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SPECIFIC AND SENSITIVE COMBINED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC—FLOW FLUOROMETRIC ASSAY FOR INTRACELLULAR 6-THIOGUANINE NUCLEOTIDE METABOLITES OF 6-MERCAPTOPURINE AND 6-THIOGUANINE

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SUMMARY

A new non-radioisotopic technique is described for measuring rates of intracelluiar formation by human leukemic blasts of 6-thioguanine nucleotide metabolites, obligatory intermediates in the antineoplastic action of both 6-mercaptopurine and 6-thioguanine itself. The method is both specific and sensitive, and involves combined high-performance liquid chromatography and flow fluorometric detection of oxidized 6-thioguanine nucleotides in alkaline permanganate-treated cell extracts. Non-metabolized 6-thioguanine and 6-thioxanthine are also separated and quantitated in this system, permitting complementary in vivo pharmacokinetic analysis. The assay may be applied to detect resistant disease at an early stage in therapy, and thereby provides the opportunity for alternative treatments to be instituted.

INTRODUCTION

The 6-thiopurine antimetabolites 6-thioguanine (TG) and 6-mercaptopurine (MP) have found important application in the clinical chemotherapy of acute leukemia [1, 2, 3]. Both agents share the absolute requirement that they be metabolized to their ribosyl monophosphate, nucleotide, derivatives as the first step in their mechanism of action. This process, termed "lethal synthesis", necessarily occurs within the leukemic cell itself, and clinical resistance to the drugs has been associated with reduced nett rates of intracellular drug nucleotide accumulation [4]. MP nucleotide undergoes further metabolism

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to TG nucleotides, and, in terms of its mechanism of action, MP merely represents another way of administering TG to malignant cells [5, 6]. The antineoplastic activity of both thiopurines results from the ultimate incorporation of a TG deoxynucleotide metabolite into DNA in phosphodiester linkage [6, 7, 8]. Since intracellular TG nucleotide metabolites are central to the action of both drugs, it is apparent that through measuring rates of TG nucleotide formation from MP or TG by human leukemic leukocytes, either in vivo or in vitro, it may be possible to identify drug-resistant disease at the outset of therapy. In this way alternative treatment may be instituted before any significant amount of time has been wasted on ineffective therapy, and unnecessary systemic toxicity may be avoided. In the past, determinations of thiopurine disposition and nucleotide formation have utilized radioisotopically labeled drugs, and this has precluded routine measurement of drug pharmacokinetics and activation in vivo. Consequently, there have been only isolated cases where thiopurine bioavailability and cellular pharmacology have been investigated clinically.

In the present paper we describe a specific and sensitive high-performance liquid chromatographic (HPLC) technique utilizing flow fluorometric detection of oxidized TG derivatives for measuring rates of intracellular non-radioactive TG nucleotide formation from MP or TG by human leukemic cells. Development of this technique follows from the report of Finkel [9] who described a non-radioactive fluorescent assay for quantitating the free base in laboratory preparations of TG. However, the latter method cannot be applied to measurement of free TG in biological materials since the fluorescent oxidized drug is not separated from naturally occurring components which fluoresce upon oxidation. We have extended Finkel's observations by demonstrating that oxidized TG nucleotides also fluoresce strongly and may be separated and quantitated by combined anion-exchange chromatography and flow fluorometry. Unchanged TG and 6-thioxanthine are also separated and quantitated in this system, and therefore the pharmacokinetics of TG may be determined in conjunction with measurement of drug nucleotide accumulation by leukemic blasts. This approach obviates a major criticism of drug sensitivity tests based solely on in vitro methodology, where in vivo bioavailability is ignored.

Our techniques may be applied in selecting individual patients most likely to benefit from an extended course of thiopurine chemotherapy.

