Biochemical Pharmacology, Vol. 18, pp. 413–417. Pergamon Press. 1969. Printed in Great Britain

ENHANCEMENT OF 6-THIOINOSINE-5′-MONOPHOSPHATE SYNTHESIS IN SOLID L1210 LYMPHOCYTIC LEUKEMIA CELLS BY PRIOR EXPOSURE TO 6-MERCAPTOPURINE*

MARY LOU MELONI and WILLIAM I. ROGERS

Arthur D. Little, Inc., Acorn Park, Cambridge, Mass., U.S.A.

(*Received* 12 *June* 1968; *accepted* 16 *August* 1968)

Abstract—A 4-fold enhancement of 6-thioinosine-5′-monophosphate synthesis by solid L1210 tumors in BDF_1 mice occurs after the third hourly i.v. dose of 6-mercaptopurine (6-MP) over that resulting from a single dose. Two prior doses of 6-MP also cause a 3-fold enhancement of 6-MP uptake and the enhanced synthesis of 6-thioxanthosine-5′-monophosphate.

PATERSON[1] has reported that Ehrlich ascites cells contain 5–6 times as much 6-TIMP† on the basis of packed cell volume after a fifth daily i.p. dose of 6-MP as after four daily doses of saline and one of 6-MP. The data indicate that prior doses of 6-MP enhanced the ability of the cells to convert 6-MP to 6-TIMP, that ribotide formation was maximal 24 hr after pretreatment and that the number of prior daily doses, whether three, four or five, did not alter the magnitude of the effect. He has also reported that a dose of azaserine 6 hr before the i.p. dose of 6-MP caused a 2- to 3-fold enhancement of 6-TIMP synthesis.[1]

During kinetic studies of the physiological disposition of 6-MP in solid L1210 lymphocytic leukemia and a 6-MP-resistant subline implanted in contralateral axillary regions of BDF_1 mice, we found that hourly i.v. doses of 6-MP altered the metabolism of the sensitive tumor so that each succeeding dose was more effectively converted to 6-TIMP than the preceding one. It is the purpose of this communication to present data which demonstrate that, by the third dose of 6-MP, a 4-fold enhancement of 6-TIMP formation is found in the solid L1210 cells. The chemotherapeutic implications of this immediate effect of prior doses may be significant.

Atkinson *et al.*[2] have reported that 6-MP is metabolized to 6-TXMP by Ehrlich ascites cells. We find that 6-TXMP is also formed by the solid L1210. However, we did not find 6-methylmercaptopurine ribonucleotide as a metabolite of 6-MP in either the solid sensitive or solid 6-MP-resistant L1210 cells, although Allan *et al.* found the methylated derivative as a significant metabolite in H.Ep. 2 cells in culture[3] and in ascites L1210 and Ca 755 cells *in vivo* or in culture.[4] The solid 6-MP-resistant L1210 formed neither 6-TIMP nor 6-TXMP even with prior hourly doses.

* This work was sponsored by Chemotherapy, National Cancer Institute, United States Public Health Service, Contract No. PH 43-65-61.

† Abbreviations used: 6-MP, 6-mercaptopurine; 6-TU, 6-thiouric acid; 6-TIMP, 6-thioinosine 5′-monophosphate; 6-TXMP, 6-thioxanthosine 5′-monophosphate; 6-TXR, 6-thioxanthosine; PRPP, 5-phosphoribosyl-l-pyrophosphate.

