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Characterisation of urinary metabolites of temozolomide in humans and mice and evaluation of their cytotoxicity*

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Summary. The experimental antineoplastic agent temozolomide was not metabolised in vitro at a measurable rate by mouse liver fractions. In contrast, the temozolomide analogue 3-methylbenzotriazinone was metabolically *N*-demethylated by hepatic microsomes to yield benzotriazinone. The major route of excretion of [^{14}C]-labelled temozolomide in mice was via the kidneys. An acidic metabolite of temozolomide, probably a conjugate, was found in the urine of mice, but its identity could not be established unambiguously. Spectroscopic analysis and chemical tests revealed that it possesses an intact NNN-linkage. Another metabolite was found in the urine of patients but not of mice. This metabolite was identified as the 8-carboxylic acid derivative of temozolomide. Unlike the unknown species, this metabolite was cytotoxic against TLX5 lymphoma cells in vitro.

Introduction

Temozolomide (CCRG 81045, M & B 39831, 8-carbamoyl-3-methyl-imidazo-[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one; for structure see Fig. 1), the 3-methyl analogue of mitozolomide, is an experimental antineoplastic agent with activity against a broad spectrum of murine tumours [18]. The drug is currently under clinical evaluation in the United Kingdom [15]. Temozolomide is directly cytotoxic [18], but little is known about its mechanism of action. In the biophase it decomposes chemically to MTIC (Tsang et al., unpublished), the cytotoxic metabolite via which the antimelanoma agent dacarbazine is thought to exert its antineoplastic activity [12, 19]. Thus, it is possible that MTIC mediates, or contributes to, the cytotoxicity of temozolomide.

As the metabolism of temozolomide is unknown, we searched for the presence of metabolites in the urine both of mice that had received temozolomide and of patients who took part in a phase I clinical trial of the drug. Knowledge of the chemical nature and biological properties of metabolites of experimental therapeutic agents is desirable, even if they, like temozolomide, do not require metabolic activation, because it might help in the interpretation of pharmacokinetic and pharmacodynamic properties of the drug. Eventually such information can be used in the rational planning of clinical trials.

The temozolomide molecule contains an *N*-methyl moiety; thus, one might expect the drug to undergo metabolic oxidative *N*-demethylation [11], conceivably a deactivation step. In the present study we tested the hypothesis that temozolomide undergoes this metabolic pathway. To that end we compared the susceptibility towards *N*-methyl oxidation by hepatic microsomal enzymes in vitro of temozolomide with that of 3-methyl-benzotriazinone (Fig. 1), a cyclic aryltriazene that is structurally related to temozolomide.

Materials and methods

Compounds. Temozolomide and [methyl-^{14}C]temozolomide were supplied by May and Baker, Ltd. (Dagenham, UK). 3-Methyl-benzotriazinone, benzotriazinone [16] and 3-methyl-2,3,dihydro-4-oxoimidazo[5,1-*d*]tetrazine-8-carboxylic acid [8] were synthesized according to published methods.

In vivo metabolism and excretion studies. Male BALB/c mice (18–22 g) were obtained from Bantin and Kingman Ltd. (Hull, UK). Animals were fed on a Heygate modified 41B breeding diet (Pilsbury Ltd, Birmingham, UK) and allowed access to water ad libitum. Mice were housed in metabolic cages either in groups of two or individually 24 h prior to dosing, and control urine was collected. At 9 a.m., mice received temozolomide or [^{14}C]-temozolomide (40 mg/kg, 6–8 μCi/mouse, i.p.) dissolved in saline with 10% dimethylsulfoxide (DMSO, 0.2 ml). This dose does not cause toxicity in mice; it was optimally effective against the

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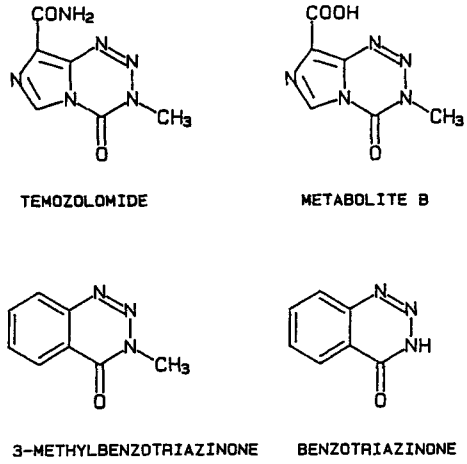

Fig. 1. Chemical structures of temozolomide and 3-methylbenzotriazinone and their metabolites

TLX5 lymphoma when given on 5 consecutive days [18]. Collection flasks containing 0.5 ml HCl (1 *N*) for stabilisation of the imidazotetrazinones were immersed in a solution of antifreeze (–5°––15° C). Urine and faeces were collected at 8-h intervals for 72 h. In some experiments, air was drawn through metabolic cages linked to bottles containing Ca_2SO_4 for removal of moisture and soda lime for removal of CO_2 before the animals entered the cage. $^{14}CO_2$ was trapped in two consecutive flasks containing a mixture (20 ml) of ethanolamine/ethoxyethanol (1 : 4, v/v).