EXPERIMENTAL

Sample preparation

TG or MP treated leukemic cells were pelleted by centrifugation and extracted with 4% perchloric acid at 4° using [8-^{14}C] adenine as internal standard; extracts were neutralized with KOH. Serum samples from patients receiving TG chemotherapy were deproteinized by ultrafiltration through Centriflo membrane cones (Amicon, Lexington, Mass., U.S.A.). Neutralized cell extracts and deproteinized serum samples were oxidized with alkaline potassium permanganate [9]; 0.1 ml of sample was mixed with 0.1 ml 0.1 *M* sodium carbonate—sodium bicarbonate buffer pH 10.1, and 0.1 ml 0.24% potassium

permanganate solution, and oxidation was allowed to proceed for 5 min. Excess permanganate was reduced by addition of 10 μl 30% hydrogen peroxide solution, and the brown precipitate of manganese dioxide was removed by centrifugation.

Chromatography

Oxidized TG and its derivatives were separated by anion-exchange chromatography on a Model 7000B chromatograph (Micromeritics, Norcross, Ga., U.S.A.) using a Reeve Angel Partisil-10 SAX strong anion-exchange column, 25 cm × 4.6 mm I.D. × ¼ in. O.D. (Whatman, Clifton, N.J., U.S.A.), and a 15-min concave buffer gradient ranging from 5 m*M* potassium phosphate (pH 3.5) to 250 m*M* potassium phosphate—500 m*M* potassium chloride (pH 4.5), at a constant flow-rate of 5 ml/min and a pressure of approximately 2200 p.s.i. The separated TG derivatives in the column effluent were detected by their fluorescence, using an FS-970 flow spectrophotofluorometer (Schoeffel, Westwood, N.J., U.S.A.) set at an excitation wavelength of 330 nm, installed with an auxillary Corning 7-54 prefilter for excitation, and employing a 389-nm cut-off emission filter. In addition to recording the photomultiplier output on a 10-mV chart recorder, the 5-V full scale output at the rear of the instrument was interfaced to a PDP 11/34 minicomputer (Digital Equipment Corp., Maynard, Mass., U.S.A.) through the computer's LPS11 system, and the chromatographic data were acquired in real-time at 2-sec intervals using the 12-bit A/D converter in the LPS11 module. Sequential digitized detector output values were stored on IBM Diskettes and were subsequently analyzed by a user written Fortran program which performed peak area integration. Peak areas were converted to absolute quantities using predetermined calibration curves. Known amounts of [^{14}C]adenine were added to cell pellets as internal standard during acid extraction, and radioactivity determinations were used to correct for losses of material during extract processing.

The mono-, di-, and triphosphates of adenosine, guanosine, uridine and cytidine were also separated under the chromatographic conditions described above, and for cell extracts these constituents were detected simultaneously in the column effluent by their UV absorption at 254 nm, using a Chromonitor 785 flow spectrophotometric detector (Micromeritics) connected in series with the flow fluorescence detector.

Materials

TG, 6-thioxanthine, DNase I, purine ribonucleotides, pyrimidine ribonucleotides, crotalus adamanteus venom and venom phosphodiesterase were purchased from Sigma (St. Louis, Mo., U.S.A.). 6-Thioguanosine was supplied by Dr. H.B. Wood of Drug Research and Development (Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., U.S.A.). Synthetic TG nucleotide standards were a gift from Dr. G.A. LePage of University of Alberta Cancer Research Unit (McEachern Laboratory, Edmonton, Canada). [8-^{14}C]-Adenine (50 mCi/mmol) was obtained from Amersham (Oakville, Canada).

