Cancer Chemother Pharmacol (1991) 27: 342–346

0344570491000185

Cancer Chemotherapy and Pharmacology

Comparison of the cytotoxicity in vitro of temozolomide and dacarbazine, prodrugs of 3-methyl-(triazen-1-yl)imidazole-4-carboxamide*

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Received 1 August 1990/Accepted 7 November 1990

Summary. The present study tested the hypothesis that the experimental antineoplastic imidazotetrazinone temozolomide degrades in the biophase to 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC) and exerts its cytotoxicity via this species. MTIC is a metabolite of the anti-melanoma agent dacarbazine and is thought to be responsible for the antineoplastic activity of the latter. Cytotoxicity in vitro was investigated in TLX5 murine lymphoma cells. MTIC and temozolomide were cytotoxic in the absence of mouse-liver microsomes, whereas dacarbazine required metabolic activation. The generation of MTIC from either dacarbazine, its primary metabolite 5-[3-(hydroxymethyl)-3-methyl-triazen-1-yl]-imidazole-4-carboxamide (HMMTIC) or temozolomide was studied by reversed-phase high-performance liquid chromatography in incubation mixtures under the conditions of the cytotoxicity assay. MTIC was found in incubations of temozolomide with or without microsomes. Dacarbazine yielded MTIC (and HMMTIC) only when microsomes were included in the incubation mixture. Although the mode of action of temozolomide seems to be similar to that of dacarbazine, the results obtained in this study show that these agents differ markedly in their ability to generate the active species MTIC.

Introduction

Temozolomide (8-carbamoyl-3-methyl-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one, CCRG 81045, M&B 39831; Fig. 1) is an antineoplastic imidazotetrazinone that is structurally related to both the experimental antitumour agent mitozolomide and the clinically used drug dacarbazine (5-[3,3-dimethyl-triazen-1-yl]imidazole-4-carboxamide, DTIC; Fig. 1). Dacarbazine is thought to exert its cytotoxicity via 5-(3-methyl-triazen-1-yl)imidazole-4-carboxamide (MTIC, Fig. 1) generated by metabolic hydroxylation of the *N*-methyl moiety of dacarbazine to afford 5-[3-(hydroxymethyl)-3-methyl-triazen-1-yl]-imidazole-4-carboxamide (HMMTIC, Fig. 1) with subsequent loss of formaldehyde (for a review see [5]). The mechanism by which temozolomide causes cytotoxicity is unknown, but it has been postulated that MTIC is involved, as it is almost certainly a product of the pH-dependent chemical decomposition of temozolomide [1, 12, 13].

In the present study the following two hypotheses concerning the relationship between temozolomide and MTIC were tested: (1) MTIC is generated from temozolomide in biological mileaux, and (2) MTIC is responsible for the in vitro cytotoxicity of temozolomide. We also wished to compare the cytotoxic potency of temozolomide with that of dacarbazine after metabolic activation. The cytotoxicity of temozolomide, dacarbazine and MTIC towards murine TLX5 lymphoma cells was investigated in the presence and absence of mouse-liver microsomes. The generation of MTIC from temozolomide and dacarbazine was followed by HPLC analysis. The overall aim of this study was to contribute to the knowledge that might eventually help judge whether there is a therapeutic advantage associated with the administration of temozolomide as compared with dacarbazine.

Materials and methods

Compounds. Temozolomide was kindly supplied by May and Baker Ltd. (Dagenham, UK). The following compounds were synthesised according to published methods: dacarbazine and MTIC [9, 10] in our laboratories, and HMMTIC [7] by Drs. K. Vaughan and R. LaFrance (Department of Chemistry, St. Mary's University, Halifax, Nova Scotia, Canada). Drugs were dissolved in dimethylsulphoxide (DMSO) before their addition to

* Part XXIII in the series: Antitumour imidazotetrazines (for part XXII, see Tsang et al. [15])

