INTRODUCTION

The interest generated by the imidazotetrazinone drug mitozolomide (1) (NSC 353451, Fig. 1) has been attributed to its potent activity against a number of murine tumor model systems, among them the L1210 and P388 leukemias, the TLX5 lymphoma, B16 melanoma, Lewis lung carcinoma, Colon 26 tumor and the M5076 sarcoma (2), and against xenografts from human melanoma, sarcoma, and lung and colon carcinomas (3). Clinical trials of mitozolomide are currently underway and preliminary results indicate that the dose-limiting toxicity is delayed thrombocytopenia (4). The results of these trials might eventually help to assess the value of the conventional tumor models as predictors for antitumor activity in the clinic. Chemical (5) and biochemical studies (6) suggest that the mode of action of mitozolomide is associated with its decomposition to the exceedingly reactive monochloroethyltriazene MCTIC. MCTIC is a powerful alkylating species with antitumor activity in its own right (7).

A large number of analogues of mitozolomide have been synthesized in order to help to understand which chemical features of the drug molecule are essential for its cytotoxic and antineoplastic properties. Recently the antitumor activity and cytotoxicity of mitozolomide derivatives have been reported in which the chloroethyl group in position 3 has been replaced by other alkyl moieties (8). Further chemical and biological studies have focused on the importance of substitutions in position 8 for cytotoxicity. This paper reports on observations which suggest that in analogues bearing a carboxamide group on carbon 8 of the imidazotetrazinone ring, a N-H moiety is required for maximum cytotoxicity. It is shown that the dimethylcarboxamyl analogue of mitozolomide (Fig. 1) was markedly less cytotoxic than mitozolomide and its monomethyl analogue (Fig. 1); nevertheless the antitumor activity of the three agents in mice bearing the TLX5 lymphoma has been reported to be very similar (2, 9). To explain this discrepancy the hypothesis has been tested that dimethylmitozolomide undergoes metabolism to a more cytotoxic analogue. In fulfillment of this objective three experimental avenues have been pursued: (a) The cytotoxicity of dimethyl and monomethyl mitozolomide have been measured in the presence of hepatic microsomes; (b) the in vitro metabolism of these compounds has been studied using HPLC analysis, in suspensions of microsomes; and (c) the murine pharmacokinetics of the mono and dimethyl analogues have been investigated to determine the in vivo exposure of solid tumor cells to the administered drug and its metabolite(s).
was added to the culture medium so that the final concentration of DMSO in the medium did not exceed 1%. Growth inhibition was calculated in cultures which were in linear growth phase. Cells were counted after 72 h using a Coulter Counter ZM (Coulter Electronics, Luton, UK).

Metabolism Studies. The preparation of liver microsomes was performed under aseptic conditions. Livers were excised from CBA/Ca mice, the strain used in the antitumor tests. Livers were homogenized in phosphate buffer (0.1 M, pH 7.4) or cell culture medium and microsomes prepared by differential centrifugation in the usual way (11) using an MSD Pegasus ultracentrifuge (MSD, UK). Microsomes were suspended in cell culture medium and added together with NADPH (0.5 μmol/ml) and magnesium chloride hexahydrate (0.325 μmol/ml) to sterile tubes (Sterlin, UK) containing 4 × 10⁷ cells. The final incubation volume was 5 ml and the tubes were capped and incubated under vigorous shaking at 37°C for 90 min. To ensure adequate oxygen supply the tubes were gassed for 1 min with 10% CO₂/air at 20-min intervals. During the incubation the pH did not vary by more than 1.5%. Preliminary experiments showed that a concentration of microsomes equivalent to 50 mg of liver per milliliter was optimal, and this concentration was used in the experiments described here. At the end of the incubation period cells were centrifuged, resuspended, seeded, and incubated as described under “Cytotoxicity Assay.”

Stability Studies. The stability of the drugs in RPMI 1640 and deactivated microsomes was investigated by incubation over a period of 90 min with sampling every 15 min. The incubation conditions in RPMI 1640 were as described under “Cytotoxicity Assay.” In the experiments with deactivated microsomes, microsomes were inactivated by heating at 95°C for 10 min and then incubated under identical conditions to those described under “Metabolism Studies.”