RESULTS AND DISCUSSION

The bottom panel in Fig. 1 illustrates the HPLC separation of an oxidized synthetic standards mixture of TG, 6-thioxanthine, 6-thioguanosine mono-, di-, and triphosphate, using fluorescence detection of the column effluent. The top panel in Fig. 1 shows the separation of a standard mixture of the mono, di-, and triphosphates of adenosine, guanosine, cytidine, and uridine, visualized by monitoring the UV absorption of the column effluent at 254 nm, under identical chromatographic conditions to those employed in the separation of TG derivatives below. The alkaline potassium permanganate treatment did not affect the physiological ribonucleotides and the sole reaction with the thiopurines was at the sulfhydryl group, where oxidation resulted in the quantitative formation of fluorescent 6-sulfonate derivatives. By oxidizing

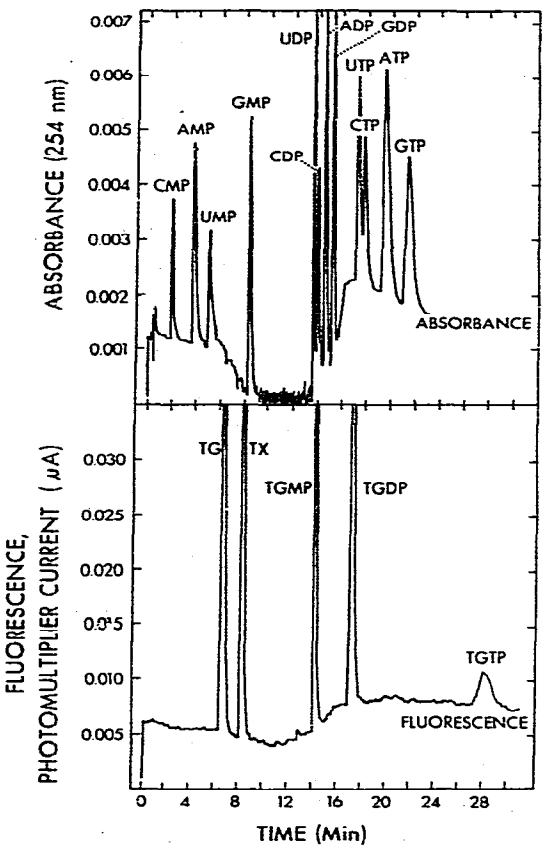


Fig.1. Separation of purine and pyrimidine ribonucleotides and oxidized 6-thiopurine derivatives by HPLC. Top: separation of a standards mixture of purine and pyrimidine ribonucleotides; absorbance profile at 254 nm. Bottom: separation of an oxidized standards mixture of 6-thiopurine derivatives; fluorescence emission profile. Abbreviations: TG = 6-thioguanine; TX = 6-thioxanthine; TGMP = 6-thioguanosine monophosphate; TGDP = 6-thioguanosine diphosphate; TGTP = 6-thioguanosine triphosphate. The actual species separated are the 6-sulfonate derivatives of the indicated thiopurines. Separation conditions as described in Experimental section.

standards mixtures it was demonstrated that this treatment did not affect the relative distribution of TG nucleotides amongst the three levels of phosphorylation. Prior to oxidation, samples of standard mixtures of TG nucleotides were separated at high concentration and quantitated by UV absorption at 340 nm (data not shown). Following oxidation the mixtures were diluted to permit more sensitive fluorescence measurements and the separation repeated. The relative distribution of TG nucleotides before and following oxidation were identical. No dephosphorylation occurred during oxidation. Oxidation did result in coordinated shifts of the thiopurine components to higher retention times, consistent with the generation of an additional negative charge on the molecules through formation of the sulfonate group. UV absorption measurements demonstrated that MP underwent a similar oxidation reaction, but the oxidized MP product did not exhibit detectable fluorescence under the conditions employed. Oxidation of 6-thioxanthine resulted in a fluorescent product, however, the intensity of the fluorescence was only approximately 10% of that exhibited by TG at the selected wavelengths for excitation and emission. Oxidation of pure synthetic TG deoxynucleoside monophosphate resulted in a fluorescent product with a retention time identical to that of the ribonucleoside monophosphate.

