Comparison of in Vivo and in Vitro Effects of Continuous Exposure of L1210 Cells to 6-Thioguanine

Jonathan Maybaum, Catherine W. Morgans, and Laura A. Hink
Department of Pharmacology, Upjohn Center for Clinical Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0504

ABSTRACT

In this study the cytokinetic and antitumor effects of 12-h continuous treatment with 6-thioguanine (TG) were studied in L1210 cells in vivo and in vitro. Loss of clonogenicity in vitro was maximized at a drug concentration of 0.2 μM. Higher drug concentrations produced less cell kill, and the surviving fraction observed after exposure to 25 μM TG was 1 log higher than at 0.2 μM (2% versus 0.2% of control cloning efficiency, respectively). Delayed G2 arrest in vitro was also found to be most pronounced at 0.2 μM, with G2 arrest more predominant at higher concentrations.

Studies in vivo were conducted using C57BL × DBA/2 F1 mice, with or without advanced L1210 ascites tumor. In initial experiments performed on animals without tumor, the 50% lethal dose for 12-h s.c. infusions of TG was approximately 0.8 μmol/kg/min. Correlation of steady-state TG plasma levels with infusion rate revealed a linear relationship up to 0.62 μmol/kg/min, above which the TG plasma concentration increased disproportionately to input rate. Total body clearance of TG, calculated from the linear portion of this curve, was 123 ml/kg/min. The antimitotic effects of TG infusions were correlated with steady state plasma concentrations achieved in each individual animal, and it was found that dose rates yielding levels from 1 to 10 μmol increased survival time by about 40%, with no apparent optimum plasma level in this range. Examination of the cytokinetic effects caused by TG infusions at the low and high ends of this maximally therapeutic range showed that, as was the case in vitro, lower concentrations of TG caused delayed G2 arrest, while higher concentrations induced more rapid G2 arrest. On the basis of these, as well as previous findings, we propose that the operative mechanism of cell kill by TG in vivo may be dose dependent and may be reflected by the relative degree of G2 versus G1 arrest. We also suggest that the appropriate strategy for the clinical use of TG is to determine the drug concentration which produces maximum G2 arrest of tumor cells, and to infuse continuously at a rate to achieve that level for the maximum time tolerated by the patient, rather than to select an arbitrary length of infusion followed by escalation to maximum tolerated drug concentration.

INTRODUCTION

The thiopurines TG and MP have been in clinical use as antileukemic agents since the late 1950s. Although the biochemical disturbances caused by these agents are complex (reviewed in Ref. 1), it has usually been concluded that the events most closely associated with their cytotoxicity in vitro are depletion of purine nucleotides or incorporation into nucleic acids as TG. As is the case with most antimetabolites, the predominance of one of several potential cytotoxic mechanisms probably depends on many factors. For example, Tidd and Paterson (2) concluded that in L5178Y cells the major mechanism of both TG- and MP-induced cytotoxicity was incorporation into DNA. Nelson et al. (3) made a similar conclusion concerning the action of these agents in Hep 2 cells. In L1210 cells in vitro, however, Worthing and Rosi Roti (4) found that cells treated for 12 h experienced delayed G2 arrest (associated with effects of incorporation of the drug into DNA; Refs. 5 and 6), while continuous treatment with TG caused G1-S arrest (symptomatic of purine starvation). More recently it has been demonstrated in CHO cells that TG seems to be cytotoxic through DNA incorporation, while MP cytotoxicity is more likely due to purine starvation (6, 7). Although the incorporation of TG into RNA has been less intensively studied, this too may contribute to cytotoxicity (8). In view of the variability of the results regarding the major mechanism of thiopurine cytotoxicity in vitro, we concluded that the operative mechanism by which TG or MP acts in vivo cannot be inferred from in vitro experiments.

In an effort to gain insight into the in vivo mechanism of TG cytotoxicity we performed the present studies, in which mice bearing L1210 ascites tumor were infused with TG for 12 h, after which we determined TG plasma levels, survival time, and cytokinetic effects of the drug treatment on tumor cells removed from treated animals at various points after treatment. In so doing we were able to observe dose-dependent changes of tumor cell cycle distributions, which we interpret to reflect an apparent dose dependency of the operative cytotoxic mechanism. In addition, we provide information on the pharmacology of TG administered to tumor-bearing animals by continuous infusion, which has not been previously reported.

MATERIALS AND METHODS

Animals and Tumors. Male C57BL × DBA/2 F1 (hereafter called BD2F1) and DBA/2 mice, 42 days old, were obtained from Charles River Laboratories. The mouse L1210 ascites cells were obtained from the National Cancer Institute and were maintained in vitro by inoculating 4 DBA/2 mice each week with 106 cells from previously inoculated DBA/2 mice. L1210 cells carried in vitro were obtained from Dr. William Mancini. These cells were grown in RPMI 1640 with 10% horse serum at 37°C with a humidified 5% CO2 atmosphere.