Pharmacokinetic Studies. Drugs were administered as detailed above and blood samples obtained as previously described (8). Quality control procedures ensured that the dosing accuracy was within acceptable limits. At least four samples were obtained for each time point. Values for the elimination rate constants were estimated from the linear regression analysis of the plots of the logarithm of the mean plasma concentration versus time. AUC values were obtained using the trapezoidal rule over 0–2.5 h. The clearance was obtained by dividing the dose by the AUC.

Chromatographic Analysis. For the analytical determination, samples (0.5 ml) were removed from the microsomal incubation mixtures at appropriate time intervals and immediately transferred into 0.5 ml HCl (1.0 M) to prevent further decomposition of compounds. Compounds were extracted with ethyl acetate and analyzed by HPLC using a Waters 840 Data system controller, a Waters 510 pump, a WISP 710B, and a 480 UV detector. The details of the extraction procedure and the HPLC assay were similar to those described previously for mitozolomide. The points are the mean ± SD of three separate experiments.

Table 1 Cytotoxicity of mitozolomide and its methyl and dimethyl derivatives against TLX5 lymphoma cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>ID₅₀ (μM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitozolomide</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Methylmitozolomide</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>Dimethylmitozolomide</td>
<td>14.6 ± 1.1</td>
</tr>
</tbody>
</table>

* Values are the mean ± SD of three separate experiments.

RESULTS

The cytotoxic potential of dimethylmitozolomide towards TLX5 cells was lower than that of mitozolomide with a ratio of IC₅₀ (dimethylmitozolomide)/IC₅₀ (mitozolomide) of 6.4. Methylmitozolomide was almost as cytotoxic as mitozolomide (Table 1). In contrast, the antitumor efficacy of the three derivatives against the TLX5 lymphoma in vivo has been reported previously to be comparable (9). In order to investigate whether this difference was due to metabolic activation of dimethylmitozolomide, TLX5 cells were incubated with the agent in the presence or absence of mouse liver microsomes and NADPH. At the end of the incubation period cells were seeded and their growth was measured. Fig. 2A shows that dimethylmitozolomide was much more cytotoxic in the presence of microsomes than in their absence. This bioactivation was dependent on the addition of NADPH to the microsomal incubation mixture (Fig. 2B). Neither mitozolomide (Fig. 2C) nor its monomethyl analogue (Fig. 2B) were bioactivated under these conditions.

The rate of disappearance from microsomal suspensions was determined for dimethyl and monomethylmitozolomide and mitozolomide. Figs. 3 and 4 summarize the analytical chemical data which suggests also that dimethylmitozolomide underwent rapid metabolism in suspensions of hepatic microsomes. HPLC analysis (Fig. 3) afforded a peak which coeluted with methyl-DIMETHYLMITOZOLOMIDE BIOACTIVATION
mitozolomide. This peak appeared almost immediately after addition of the drug to the incubate and its maximal concentration was observed after incubation for 45 min (Fig. 4). In samples of the microsomal suspension obtained within 30 min of the incubation period small amounts of two other metabolites, X & Y, were eluted from the column. However collection and concentration of the eluate did not yield sufficient material for mass spectral characterization. One of these metabolites, X, was also produced following incubation of mitozolomide with heat-inactivated microsomes. On incubation with heat-inactivated microsomes, or in the absence of NADPH, monomethylmitozolomide and the other metabolites were not formed. In further experiments the rate of disappearance from microsomal suspensions was determined for dimethyl and methylmitozolomide as well as mitozolomide. The half-life values obtained (Table 2) were similar suggesting that there is no inherent difference in stability between the metabolites and the parent compound. A similar investigation was carried out with methylmitozolomide. The time course of its disposition is illustrated in Fig. 5. Methylmitozolomide was absorbed rapidly and had an elimination half-life of 0.16 h with a plasma AUC of 4.09 mg-h/liter. This compound was rapidly distributed to the tumor with a maximum concentration of 8.01 μg/g achieved at 0.17 h and a tumor-tissue AUC of 3.77 μg·h/g.

Following i.p. administration of dimethylmitozolomide the drug was rapidly absorbed and had an elimination half-life of 0.15 h with a plasma AUC of 2.34 mg-h/liter and a tumor AUC of 2.38 μg·h/g. The tumor exposure to the monomethyl metabolite was similar to that observed on i.p. administration of monomethyliermitolamide with a maximum concentration of 6.09 μg/g achieved 0.34 h following administration of the dimethylmitozolomide. The tumor AUC of the monomethyl metabolite was 4.55 μg·h/g which was slightly larger than that achieved on administration of the monomethyliermitolamide itself. By comparison of the AUC and clearance values (13) obtained for both mono- and dimethylmitozolomide it was found that 89% of the dimethylmitozolomide was metabolized via the monomethyl derivative.