The amount of temozolomide excreted in the urine was analysed by HPLC. Radioactivity in the urine, faeces or breath was analysed by scintillation counting using a Tricarb Liquid Scintillation Analyzer (Canberra Packard, Pingbourne, UK). Counts were corrected for quenching by the external standard ratio; the external standard used was [^{14}C]-hexadecane. The resulting values represent the mean ± SD of six mice.

Patients. Urine was obtained from patients who participated in a phase I clinical trial coordinated by Dr. G. R. P. Blackledge (Queen Elizabeth Hospital, Birmingham). Temozolomide was given orally (capsules) at doses of 200–920 mg/m^2.

In vitro metabolism studies. A liver homogenate (1 g/ml) was prepared in ice-cold 0.1 M phosphate buffer (pH 7.4) and centrifuged at 10,000 *g* for 10 min in an MSE Pegasus ultracentrifuge (MSE, Crawley, UK) at 4° C. The 10,000 *g* supernatant was in turn centrifuged at 100,000 *g* for 1 h, which furnished microsomes. Incubation mixtures contained liver homogenate or 10,000 *g* supernatant or microsomes equivalent to 50 mg liver/ml, magnesium chloride hexahydrate (0.33 mM), reduced nicotinamide adenine dinucleotide phosphate (NADPH, 0.5 mM) and temozolomide or 3-methylbenzotriazinone (0.58 mM) in a final volume of 5 ml 0.1 M phosphate buffer. Mixtures were incubated in scintillation vials at 37° C for 60 min under shaking and with access of air.

HPLC analysis. Sample preparation and analysis of imidazotetrazinones were performed as previously described [13, 14]. In short, samples of urine or of the hepatic incubation mixture were acidified and extracted with ethylacetate. Analysis of temozolomide and its metabolites was carried out using a Lichrosorb C18 RP select B column and a Lichrosorb guard column attached to a Waters chromatography system (with a Waters 840 data station, an M710B WISP autosampler, an M510 solvent pump and a Lamda max 480 variable wavelength detector; Waters Associates, Milford, Mass., USA).

For the quantitative determination of temozolomide, 8-carbamoyl-3-ethylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one was used as an internal standard. The mobile phase for the analysis of urinary metabolites was 5% methanol in 0.5% acetic acid at a flow rate of 1 ml/min. For in vitro metabolism experiments, the mobile phase was 10% methanol in 0.5% (v/v) acetic acid and the flow rate, 1.5 ml/min. Detection was carried out by UV spectrophotometry at 325 nm. The efficiency of extraction of temozolomide from urine was 58.4% ± 2.3% (n = 6). The respective values for the inter-day and within-day reproducibility of temozolomide were 7.5% and 1.8% at 35 mg/l; 6.8% and 1.5% at 4.3 mg/l; 8.2% and 3.7% at 0.4 mg/l.

Isolation of metabolites. For the isolation of metabolite B, samples (0.2–0.3 l) of acidified human urine were continuously extracted for 3 days under reflux with ethylacetate. For the isolation of metabolite A, urine samples were freeze-dried and the residue was reconstituted with 0.5% acetic acid and then extracted 7–10 times with ethylacetate at room temperature. The ethylacetate extracts were purified using C_{18} Sep-pak cartridges. For metabolite A, this isolation step was followed by anionic-exchange chromatography using a Dowex Cl$^-$ resin (mesh size, 100–200 µm) that had been loaded with formate. The metabolite was eluted from the column with formate buffer (0.1 M, pH 4). Finally, both metabolites were further purified by repeated HPLC.

Spectral analysis. Mass spectra were measured on either a VG micromass 12B single-focussing instrument or a VG70SEQ spectrometer (VG Instruments, Wythenshawe, UK) using a direct insertion probe. Data were processed using a VG11/250 data system. Isobutane was used as a reagent gas for spectral analysis in the chemical ionisation mode. In the electron-impact mode, mass spectra were measured at 70 eV with an inlet temperature of 230° C.

For fast-atom-bombardment mass spectra, samples were dissolved in the matrix, either glycerol or thiodiglycol. Spectra were run at a scan rate of 3 s/decade.

High-field [^{1}H]-NMR spectra were obtained on a Bruker AC-300 instrument (Bruker UK Ltd., Coventry) equipped with an Aspect 3000 computer operating at 300.13 MHz. Compounds were dissolved in DMSO-d_6 or D_2O prior to analysis. Sodium 3-(trimethylsilyl)-1-propanesulphonate was used as an internal standard. Spectra were recorded at room temperature using 32,768 data points over a sweep width of 2,994.012-Hz and an acquisition time of 5.472 s. In the case of metabolite I, the water signal was suppressed by continuous secondary irradiation. UV spectra were recorded using a Hewlett Packard HP 8451A diode-array spectrophotometer (Hewlett Packard, Corvallis, Ore., USA).