Abbreviations: AUC, area under the curve; HMMTIC, 5-[3-(hydroxymethyl)-3-methyl-triazen-1-yl]imidazole-4-carboxamide; HPLC, high-performance liquid chromatography; MTIC, 3-methyl-(triazen-1-yl)imidazole-4-carboxamide

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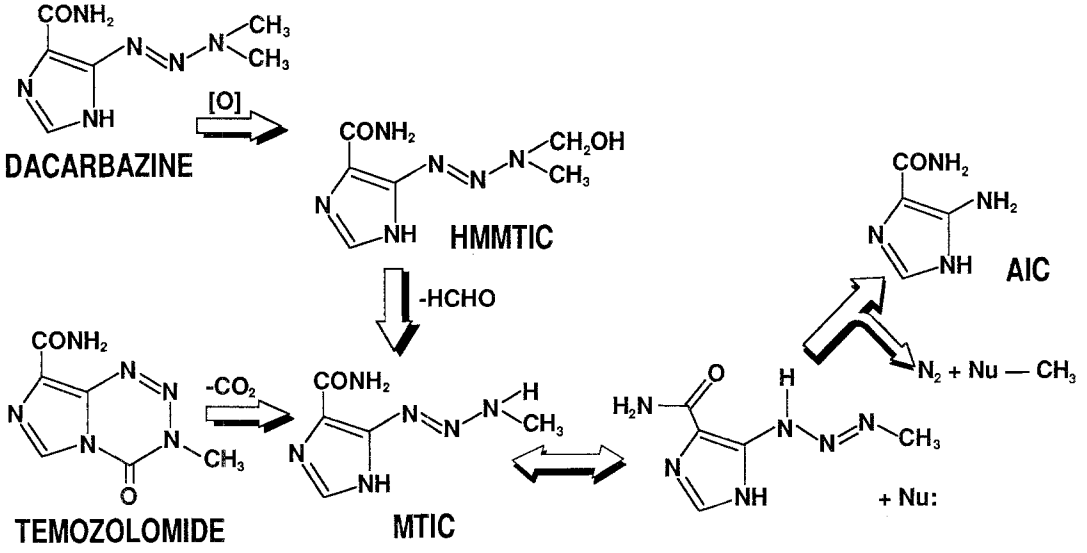

Fig. 1. Pathway of decomposition of temozolomide and route of metabolism of dacarbazine

cell-culture medium, the final concentration of DMSO not exceeding 1%.

Metabolism studies. Mouse-liver microsomes were isolated and used as previously reported [6]. Procedures were conducted under aseptic conditions. Male CBA/Ca mice (18–22 g, obtained from Bantin and Kingman Ltd., Hull, UK) were used as the source of liver. Animals were kept for 1 week prior to their use, were fed Heygate modified 41B pellets and allowed access to water ad libitum. Mice were killed by cervical dislocation and livers were excised and homogenised in cell-culture medium. Microsomes were prepared by differential centrifugation in the usual way using a MSE Pegasus ultracentrifuge. Microsomes (equivalent to 50 mg liver/ml) or heat-inactivated microsomes (10 min, 90° C), and TLX5 cells (4×10^4 ml) were incubated in Sterilin tubes (Sterilin, Feltham, UK) with magnesium chloride (0.33 mM) and in the presence or absence of reduced nicotinamide adenine dinucleotide phosphate (NADPH, 0.5 mM) and triazene derivative. In control experiments triazenes were replaced by cyclophosphamide. The final incubation volume was 5 ml. The tubes were capped and incubated with gentle shaking at 37° C for 60 min. At 15-min intervals, mixtures were gassed for 1 min with air containing 10% CO_2. During the incubation period the pH of the medium did not change by more than 2%.