A calibration curve for oxidized TG is presented in Fig. 2 on a double logarithmic scale over the range of photomultiplier current settings. Peak area represents the product of the baseline corrected, computer-integrated A/D counts over the peak, and the value of the photomultiplier current producing full-scale output at the particular instrument setting corresponding to

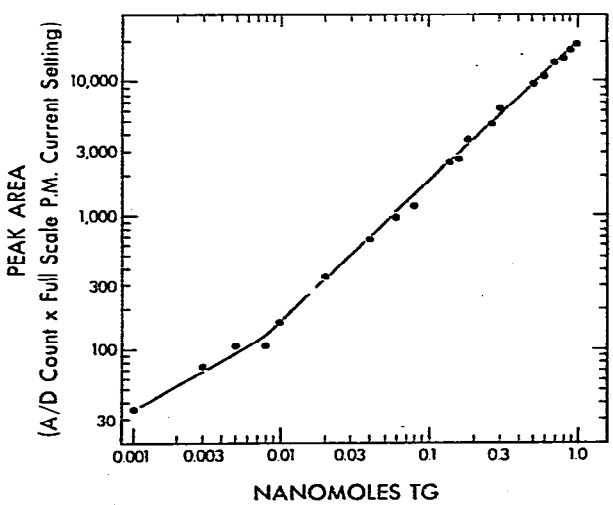


Fig.2. Chromatographic fluorescent peak areas of oxidized 6-thioguanine standards. Baseline corrected peak areas were determined by computer integration of the spectrophotofluorometer detector output signal acquired in real time. Areas are normalized as the product of the actual peak area and the detector photomultiplier current (μA) producing full-scale output at the signal amplification selected for each individual measurement. Chromatographic separation conditions were the same as those for Fig. 1 except that it was unnecessary to continue the gradients to completion.

each measurement. The abscissa represents the quantity of oxidized TG injected onto the column. The system is capable of measuring amounts of TG as low as 4 pmoles, or somewhat less than a nanogram. Quantities of TG nucleotides in cell extracts were derived from a TG calibration curve and are expressed as TG equivalents. Fig. 3 illustrates the simultaneous UV absorption and fluorescence emission chromatographic profiles for an oxidized acid extract of cultured mouse lymphoma L5178Y cells exposed to 3 μM TG. These cells are demonstrably sensitive to the cytotoxic effects of TG [5, 6]. The data correspond to an extract of 6.6×10^5 cells. TG, and TG nucleoside mono-, di-, and triphosphates are evident in the fluorescence emission profile (cf. Fig. 1). The peak immediately following TG represented a component of the tissue culture medium and was displaced from authentic 6-thioxanthine standard added to the cell extract (data not shown). In fact, 6-thioxanthine added during extract preparation may be used as internal standard in determinations of initial rates of TG nucleotide formation by human leukemic blasts (see Fig. 7). The triphosphates of uridine, cytidine, adenosine and guanosine are seen in the simultaneous UV absorption trace. Pretreatment of the acid extracts with crude crotalus adamanteus venom prior to oxidation resulted in complete nucleotide dephosphorylation and