Determination of TG Plasma Concentrations. During the last hour of infusion, blood samples were taken by cutting 0.25 inch off the tail of each mouse and collecting the blood in a 1 ml heparinized capillary tube. The tubes were spun for 5 min in a microcapillary centrifuge. The plasma was then collected and stored at −20°C until assayed for TG, using a modification of the method of Tidd and Dedhar (9). Samples were prepared as follows: 10 μl plasma, 80 μl H2O, and 10 μl 4 N HCl were vortexed in a microcentrifuge tube and then spun in a microcentrifuge for 30 s; 90 μl of the supernatant were transferred to a new microcentrifuge tube and extracted with 90 μl of amine-Freon solution (2.2 ml tri-n-octylamine plus 7.8 ml Freon) (10). Seventy μl of the top layer were transferred to a d-S arrest bearing LI210 ascites tumor were infused with TG for 12 h, after which we determined TG plasma levels, survival time, and cytokinetic effects of the drug treatment on tumor cells removed from treated animals at various points after treatment. In so doing we were able to observe dose-dependent changes of tumor cell cycle distributions, which we interpret to reflect an apparent dose dependency of the operative cytotoxic mechanism. In addition, we provide information on the pharmacology of TG administered to tumor-bearing animals by continuous infusion, which has not been previously reported.

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Determination of TG Plasma Concentrations. During the last hour of infusion, blood samples were taken by cutting 0.25 inch off the tail of each mouse and collecting the blood in a 100-μl heparinized capillary tube. The tubes were spun for 5 min in a microcapillary centrifuge. The plasma was then collected and stored at −20°C until assayed for TG, using a modification of the method of Tidd and Dedhar (9). Samples were prepared as follows: 10 μl plasma, 80 μl H2O, and 10 μl 4 N HCl were vortexed in a microcentrifuge tube and then spun in a microcentrifuge for 30 s; 90 μl of the supernatant were transferred to a new microcentrifuge tube and extracted with 90 μl of amine-Freon solution (2.2 ml tri-n-octylamine plus 7.8 ml Freon) (10). Seventy μl of the top layer were transferred to a new microcentrifuge tube and 70 μl each of 0.1 M NaHCO3 (pH 10.0) and 0.24% KMnO4 were added and, after a 10-min incubation at room temperature, 10 μl of 30% H2O2 were also added. The tube was spun for 30 s in a microcentrifuge to remove particulate matter and aliquots of the supernatant were analyzed by high performance liquid chromatography.

The oxidized TG product, TG sulfonate, was quantitated by reverse phase chromatography by using an ion-pairing reagent and fluorescence detection. A Regis VAL-U-PAK 5-μm octadecylsiline column was used with a mobile phase consisting of 5 mM NaH2PO4 plus 5 mM tetra-...
RESULTS

Cytotoxicity of TG to L1210 Cells in Vitro. Exposure of L1210 cells to TG for 12 h in vitro resulted in maximal loss of clonogenicity at 0.2 ¿M (Fig. 1). At higher concentrations cytotoxicity decreased, and exposure to 25 ¿M TG resulted in a surviving fraction 1 log higher than 0.2 ¿M (2% versus 0.2% of control cloning efficiency). Such apparently paradoxical relationships have previously been observed to be caused by TG (6, 12) and MP (13).

Fig. 1. Cytotoxicity of TG to L1210 cells in vitro. L1210 cells were exposed to the indicated concentrations of TG for 12 h, after which the drug was washed out and an appropriate number of cells were plated in semisolid medium. Colonies (ovy 0.5 mm in diameter) were counted 10–14 days later and are normalized to the cloning efficiencies of control cells. The plating efficiency of control cells was typically 50–70%.

Table 1 Toxicity of TG infusions

<table>
<thead>
<tr>
<th>Infusion rate (¿mol/kg/min)</th>
<th>Survivors/animals tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ¿mol/kg/min</td>
<td>4/4</td>
</tr>
<tr>
<td>0.62 ¿mol/kg/min</td>
<td>8/8</td>
</tr>
<tr>
<td>0.83 ¿mol/kg/min</td>
<td>7/15</td>
</tr>
<tr>
<td>1.04 ¿mol/kg/min</td>
<td>7/16</td>
</tr>
<tr>
<td>1.25 ¿mol/kg/min</td>
<td>0/4</td>
</tr>
</tbody>
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Fig. 2. Cytokinetic effects of TG on L1210 cells in vivo. Cells in suspension at an initial density of 5 x 10^5/ml were exposed to the indicated concentrations of TG for 12 h, at which time they were washed free of drug and allowed to grow in the absence of TG. Samples were taken at 0, 24, or 48 h after the end of drug treatment and were analyzed by flow cytometry as described. Histograms are shown with fluorescence (DNA content) on the abscissa and frequency on the ordinate axis.
Frequently observed symptoms of toxicity were weight loss, ruffled appearance, and facial swelling, occurring usually 4–7 days postinfusion. Rectal bleeding was occasionally seen. Necropsy of a TG-treated animal with facial swelling revealed septicemia and inflammation of the salivary glands.