Cell culture and cytotoxicity assay. The TLX5 lymphoma was routinely passaged in CBA/Ca mice as previously described [13]. For in vitro culture, ascites cells were harvested from animals and rinsed with cell-lysis buffer [3]. Cells were routinely cultured in RPMI 1640 medium supplemented with 17% horse serum at 37° C under air: CO_2 (9:1, v/v). The cells used in these experiments had been subcultured between 10 and 20 times after their removal from the animals; these cells are known to retain their tumourigenic potential in vivo when reinjected into mice [2]. The doubling time of these cells was 14 ± 2 h. Cells were incubated with each compound in either the presence or the absence of microsomes. At the end of the incubation period, cells were pelleted by centrifugation, resuspended in fresh medium and seeded in Nunclon 24-well dishes (Gibco, Paisley, UK). After 72 h, cells were counted using a Coulter Counter ZM (Coulter Electronics, Luton, UK). Growth potential was assessed in cultures that were in the linear growth phase.

Sample preparation and HPLC analysis. An aliquot (0.1 ml) of the incubation mixture was placed in a centrifuge tube to which chilled acetonitrile or a mixture of acetonitrile/methanol (0.2 ml) containing the internal standard (see below) had been added. Samples were mixed and protein was removed by centrifugation (2,500 rpm) at –20° C for 10 min. The supernatant was injected onto the HPLC column. Chromatographic analysis, which was partially based on a method published previously [11], was performed on a Waters system consisting of the Waters 840 Data System, an M510 solvent pump and a Lamda max 480 variable wavelenght detector (Waters Chromatography Division, Millipore UK Ltd., Watford, UK). Samples were injected using a 0.02-ml injection loop. Compounds were detected on the basis of their UV absorbance at 323 nm.

MTIC and temozolomide were separated using a 12.5-cm Lichrosorb C18 RP select B column with a Lichrosorb guard column and a mobile phase containing 5% acetonitrile in 0.05 M ammonium acetate (pH 6.7), which was pumped through the column at a flow rate of 1.5 ml/min; hydroxyethylbenzotriazinone was used as the internal standard. Separation of MTIC, HMMTIC and dacarbazine was achieved using a column similar to that described above but with a length of 25 cm. The mobile phase in this instance was 3.25% acetonitrile/1.75% methanol in 0.05 M ammonium acetate (flow rate, 1.5 ml/min); metronidazole was used as the internal standard. Recoveries were as follows: temozolomide, $93\% \pm 10\%$; MTIC, $96\% \pm 9\%$; dacarbazine, $90\% \pm 7\%$; HMMTIC, $94\% \pm 8\%$ (mean $\pm$ SD, $n = 6$ in each case).

The reproducibility of the HPLC assay used was investigated using six samples for each compound. The coefficients of variation were as follows for within-day and between-day reproducibility, respectively: 20.3 mg/l temozolomide, 12% and 12%; 2.5 mg/l tenozolomide, 9% and 10%; 20.4 mg/l MTIC, 16% and 15%; 2.6 mg/l MTIC, 14% and 18%; 20.3 mg/l HMMTIC, 13% and 14%; 2.5 mg/l HMMTIC, 15% and 17%. The coefficients of variation for replicate injections of one sample each of temozolomide, MTIC or HMMTIC were 2%, 4% and 5%, respectively ($n = 6$). The limit of detection for these compounds was between 0.2 and 0.3 mg/l, equivalent to 4–6 ng on the column. The compounds in the samples prepared for analysis were stable for at least 8 h at –15° C.

Results

Cytotoxicity

The cytotoxic potential of temozolomide, dacarbazine and its metabolites MTIC and HMMTIC was investigated in TLX5 lymphoma cells in the absence or presence of mouse-liver microsomes (Fig. 2). The viability of the microsomal preparation used was tested in parallel experiments by assessment of the ability of microsomes to biotransform cyclophosphamide to cytotoxic species. Growth of cells that had been exposed to cyclophosphamide (5 mg/l) for 1 h in the presence of microsomes was decreased by $87\% \pm 7\%$ (mean $\pm$ SD, $n = 4$) as compared with cells that had been incubated with cyclo-