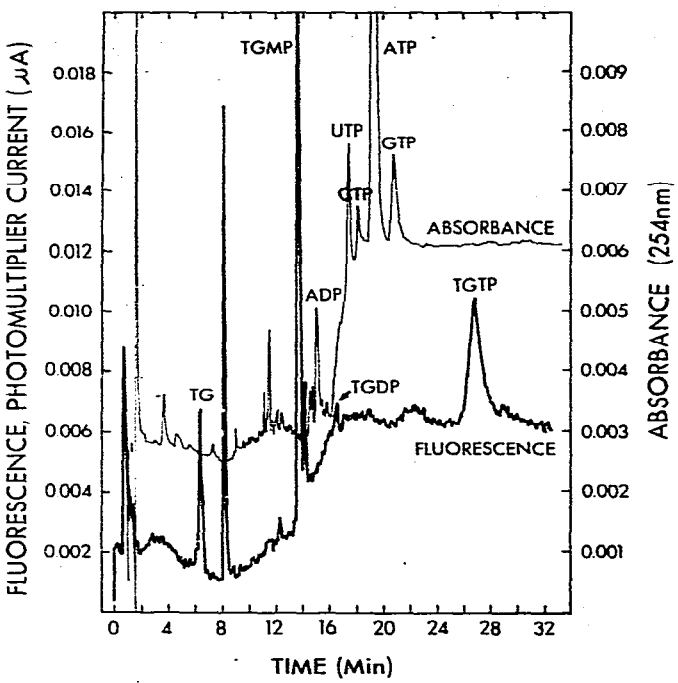


Fig.3. HPLC separation of an oxidized acid extract of cultured L5178Y cells exposed to TG. Simultaneous absorbance (254 nm) and fluorescence emission detection of the column effluent. The data represent 6.60×10^5 cells exposed to 3 μM TG for 13 h. Abbreviations: TGMP = 6-thioguanosine monophosphate; TGDP = 6-thioguanosine diphosphate; TGTP = 6-thioguanosine triphosphate. The actual thiopurine species separated are the 6-sulfonate derivatives of the indicated compounds. Separation conditions were the same as those for Fig. 1.

the disappearance of the nucleotide peaks in both profiles. In the case of the fluorescence emission profile, a new peak of equivalent area appeared with retention time slightly less than that of oxidized TG and identical to that of oxidized 6-thioguanosine. This result confirmed the nucleotide identity of the fluorescent peaks. Fig. 4 reproduces the fluorescence emission trace obtained on chromatographic separation of an oxidized extract of 1.65×10^6 cultured L5178Y cells exposed to 25 μM MP. The profile is very similar to that of Fig. 3 for cells exposed to TG, and demonstrates the formation of TG and TG nucleoside mono-, di- and triphosphate nucleotide metabolites from MP by L5178Y cells. Formation of TG nucleotide metabolites is essential to the cytotoxic activity of MP [6]. Oxidized MP did not exhibit any detectable fluorescence under the conditions employed to detect oxidized TG and its derivatives. Following acid extraction of TG-treated cells, the acid insoluble nucleic acid plus protein residues were subjected to alkaline hydrolysis at 37°. Acid soluble breakdown products of RNA released by this procedure were oxidized and chromatographed. Fig. 5 presents the simultaneous absorbance and fluorescence profiles of the column effluent obtained with one such hydrolysate. The absorbance trace shows a partial separation of the mixture of normal ribonucleoside 2′- and 3′-monophosphate derivatives produced by alkaline hydrolysis of RNA. The fluorescence trace demonstrates the incorporation of TG into RN— in phosphodiester linkage and its release

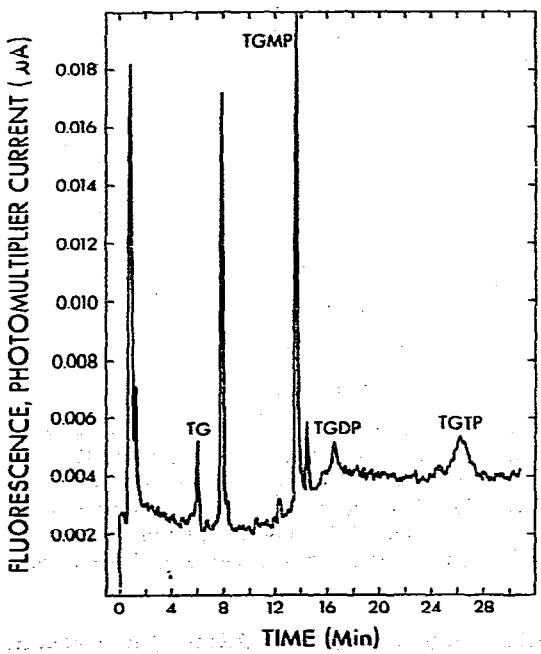
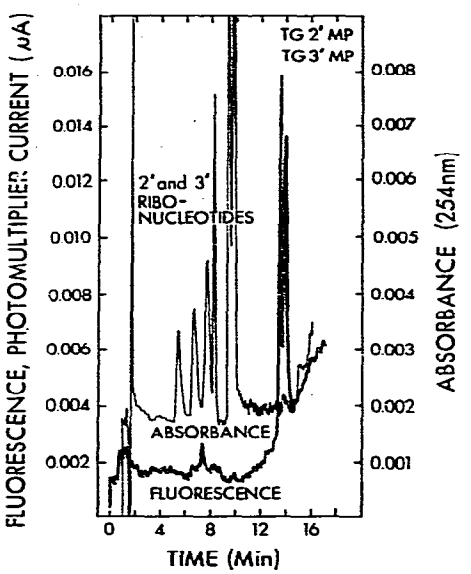