Fig. 3 depicts the relationship between infusion rate and steady state plasma concentration of TG following administration to tumor-bearing mice. It appears that TG clearance is linear up to 0.62 μmol/kg/min. The only infusion rate studied which was higher than this, 0.83 μmol/kg/min, yielded plasma levels higher than expected from a linear system, suggesting that saturation may begin to occur at this level. By performing a linear regression on the points below 0.83 μmol/kg/min we calculate a total body clearance of 123 ml/kg/min. This value is approximately twice the hepatic blood flow of the mouse (14).

Antitumor Effects of Continuous TG Infusions. The ability of 12-h TG infusions to prolong survival of L1210-bearing mice is described in Fig. 4. Infusions resulting in plasma concentrations below 1 μM had variable effectiveness, prolonging the survival of some animals but not others. Increase in survival appears to be constant in the range of 1–10 μM, with an ILS of about 40%, and begins to decline above 10 μM.

Cytokinetic Effects of TG Infusions. Infusion rates of 0.13 and 0.62 μmol/kg/min were chosen to represent the low and high ends of the concentration range of maximally effective infusions. Cells obtained from mice infused with vehicle had a distribution typical of unperturbed exponentially growing cells (Fig. 5). This pattern was not drastically altered during the 2 days following infusion, although there did appear to be a decrease in the fraction of cycling cells at these times. Such a pattern is consistent with the tumor cells approaching plateau phase, as might be expected in mice within 24 hr of death. In mice treated with the lower drug concentration we saw no difference from control immediately after termination of the infusion. At 2 days postinfusion we observed a substantial shift of the population away from Go and S, and toward G2. This pattern of delayed G2 arrest is very similar to those seen previously in vitro (4–6). Cells isolated from the mice receiving the higher dose of TG did not undergo delayed G2 arrest. Rather, these cells exhibited G1 or G1-S arrest which was clearly apparent 1 day postinfusion and even more striking at 2 days postinfusion.

DISCUSSION

Relationship of Cytokinetic Disturbances to Mode of TG Action. Although it has been recognized since the early 1960s that purine nucleotide depletion and DNA incorporation are important consequences of TG treatment, the cytokinetic disturbances associated with these effects were first described by Baranco and Humphrey in 1971 (15) and, in more detail, by Wotring and Roti Roti in 1980 (4). The latter study indicated that the lethal action of TG in L1210 cells in vitro was associated with delayed G2 arrest, and presumably was related to incorporation of the analogue into DNA. It was also concluded in this study that a secondary, less lethal effect, was associated with relatively rapid G1/S phase arrest, and that this was likely to be due to purine nucleotide starvation. This interpretation is consistent with findings we subsequently made using the CHO cell line, where we concluded that cytotoxicity due to DNA incorporation of TG was accompanied by delayed G2 arrest and unilateral chromatid damage, while rapid G1-S arrest was associated with purine nucleotide depletion (in that case caused by MP, rather than TG) (6).

In the present study we were able to induce either delayed G2 arrest or G1-S arrest in L1210 cells in vitro, depending on the concentration of TG used (Fig. 2). We observed maximum G2 arrest at 0.2 μM TG, with G1-S arrest being predominant at higher concentrations. This result is quantitatively different from previous work (4), in which G2 arrest occurred at 10 μM, although the qualitative nature of the histogram is very similar. The discrepancy in drug concentrations may be attributable to properties of the particular sublines of L1210 cells used, or to differences in medium or serum components. Interestingly, the
drug concentration which caused maximum G2 arrest also induced the greatest loss of clonogenicity (Fig. 1). A pattern of cytotoxicity like that in Fig. 1, in which cytotoxicity peaks and then declines with increasing TG concentrations, was also seen by Christie et al. (12), who additionally determined that the gross extent of TG incorporation into cellular DNA responded similarly to cytotoxicity, reaching a maximum in the low micromolar region and then decreasing with higher TG concentrations. This finding further suggests that the relative significance of DNA incorporation and purine starvation, as well as the interaction between these effects, can vary with drug concentration. On the basis of all of these studies, we decided that the qualitative nature of cytokinetic perturbations (i.e., G1-S versus G2 arrest) would be the most reasonable parameter to use to assess the primary mechanism of thio-uracil action in vivo.