Table 2 Chemical stability of mitozolomide and its methyl and dimethyl derivatives in RPMI, deactivated microsomes and in a metabolically active microsomal preparation

<table>
<thead>
<tr>
<th>Compound</th>
<th>Half-life (min)</th>
<th>RPMI</th>
<th>Deactivated microsomes</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitozolomide</td>
<td>34</td>
<td>43</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Methylmitozolomide</td>
<td>44</td>
<td>47</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Dimethylmitozolomide</td>
<td>46</td>
<td>57</td>
<td>55</td>
<td></td>
</tr>
</tbody>
</table>

* Values are the mean of two experiments.

It was considered possible that the increase in cytotoxicity of dimethylmitozolomide seen in the presence of microsomes was at least partially due to the formaldehyde generated as the ultimate oxidation product of the dimethylmitozolomide N-methyl moiety. To test this hypothesis the cytotoxicity of formaldehyde against TLX5 cells was measured. The growth of the cells was not affected by exposure for 90 min to 10 nmol/ml formaldehyde, the maximal concentration theoretically achievable by metabolic N-demethylation of 28% of the dimethylmitozolomide (Fig. 4).

In order to test the hypothesis that N-demethylation of dimethylmitozolomide was an important route of metabolism in vivo, plasma and tumor samples were analyzed from mice which had received dimethylmitozolomide. Methylmitozolomide was indeed found as a metabolite in plasma and tumor tissue and Fig. 5 shows the time course of the appearance of the monomethyl metabolite together with the disposition of the parent compound. A similar investigation was carried out with methylmitozolomide. The time course of its disposition is illustrated in Fig. 6. Methylmitozolomide was absorbed rapidly and had an elimination half-life of 0.16 h with a plasma AUC of 4.09 mg·h/liter. This compound was rapidly distributed to the tumor with a maximum concentration of 8.01 μg/g achieved at 0.17 h and a tumor-tissue AUC of 3.77 μg·h/g.

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DISCUSSION

The chemical synthesis and biological testing of analogues of a novel experimental agent aids antitumor drug development in at least two ways: (a) As a strategy to find derivatives with a better therapeutic ratio than that of the original molecule, and (b) as a way to increase the understanding of the chemical features which impart antineoplastic activity or cytotoxicity on the molecule. The experiments described here have been conducted on analogues of mitozolomide which are themselves unlikely to constitute a therapeutic advantage over mitozolomide. However, the results suggest that these compounds, the mono- and dimethylcarboxamido derivatives, help to identify a structural characteristic of the mitozolomide molecule which determines its cytotoxic properties. The replacement of both hydrogen atoms by methyl substituents in the 8-carboxamido function of the molecule affords a compound with reduced cytotoxic potential. Nevertheless, dimethylmitozolomide possesses antitumor activity in vivo comparable to that of mitozolomide. The following results suggest that this is due to metabolic activation of dimethylmitozolomide: (a) unlike mitozolomide, or the monomethyl derivative, dimethylmitozolomide was rapidly metabolized by microsomes to a more cytotoxic species; (b) the major metabolite of dimethylmitozolomide, in vivo and in vitro, was monomethylmitozolomide, the cytotoxicity of which is comparable to that of mitozolomide. Dimethylmitozolomide was an excellent substrate for the microsomal metabolizing enzymes, as N-demethylation was rapid and extensive. The dependence of the bioactivation reaction on the presence of NADPH suggests that the enzymes involved belong to the family of the cytochrome P450 monoxygenases. The pharmacokinetic investigations indicate that dimethylmitozolomide is probably acting as a prodrug for the monomethyl derivative as the solid tumor had a greater exposure to monomethylmitozolomide following administration of the dimethyl derivative as the solid tumor had a greater exposure to monomethylmitozolomide following administration of the dimethyl derivative as the solid tumor had a greater exposure to monomethylmitozolomide following administration of the dimethyl

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Fig. 7. Proposed scheme for the metabolic activation of dimethylmitozolomide.

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Metabolism and Murine Pharmacokinetics of the 8-(N,N-Dimethylcarboxamide) Analogue of the Experimental Antitumor Drug Mitozolomide (NSC353451)

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