Fig.4. HPLC separation of an oxidized acid extract of cultured L5178Y cells exposed to MP. Fluorescence emission trace of the column effluent. Represented are data obtained with 1.65×10^6 cells exposed to 25 μM MP for 15.5 h. Abbreviations: TGMP = 6-thioguanosine monophosphate; TGDP = 6-thioguanosine diphosphate; TGTP = 6-thioguanosine triphosphate. The actual chemical species separated are the 6-sulfonate derivatives of the indicated compounds. Separation conditions were the same as those for Fig. 1.

by hydrolysis as a mixture of TG ribonucleoside 2'- and 3'-monophosphates. The acid-insoluble DNA plus protein residues of alkaline hydrolysis were suspended in 0.5 *M* Tris buffer (pH 7.0) and subjected to enzymatic hydrolysis of the DNA with pancreatic deoxyribonuclease (DNase I) and purified crotalus adamanteus venom phosphodiesterase. Ultrafiltrable hydrolysis products generated by this treatment were oxidized and separated on the chromatograph. Fig. 6 depicts the simultaneous absorbance and fluorescence profiles observed upon separation of a crude DNA hydrolysate prepared from TG treated L5178Y cells. The normal deoxyribonucleotide constituents of DNA are apparent in the UV absorbance trace and fluorescence detection of the column effluent demonstrated the presence of TG deoxyribonucleoside 5'-monophosphate, derived from TG which was incorporated into the DNA in internucleotide phosphodiester linkage. Incorporation of the TG deoxyribonucleotide metabolite into DNA is responsible for the cytotoxic action of both TG and MP [6, 7, 8].



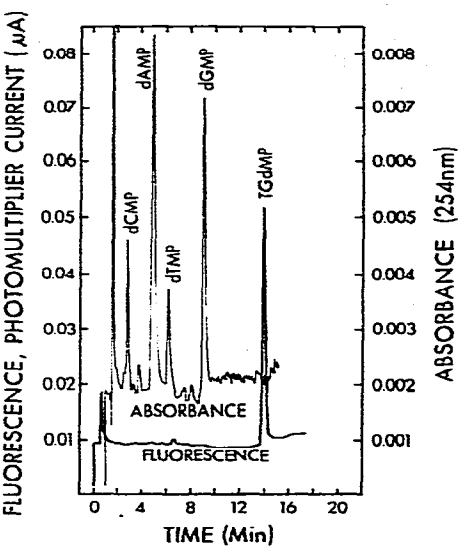


Fig.5. HPLC separation of an oxidized alkaline hydrolysate of the insoluble residue of acid extracted L5178Y cells exposed to TG. Simultaneous absorbance (254 nm) and fluorescence emission detection of the colum effluent. The data represent 1.20 × 10^5 cells exposed to 3 μM TG for 13 h. Abbreviations: TG2'MP, 6-thioguanosine 2'-monophosphate; TG3'MP, 6-thioguanosine 3'-monophosphate. The actual TG-related species separated are the 6-sulfonate derivatives of the indicated compounds. Separation conditions were the same as those for Fig. 1.