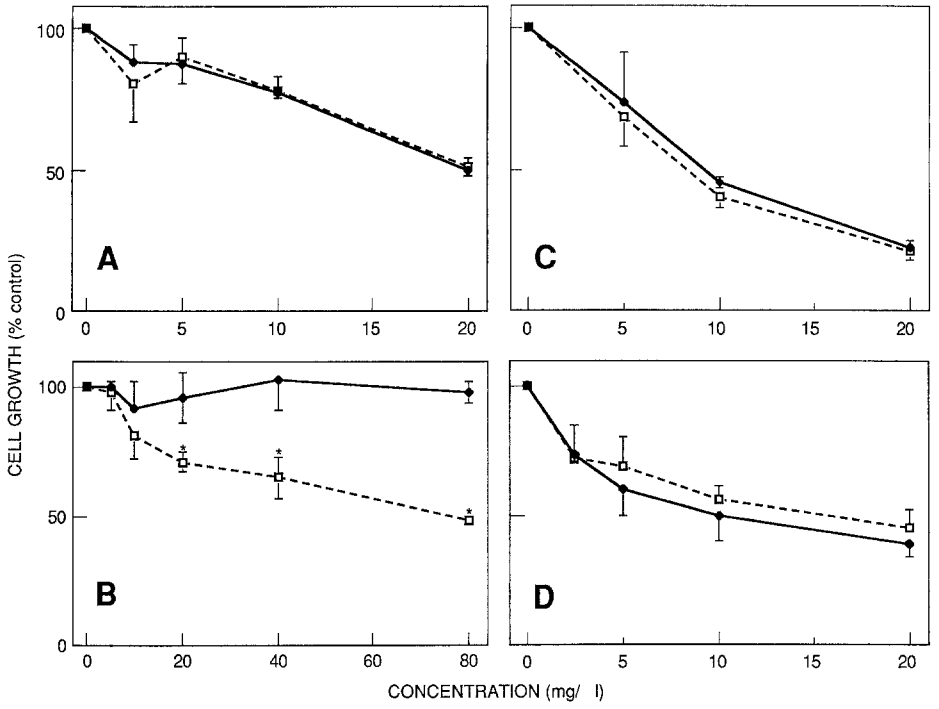

Fig. 2 A–D. Cytotoxicity of **A** temozolomide, **B** dacarbazine **C** HMMTIC and **D** MTIC against murine TLX5 lymphoma cells in the presence (*open squares*) or absence (*closed rhombi*) of microsomes. Incubation conditions are described in Materials and methods. Values represent the mean ± SD of 4–12 experiments. The asterisk indicates a significant difference (P <0.001, Student's t-test) between the presence and the absence of microsomes