MATERIALS AND METHODS

Female BDF_1 mice weighing 20 g were implanted with sensitive and 6-MP-resistant L1210 solid tumors (40-mg fragments) s.c. in contralateral axillary regions. After 5 days, the animals were injected i.v. with 8-^{14}C-6-MP (caudal vein) as single doses of 60 mg/kg (180 mg/m^2). 8-^{14}C-6-mercaptopurine hydrate was purchased from New England Nuclear Corp. 6-Mercaptopurine was obtained from the Cancer Chemotherapy National Service Center (NSC 755). Six mice were sacrificed at each time interval to determine the drug and metabolites in tumors. The 6 g of tumor obtained were pooled and chilled immediately, homogenized with cold distilled water and adjusted to 0·4 N with $HClO_4$ to extract the acid-soluble components. All operations were carried out at about 4°. The insoluble residues were extracted twice more with 0·2 N $HClO_4$. The extracts were combined, carefully neutralized with 5 N KOH to pH 7 and centrifuged to remove the insoluble $KClO_4$. The radioactivity of aliquots was determined in Bray's solution with a Nuclear-Chicago Mark I scintillation counter so that data on recovery of radioactivity from chromatographs could be calculated. Unless 90 per cent of the radioactivity was accounted for, the experiment was repeated. Other aliquots were applied to Dowex-1-formate columns (16·0 × 0·5 cm) and eluted with an asymptotic formic acid gradient with 10 M formic acid in the reservoir and 300 ml H_2O in the mixing chamber, as described by Hurlbert *et al.*[5] Fractionation and monitoring of column eluates were performed on a Gilson Fractionator with a u.v. absorption meter fitted with a 313 mμ filter. Fractions (2–3 ml) were collected at a rate of 0·25 ml/min. The order of elution of components was: 6-MP, dethiolated ^{14}C-purine* (presumably hypoxanthine or adenine or both), 6-TU, 6-TX, 6-TIMP and 6-TXMP. After appropriate fractions were pooled and concentrated *in vacuo*, metabolites were quantitated by using data from the literature on ultraviolet absorption and molar extinction coefficients. Radioactive quantitation was also employed. The identities of the metabolites were confirmed on descending paper chromatography in a solvent system of equal volumes of 5% Na_2HPO_4 (pH 7·0) and isoamyl alcohol. In most cases the paper chromatograms were radioautographed to verify that the spot located by inspection under u.v. light was also the only radioactive spot in that fraction Any degradation of 6-MP or metabolites that was of sufficient magnitude to affect the results would have been detected. No degradation occurred under the conditions described.

RESULTS

The data in Fig. 1 indicate the disposition of 6-MP and its metabolites in the L1210 tumor and in the 6-MP-resistant subline and show that the concentration of 6-TIMP reached a maximum in the sensitive cells 1 hr after a single i.v. dose of 6-MP. In the sensitive tumor, the half-life of the nucleotide in the acid-soluble fraction appeared to be about 1 hr; the concentration of 6-MP and the dethiolated purine formed from the 6-MP also reached their maximum concentrations at about 1 hr. All three compounds left the acid-soluble fraction of the sensitive cells at about the same rate. Resistant

* The thiopurines absorb maximally above 290 mμ, but this fraction, which chromatographs like hypoxanthine or adenine on Dowex-1 under the conditions described above or on paper chromatography under the conditions described below, absorbs maximally at 260 mμ at pH 10·8 and at 256 mμ at pH 6·4 or pH 1·0 and, therefore, must be a dethiolated purine. In addition to the spectral characteristics of a purine, it contains the radioactive 8-carbon of 6-MP.

cells, on the other hand, attained maximal concentrations of 6-MP earlier than sensitive cells, dethiolated 6-MP more effectively and produced no 6-TIMP. Although 6-TU was present in sensitive and resistant cells, it probably was synthesized elsewhere in the animal because L1210 ascites cells grown in BDF_1 mice, but incubated with 6-MP *in vitro*, produced no 6-TU. However, the L1210 ascites cells did dethiolate 6-MP and did produce 6-TIMP, 6-TXMP and 6-TXR.*

The data in Table 1 include the 1-hr data from Fig. 1 in the first column and show that, when a second intravenous dose was given 1 hr after the first and the solid tumors were analyzed 1 hr later the concentration of 6-TIMP present had more than doubled. It is also seen in Fig. 1 that by 2 hr after the first dose only 13 μg 6-TIMP remained

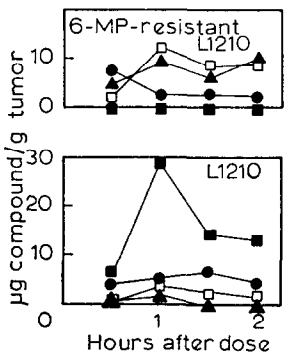

FIG. 1. The presence of 6-MP and metabolites in sensitive and 6-MP-resistant solid L1210 after a single dose of 6-MP (60 mg/kg). The data are averages from 3 separate experiments.