Fig.6. HPLC separation of an oxidized pancreatic deoxyribonuclease (DNase I) plus venom phosphodiesterase hydrolysate of DNA present in the acid insoluble residue following alkaline hydrolysis of L5178Y cells previously exposed to TG. Simultaneous absorbance (254 nm) and fluorescence emission detection of the column effluent. The data represent 2.14 × 10^5 cells exposed to 3 μM TG for 13 h. Abbreviations: TGdMP = 2'-deoxy-6-thioguanosine monophosphate. The actual TG-related compound separated is the 6-sulfonate of TGdMP. Separation conditions were the same as those for Fig. 1.

The procedures described in this paper were developed as a means of predicting thiopurine resistance in human leukemia, based upon the accumulation of TG nucleotides by blast cells either in vivo or in vitro. The data of Fig. 7 demonstrate the initial rates of TG nucleoside monophosphate formation from TG (50 μM) in vitro by freshly isolated leukemic cells of two patients with acute myelogenous leukemia. In these patients' blast cells, the extent of monophosphate accumulation from TG within 15 min was 25—50 times less than that observed with thiopurine sensitive cultured mouse leukemia L1210 and L5178Y cells (data not shown). In addition to possible differences in phosphoribosyltransferase activity, this lower nett rate of drug nucleotide synthesis was probably at least partially the result of a considerably higher dephosphorylation activity in the human vs. the mouse leukemic cells. When cells of patient 1 were washed free of drug at 15 min and incubated further at 37° in the absence of TG, intracellular TG nucleotide decayed rapidly and was essentially undetectable within 15 min. Therefore it is likely that during incubation with TG much drug nucleotide was degraded as soon as it was formed by the human cells, leading to the low overall rates of nucleotide accumulation. In this context, it is pertinent that a high activity of mem-

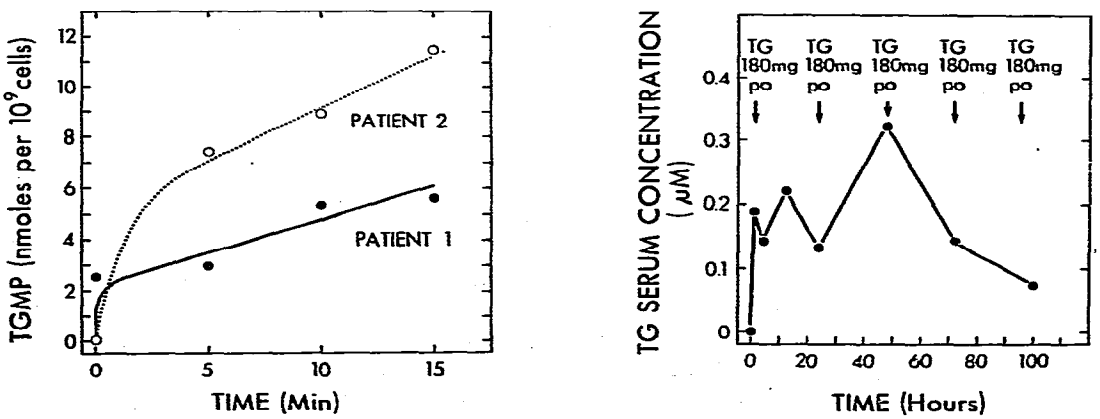


Fig.7. Initial rates of accumulation of TG nucleoside monophosphate, in vitro, by freshly isolated blast cells of human acute myelogenous leukemias. Cells (2 x 10^7 per ml, 2 ml per point) were incubated at 37° in MEM medium, supplemented with 15% fetal calf serum, and were exposed to TG (50 μM) for the indicated time intervals. Acid extracts of cell pellets were prepared, oxidized, and the 6-thioguanosine monophosphate (TGMP) separated and quantitated by HPLC with flow fluorescence detection. Separation conditions were the same as those for Fig. 1. Amounts of TGMP are expressed as nmoles TG equivalent, determined from a TG calibration curve using [8-^{14}C]adenine as internal standard for extract recovery.