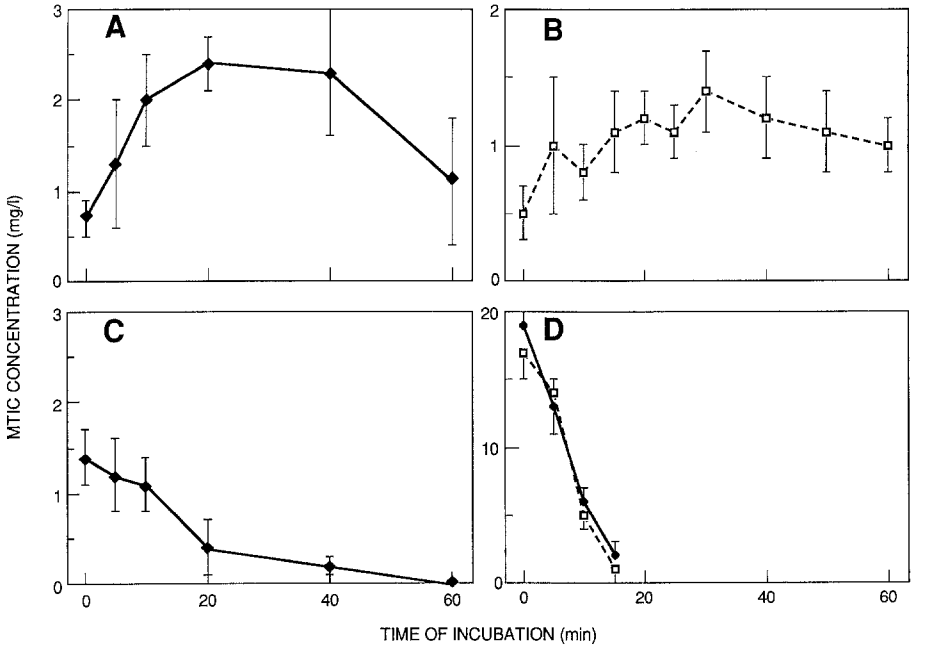

Fig. 3 A–D. Time course of the appearance and disappearance of MTIC from cellular incubates with **A** temozolomide (20 mg/l), **B** dacarbazine (40 mg/l) **C** HMMTIC (20 mg/l) or **D** MTIC (20 mg/l). Microsomes were absent in **A** and **C** but were present in **B**. Results obtained in incubates of temozolomide or HMMTIC with microsomes were indistinguishable from those shown in **A** and **C**, respectively. When dacarbazine was incubated without microsomes, MTIC was not detected. Values in **D** were obtained in the presence (*open squares*) or absence (*closed rhombi*) of microsomes. Values represent the mean ± SD of 4 experiments. For details of microsomal incubations, see Materials and methods

phosphamide in the absence of microsomes. Likewise, the cytotoxicity of dacarbazine was markedly increased in the presence of microsomes (Fig. 2B). In contrast, microsomes did not affect the cytotoxic potency of temozolomide, HMMTIC or MTIC (Fig. 2A,C,D). The cytotoxicity caused by dacarbazine and cyclophosphamide was dependent on the presence of NADPH in the mixture and was abolished when viable microsomes were replaced by heat-inactivated ones.

These findings are consistent with the involvement of hepatic mixed-function oxygenases in the bioactivation of these drugs. It is unlikely that the observed cytotoxicity of dacarbazine was due to metabolically generated formaldehyde, because the growth of the cells was not retarded by exposure to formaldehyde at concentrations of up to 0.132 mM; this formaldehyde concentration would be achieved if 24% of the dacarbazine at the highest concentration (0.55 mM) were metabolically N-demethylated.

Degradation and metabolism

To find out whether MTIC was generated in incubations of temozolomide, dacarbazine or HMMTIC, we analysed extracts of incubation mixtures with or without microsomes by HPLC. MTIC was found in incubation mixtures of

temozolomide in both the presence and the absence of microsomes (Fig. 3A). The rate of decomposition of temozolomide was not affected by the presence of microsomes, the decomposition half-life of the drug being 50 ± 4 and 52 ± 5 min (mean $\pm$ SD, $n = 3$) in the presence and absence of microsomes, respectively. However, in incubates with dacarbazine, MTIC was detected only when microsomes were included (Fig. 3B). An additional peak in the chromatogram of the microsomal incubate of dacarbazine coeluted with HMMTIC.

The generation of HMMTIC and MTIC from dacarbazine was dependent on the presence of viable microsomes and NADPH in the incubate and air in the atmosphere above the incubate (data not shown). Authentic HMMTIC and MTIC degraded identically with or without microsomes. HMMTIC decomposed at a half-life of 16 ± 2 min (mean $\pm$ SD, $n = 3$) and generated MTIC (Fig. 3C). HMMTIC was more stable than MTIC, which exhibited a decomposition half-life of 5.5 ± 0.5 min (mean $\pm$ SD, $n = 4$; Fig. 3D). It must be stressed that inferences as to the identification of MTIC and HMMTIC as metabolites or degradation products of dacarbazine and temozolomide were made only on the basis of co-chromatography using authentic material; in view of their considerable chemical instability, attempts to isolate and characterise the products further were not made.