Quantitation is described in the legend for Table 1. ●, 6-MP; ■, 6-TIMP; ▲, 6-TU; □, ^{14}C-dethiolated purine†.

TABLE 1. STIMULATION OF 6-MP ANABOLISM BY PRIOR DOSES OF 6-MP SOLID L1210 LYMPHOCYTIC LEUKEMIA‡

Time (hr) Zero One Two Three	Action dose sac	Action dose dose sac	Action dose dose dose sac	Action dose§ dose§ dose sac	
				Estimated by u.v.	^{14}C
6-MP	5·6	22	32	28	19
6-TU	2·3	5·3	4·4	2·3	0·4
6-TIMP	30	70	140	147	123
6-TXMP	0	2·6	8·3	14	12
6-TXR	0	2·1	10·4	14	13
^{14}C-Purine†	5·8	5·8	17	32	16

‡ Values are given as μg compound/g solid tumor. Each dose (60 mg 6-MP/kg or 180 mg/m^2) was given to female 20-g BDF^1 mice with solid L1210. For abbreviations, see text.

§ The first two doses of 6-MP were unlabeled; elsewhere in this table, dose refers to the injection of 6-MP-8-^{14}C.

† Radioactive, dethiolated purine is a ^{14}C-labeled fraction with maximal u.v. absorbance of 260 mμ. It is probably hypoxanthine and/or adenine. For purposes of quantitation, an average of hypoxanthine and adenine molar extinction coefficients was used (E = 15,300).

* M. L. MELONI and W. I. ROGERS, unpublished data.

per gram of tumor. Therefore, it is estimated that about 57 of the 70 μg was due to the second dose. The effect of a third hourly dose is given in the third column of data. The formation of 6-TIMP was further enhanced by the second dose, and the concentrations of 6-TXMP and 6-TXR continued to rise.

Because of the uncertainty in estimating how much of the 6-TIMP from the first and second doses still remained in the tumors, an experiment was done to take advantage of the fact that the drug and metabolites are measured quantitatively by both spectrophotometry and radioactivity after chromatographic isolation. The data in the right-hand column, Table 1, are from an experiment in which the first two doses of 6-MP were unlabeled; the third dose was 8-^{14}C-6-MP. In this experiment, 1 hr after the third dose the total concentrations of 6-MP, 6-TU, 6-TIMP, 6-TXR and dethiolated purine in the cells, when estimated by ultraviolet spectrophotometry only, were virtually the same as when estimated by u.v. and radioactivity in the previous experiment (third column). It is seen that 123 of the 147 μg 6-TIMP per gram of tumor found 1 hr after the third dose was due to the last dose. In comparing this 123 μg 6-TIMP found 1 hr after the third dose of 6-MP with the 30 μg 6-TIMP found 1 hr after the first dose (first column), it is clear that a 4-fold potentiation of capability to synthesize 6-TIMP was a result of the two prior doses. It is also of interest that 19 μg 6-MP per gram of tumor was present from the third dose, but that only 5·6 μg 6-MP per gram of tumor was taken up after the first dose.

Fig. 1 shows that after the 6-TIMP concentration had become greatest (1 hr after the first dose), its half-life was about an hour. If the subsequent doses had no effect on this half-life, it is calculated that by the third hour after the first dose, the first and second doses would have contributed about 35 μg to the 6-TIMP pool in 1 g of tumor. This amount is comparable (within the precision of the experiments) to the 24 μg due to the first 2 (nonradioactive) doses in the experiment with 2 nonradioactive doses followed by a dose of radioactive 6-MP. Therefore, in addition to the 4-fold potentiation found for the solid L1210 cells *in vivo*, it may be concluded that the rate at which 6-TIMP left the acid soluble fraction of the cells was not substantially affected by the prior doses of 6-MP.