Fig.8. Serum concentrations of TG in an acute myelogenous leukemic patient receiving oral TG. TG at the indicated dosage was administered at the times indicated by the arrows. Where the arrows and data points are coincident, the blood samples were taken immediately prior to administration of the drug. Each point represents the value determined from 30 μl of deproteinated serum which was oxidized and subjected directly to chromatographic separation without further manipulation. TG concentrations were determined from the area of the fluorescent oxidized TG peak detected in the column effluent, using a TG calibration curve. The chromatographic separation conditions were the same as those used to separate the nucleotides except that it was unnecessary to continue the gradients to completion.

brane bound alkaline phosphatase has been associated with thiopurine resistance in an animal model tumor system [10].

The HPLC—flow fluorometric methodology may also be used to measure concentrations of free TG in biological materials since TG is separated and quantitated in this system.

The serum levels of TG in an acute myelogenous leukemic patient receiving a course of oral TG are shown in Fig. 8. The data correspond to measurements on 30 μl of deproteinated serum, oxidized and applied directly to the chromatographic column without using any extraction or concentrative procedures. It can be seen that the serum concentration of TG fluctuated around 0.2 μM in this patient. Experiments with L5178Y cells in culture demonstrated that 0.2 μM TG was cytotoxic if cells were continuously exposed to this concentration for several days. The TG assay may be used routinely to evaluate a patient's capacity for maintaining an effective concentration of drug in the circulation during therapy. Such measurements, especially in conjunction with blast cellular drug nucleotide formation data, are of obvious value in determining the potential efficacy of an extended series of treatments with TG for individual patients. TG resistant disease might occur even in patients whose leukemic cells exhibited a high rate of TG nucleotide formation in vitro, if the drug was poorly absorbed or rapidly eliminated, with the result that cytotoxic plasma concentrations were not maintained throughout the period of treatment.

CONCLUSIONS

Through determination of TG nucleotide formation from MP or TG by leukemic blasts, our HPLC—flow fluorometric method may be used to predict resistance to MP or TG in human leukemia. In addition, predictions of both positive and negative responses to TG may possibly be made by combining cellular drug nucleotide determinations and TG serum concentration measurements during an initial course of therapy. We are currently evaluating the usefulness of these tests in the clinical management of human leukemia.

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REFERENCES

1 J.H. Burchenal, M.L. Tan, R.R. Ellison, M.P. Sykes, T.C. Tan, L.A. Leone, D.A. Karnofsky, L.F. Craver, H.W. Dargeon and C.P. Rhoads, Blood, 8 (1953) 965.
2 D.R. Hales, R.W. Jerner, B.E. Hall, F.M. Willet, J. Franco and T.V. Feichtmeir, Clin. Res. Proc., 5 (1957) 32.
3 R.R. Ellison and J.H. Burchenal, Clin. Pharmacol. Ther., 1 (1960) 631.
4 D. Kessel and T.C. Hall, Cancer Res., 29 (1969) 2116.
5 D.M. Tidd and A.R.P. Paterson, Cancer Res., 34 (1974) 733.
6 D.M. Tidd and A.R.P. Paterson, Cancer Res., 34 (1974) 738.
7 G.A. LePage, Cancer Res., 20 (1960) 403.
8 G.A. LePage, Cancer Res., 23 (1963) 1202.
9 J.M. Finkel, J. Pharm. Sci., 64 (1975) 121.
10 M.K. Wolpert, S.P. Damle, J.E. Brown, E. Sznycer, K.C. Agrawal and A.C. Sartorelli, Cancer Res., 31 (1971) 1620.