Pharmacology of Constant Infusions of TG. The in vitro studies mentioned above were performed such that cells were exposed to constant concentrations of TG for intervals close to one doubling time. In order to simulate this situation in vivo it was necessary to administer the drug by continuous infusion, rather than by single or multiple bolus injections, as has been done in previous studies. Since the pharmacology of TG delivered by extended infusion had not been characterized before, we began by defining the drug level producing lethal toxicity when infused for 12 h, which is similar to the doubling time of the L1210 cell line both in vitro and in vivo (16). The appearance of lethal toxicity occurred over a relatively narrow dose range, with a 50% lethal dose of about 0.83 μmol/kg/min (Table 1). The onset of this toxicity correlated with a break in the linearity of the relationship between infusion rate and steady state plasma concentration (Fig. 3). It is possible that at input rates above 0.62 μmol/kg/min the metabolism of TG approaches saturation, resulting in extremely high circulating levels of the drug.

Comparison of in Vivo and in Vitro Cytotoxic and Cytokinetic Effects of TG. The dose-response data given in Fig. 4 are unusual in that they relate increase in survival time to the actual plasma levels attained in each individual animal, as well as to dose rate. The antitumor effects of TG infusions are in accord with the observed pattern of in vitro cytotoxicity (Fig. 1), in that both ILS and loss of clonogenicity appear to plateau at drug concentrations in the vicinity of 1 μM. From the in vitro cytotoxicity data we would have predicted that the maximum antitumor effect in vivo would occur at a plasma level of about 0.2 μM. Although one of the longest surviving animals achieved such a plasma level, there is a high degree of variability in the survival of animals receiving low dose rates of TG, and there is no clear maximum of ILS corresponding to the maximum in vitro cytotoxicity seen in Fig. 1. The clonogenicity data do indicate, however, that the range of TG concentrations which produce maximum cell kill is very narrow. Therefore, one possible reason for the difference in this aspect between the in vivo and in vitro systems may be that, since in the animal experiments our blood samples are taken from a site physiologically removed from the tumor site, the drug levels measured in plasma may not be identical to those seen by the tumor cells. Also, we infused our animals 5 days after tumor inoculation, at relatively advanced stage of disease. In this circumstance there may be metastatic cells in physiologically privileged sites (e.g., central nervous system spaces) which are exposed to subeffective drug concentrations. It is also possible, of course, that unknown host factors may cause the dose-response relationship observed in vitro to be different or absent in vivo. Nonetheless, our observation that maximum antitumor effect is reached at TG doses well below those causing host toxicity, similar to that of Sartorelli and Booth (17), who used a 6-day series of bolus injections of TG, is of potential significance with respect to clinical administration of TG, as is discussed below.

The results in Fig. 5 demonstrate that, as seen in vitro, L1210 cells in vivo can be made to arrest either rapidly in G1 or in a delayed fashion in G2 as a result of TG treatment, and that the relative concentration dependence of these effects is similar to that in vitro. The degree of G2 arrest seen at the lower dose rate in vivo was less than that observed in vitro under conditions of maximum cell kill, which is consistent with the lack of a distinct range of plasma levels resulting in maximum antitumor effect, as discussed above. To our knowledge, this is the first characterization of the cytokinetic effects of TG in vivo.

The findings presented here may hold significance insofar as the clinical administration of TG is concerned, since they suggest that the usual approach to selecting a therapeutic regimen, i.e., increasing dose to the maximally tolerated level when given by an arbitrarily chosen schedule, may overshoot the most effective antitumor concentration in the case of TG. Rather, one would want to define the optimum tumoricidal concentration by in vivo and/or in vitro approaches (e.g., adjustment of dose rate to achieve maximum G2 arrest), and then extend the length of exposure to this drug concentration to tolerated limits. These results also suggest other ways in which the therapeutic efficacy of TG might be improved. For example, the observed total body clearance of TG in this study is approximately twice the liver blood flow in a mouse (14). This relatively rapid disposition makes TG a candidate for use by extended regional infusion (18). Since there have been some responses to TG reported in patients having tumors of colorectal origin (19), we intend to explore the use of TG given by hepatic artery infusion for the treatment of hepatic tumors. Another approach which might be useful is the combination of TG with appropriate concentrations of 4-amino-5-imidazolocarboxamide which we have previously shown to potentiate TG-induced cytotoxicity in vitro by as much as 1 log (6).

In summary, we have defined the toxicity, antitumor activity, and cytokinetic effects of TG when administered to tumor-bearing mice by continuous infusion. We find that the responses of these parameters in vivo are similar in many respects to
responses obtained in vitro, and we suggest that the administration of TG by continuous infusion, either systemically or regionally, at input rates aimed to achieve specific drug levels be considered in clinical trials.

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