TLX5 cells were incubated with either MTIC, temozolomide, dacarbazine or HMMTIC, and MTIC concentrations were measured at several intervals up to 1 h. AUC values were computed from the MTIC concentration-versus-time curves using the trapezoidal rule and were plotted against the extent of cytotoxicity observed under identical incubation conditions. In view of differences in the chemical nature of the different cytotoxicants under study, conclusions reached from this kind of plot must be drawn with caution. Nevertheless, inhibition of cell growth seemed to be correlated with the AUC of MTIC, except in the case of incubations with HMMTIC (data not shown). HMMTIC appeared to possess either intrinsic cytotoxicity or toxicity mediated by species other than or in addition to MTIC.

Discussion

Temozolomide has been postulated to be a prodrug of MTIC because it can undergo chemical decomposition to MTIC [13]. The results obtained in the present study support this postulate; they show that MTIC is generated from temozolomide in the biophase. MTIC is thought to be the major cytotoxic metabolite of dacarbazine (for a review see [16]). The present results render it likely that MTIC is responsible for, or contributes to a major part of, the cytotoxic potential of temozolomide.

The antitumour activity of temozolomide observed in several murine tumour models has been reported to be comparable with or, in the case of L1210 and P388 leukaemias and B16 melanoma, superior to that of dacarbazine [13]. Like the monomethyltriazene 1-*p*-cyanophenyl-3-methyltriazene, temozolomide can induce terminal differentiation in K562 cells [14]. Furthermore, a L1210 line with acquired resistance to dacarbazine has been shown to be cross-resistant to temozolomide [13]. These results suggest a commonality of mechanism of action between temozolomide and dacarbazine.

The results of the in vitro cytotoxicity study presented herein are consistent with the suggestion that dacarbazine requires metabolic activation [4, 16]. By HPLC analysis we showed that under physiological conditions, temozolomide and dacarbazine are transformed to MTIC by chemical decomposition (temozolomide) and by oxidative metabolism (dacarbazine). It is therefore probable that MTIC plays an important role in the antineoplastic activity of both of these drugs. Intriguingly, the cell-killing ability of HMMTIC appeared to exceed that expected on the basis of its ability to generate MTIC. This result suggests that HMMTIC might well contribute substantially to the overall cytoxicity of dacarbazine in vivo. Although the concentrations of formaldehyde produced as a product of HMMTIC degradation were unlikely to be intrinsically cytotoxic, they might possibly exacerbate the cytotoxicity of the methylating agent MTIC.

There is a substantial difference between humans and rodents in their ability to catalyse the oxidative N-demethylation of dacarbazine, mice being far superior in the rate at which they metabolise the drug [8]. This difference might provide an explanation for the observed difference between species in response to this agent. If the generation of MTIC, or of MTIC and HMMTIC in the case of dacarbazine, is indeed an essential determinant of the antitumour activity of temozolomide and dacarbazine, one could argue that temozolomide might provide a pharmacokinetic advantage over dacarbazine in that the generation of the cytotoxic monomethyltriazene from the former does not require metabolism. In other words, one might be able to achieve levels of cytotoxic triazene species in a more predictable fashion using temozolomide.

In a very preliminary study in vivo of the pharmacokinetic properties of temozolomide in mice bearing the TLX5 lymphoma, the drug was shown to be extensively distributed into tumour tissue (Tsang et al., unpublished). MTIC was detected in plasma but not in tumour tissue of mice that had received temozolomide. The lack of detectability of MTIC in the tumour is probably due to the breakdown of MTIC during the manipulation of tissue before extraction. Nevertheless, it is likely that temozolomide is degraded to MTIC in the tumor as demonstrated in cell-culture medium in the present in vitro study. Whether the treatment of humann malignancies using temozolomide actually offers an advantage over dacarbazine therapy remains to be shown.

Acknowledgements. This work received generous financial support from the Cancer Research Campaign of Great Britain (programme grant SP1518). L. L. H. T. was the recipient of an Overseas Research Student Award. We thank Drs. K. Vaughan and R. LaFrance (Department of Chemistry, St. Mary's University, Halifax, Nova Scotia, Canada) for the gift of HMMTIC and Drs. D. R. Newell, B. Foster and C. Rutty (Institute of Cancer Research, Sutton, Surrey) for their advice and helpful discussions.

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