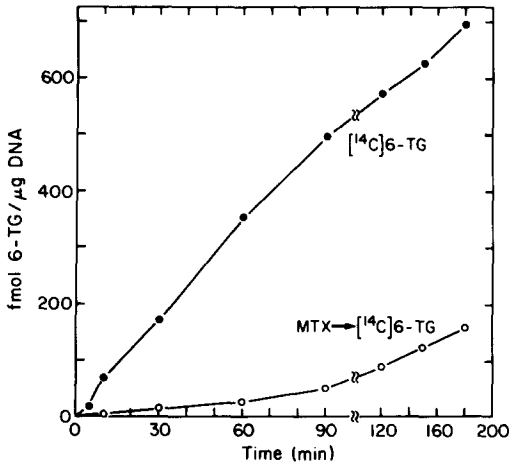

Figure 3. Incorporation of 6-TG into DNA. L1210 cells were exposed to [^{14}C] 6-TG for 5 to 180 minutes, the DNA isolated and radioactivity measured. Cells exposed to 6-TG alone (●), and cells pretreated with 1μM MTX, and then exposed to 6-TG (○). Results are representative of 4 separate experiments.

sulted in an enhanced conversion of 6-TG to 6-TG-nucleotides and an enhanced rate of 6-TG incorporation into RNA. MTX has been previously demonstrated to cause an expansion of cellular PRPP pools in L1210 leukemia cells (2). Although PRPP pools were not quantitated in the present study, the dependence of 6-TG upon PRPP-transferase for activation suggests that this was the basis for the increased conversion of 6-TG to 6-TG-nucleotides. This has been previously observed with MTX and FUra, which is also converted to nucleotide via PRPP-transferase (2,3). The mechanism mediating the cytotoxic activity of 6-TG has been reported to result from its incorporation into DNA (1). The results of the present study are in apparent contrast with this mechanism. Pretreatment of cells with MTX, although enhancing the cytotoxicity of 6-TG, markedly suppressed the incorporation of 6-TG into DNA over the drug exposure period. These results suggest that 6-TG may produce cytotoxicity in addition to , but independent of, that resulting from 6-TG incorporation into DNA. The contribution of 6-TG incorporation into RNA to cytotoxicity has been not been previously established. However, it is possible that this incorporation of an abnormal base into nascent RNA could cause alterations in RNA function. This type of effect has been observed with other antimetabolites such as 5-azacytidine and FUra (6,7). MTX pretreatment

causes a large increase in the rate of 6-TG incorporation into RNA. Considering the corresponding increase in cytotoxicity and the suppression of 6-TG incorporation into DNA that is also observed, 6-TG incorporation into RNA should be considered as a potentially cytotoxic event in the enhanced 6-TG potency following MTX pretreatment. The suppression of 6-TG incorporation into DNA following MTX pretreatment is probably related to an inhibition of the rate of DNA synthesis that results from MTX (1). Studies are currently being completed to assess both rates of RNA and DNA synthesis in relation to treatment with these agents to understand the changes in 6-TG incorporation. It should also be recognized that 6-TGRP can inhibit several enzymes involved in de novo purine biosynthesis. Our studies demonstrated that 6-TGRP pools were increased with MTX pretreatment, and this may act synergistically with MTX in depleting purine nucleotides and altering DNA synthesis.

In conclusion, our studies demonstrate that MTX can markedly modulate 6-TG cytotoxicity. Pretreatment of cells with MTX will increase 6-TG cytotoxicity, while simultaneous administration of MTX with 6-TG antagonizes 6-TG cytotoxicity. The results suggest that the effect of MTX preexposure is to enhance the activation of 6-TG via PRPP-transferase, and that the increased cytotoxicity is unrelated to 6-TG incorporation into DNA. Incorporation of 6-TG incorporation into RNA, which is enhanced by MTX pretreatment, may contribute to 6-TG cytotoxicity under these conditions. Further biochemical studies are being completed to characterize the mechanism mediating this interesting interaction. These results should be considered with use of MTX and 6-TG in the treatment of leukemia or other neoplastic diseases.

REFERENCES

1. Pratt, W.B. and Reddon, R.W. The Anticancer Drugs, pp. 98-147, Oxford University Press, 1979.
2. Cadman,E., Heimer, R. and Benz, C. J. Biol. Chem. 256: 1695, 1981.
3. Benz, C. and Cadman, E. Cancer Res. 41: 994, 1981.
4. Schneider, W.C. Methods Enzymol. 3: 680-684, 1957.
5. Weiss, J.W. and Pitot, H.C. Biochemistry, 14: 316-326, 1975.
6. Wilkinson, D.J. and Pitot, H.C. J. Biol. Chem. 218: 63-68, 1973.