It is of interest that 6-TXMP and 6-TXR were also detected in the sensitive cells after the second dose of 6-MP. There was a 5-fold increase in the concentration of 6-TXMP and of 6-TXR 1 hr after the third dose of 6-TXMP. Although this increase was substantially greater than the increase in 6-TIMP concentration, the relative fraction of each of the three compounds formed from the third, radioactive dose of 6-MP was about 86 per cent. This suggests that the formation of the analogs is via 6-TIMP, as suggested for Ehrlich ascites cells by Atkinson *et al.*[2] The solid, 6-MP-resistant line of L1210 formed no 6-TIMP, 6-TXMP nor 6-TXR after any of the doses of 6-MP, as seen in Table 2.

DISCUSSION

The enhancement of 6-TIMP formation from 6-MP after prior hourly doses of 6-MP is consistent with the hypothesis that 6-TIMP inhibits *de novo* purine biosynthesis, as shown for L1210 by Bennett *et al.*,[6] and that the phosphoribosylpyrophosphate (PRPP) pool then becomes available for reaction with 6-MP to form 6-TIMP catalyzed by guanine-hypoxanthine phosphoribosyl transferase, as shown for Ehrlich ascites tumor cells by Atkinson and Murray.[7]

Two factors tend to reinforce the increased rate of 6-TIMP synthesis. The first is that the greater the intracellular concentration of 6-TIMP, the greater the inhibition of *de novo* purine biosynthesis and the greater the potential pool of one substrate, PRPP. The second is that prior doses of 6-MP cause a greater uptake of 6-MP by the tumor cells, for instance, three times as much 6-MP was taken up after the third

TABLE 2. 6-MP AND METABOLITES IN 6-MP-RESISTANT SUBLINE OF SOLID L1210 AFTER MULTIPLE DOSES*

Time (hr) Zero One Two Three	Action dose sac	Action dose dose sac	Action dose dose dose sac
6-MP	3·4	15	19
6-TU	5·9	26	20
6-TIMP	0	0	0
6-TXMP	0	0	0
6:TXR	0	0	0
^{14}C-Purine†	19	20	38

* Values are given as μg compound/g solid tumor. Each i.v. (caudal vein) dose (60 mg 6-MP/kg or 180 mg/m^2) was administered to female 20-g BDF_1 mice with solid L1210 and 6-MP-resistant L1210 implanted in contralateral axillary regions. For abbreviations, see text.

† Quantitation is described in the legend for Table 1.

hourly dose as after the first. The higher intracellular concentration of the second substrate, 6-MP, would also tend to drive the phosphoribosyl transferase-catalyzed reaction, particularly if 6-MP competes effectively for the enzyme, as in the case of Ehrlich ascites cells.[7]

Increased intracellular concentrations of the two substrates, 6-MP and PRPP, could result in increased or enhanced conversion of 6-MP to 6-TIMP and is a possible explanation for the results reported.

This work is being extended to determine if the therapeutic response of the solid tumor to 6-MP can also be enhanced *in vivo* and to determine the effects of varying the size and frequency of prior doses on the capacity of the solid tumor to synthesize thioinosinate. It is also important, from the standpoint of potential increased drug toxicity to the host, to determine if normal tissues such as intestine and liver are similarly affected biochemically.

Acknowledgements—We wish to acknowledge the cooperation of Mr. Isidore Wodinsky and Mr. Michael Mazrimas in conducting these experiments.

REFERENCES

1. A. R. P. PATERSON, *Can. J. Biochem. Physiol.* **37**, 1011 (1959).
2. M. R. ATKINSON, G. ECKERMANN and J. STEPHENSON, *Biochim. biophys. Acta* **108**, 320 (1965).
3. P. W. ALLAN, H. P. SCHNEBLI and L. I. BENNETT, *Biochim. biophys. Acta* **114**, 647 (1966).
4. P. W. ALLAN and L. L. BENNETT, JR., *Proc. Am. Assoc. Cancer Res.* **9**, 2 (1968).
5. R. HURLBERT, H. SCHMITZ, A. BRUMM and U. R. POLTER, *J. biol. Chem.* **209**, 23 (1954).
6. L. L. BENNETT, L. SIMPSON, J. GOLDEN and T. L. BARKER, *Cancer Res.* **23**, (9), 1574 (1963).
7. M. R. ATKINSON and A. W. MURRAY, *Biochem. J.* **94**, 64 (1965).