Cytotoxicity assay. The TLX5, X-irradiation-induced thymus lymphoma was routinely passaged in CBA/CA mice as previously described [18]. Cells were harvested from animals, rinsed with cell lysis buffer and maintained in vitro in RPMI 1640 medium supplemented with 17% horse serum (Gibco Europe, Paisley, UK) at 37° C under an atmosphere of air with 10% CO_2. For all experiments, cells were taken from subcultures 10–20, during which the cells retained their tumorigenicity in vivo as shown by their reinjection into the animals [3]. The doubling time of the cells was 14 ± 2 h. Drugs were dissolved in DMSO and added to the culture medium such that the final concentration of DMSO in the medium did not exceed 1%. Cells were grown in Nunclon 24-well dishes (Nunc, Roskilde, Denmark) and counted after 72 h using a Coulter ZM counter (Coulter Electronics, Luton, UK). IC_{50} is defined as the drug concentration that causes toxicity or stasis of 50% of the cells.

Results

Urinary excretion of temozolomide and its metabolites

Initially, the fate of temozolomide was studied in mice by assessment of the excretion of drug-derived species in the urine and breath after administration of [methyl-^{14}C]-temozolomide. Figure 2 shows the time-course of the cumulative excretion of drug-derived radioactivity and of parent drug in the urine and of metabolically generated $^{14}CO_2$ in the expired air over a period of 3 days. A major portion of the dose was eliminated within the first 16 h. Renal excretion was the predominant route of elimination

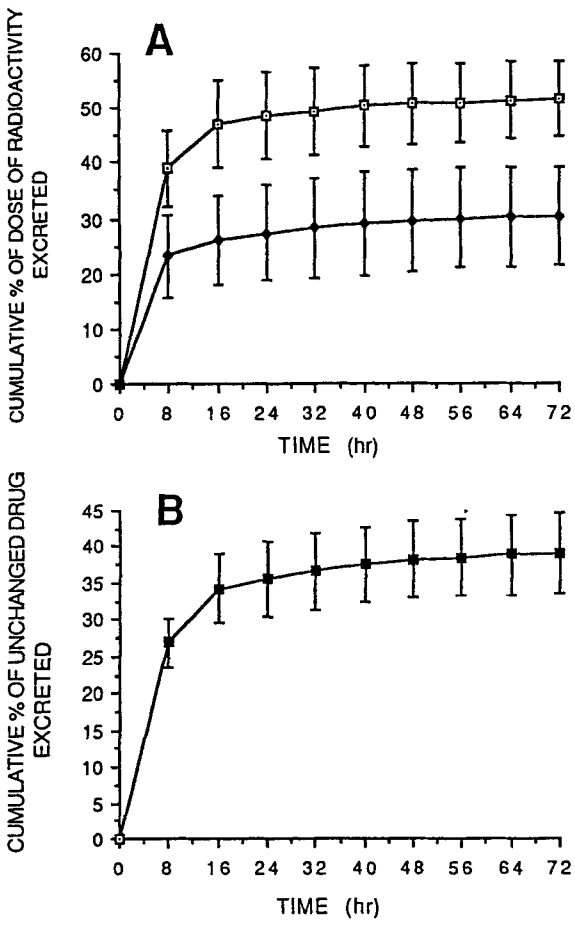

Fig. 2 A, B. Cumulative excretion of A radioactivity in the urine (-□-) and the breath (-◆-) and B unchanged temozolomide in the urine of mice that received [methyl-^{14}C]-temozolomide or unlabelled temozolomide (40 mg/kg) via the i.p. route. Values represent the mean ± SD of 6 mice

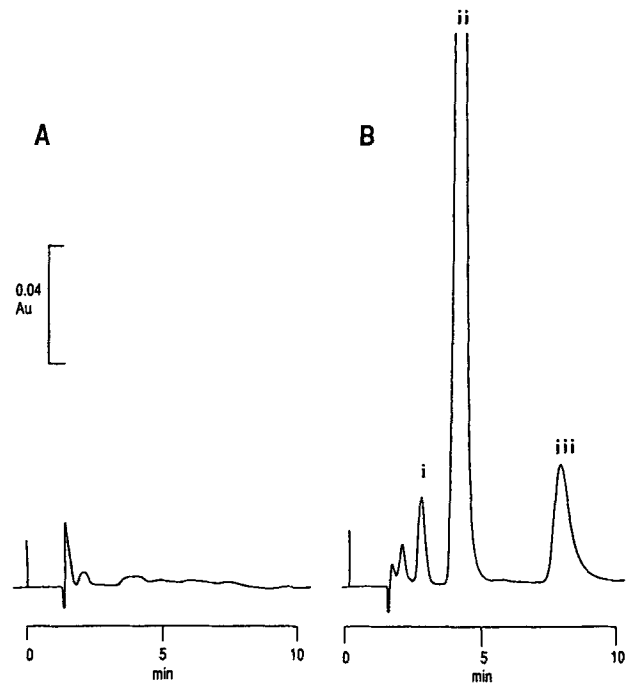

Fig. 3 A, B. HPLC chromatogram of an ethylacetate extract of a urine sample obtained from a patient A after treatment with dexamethazone and metoclopramide but prior to administration of temozolomide and B 2 h after treatment with temozolomide (700 mg/m^2) via the p.o. route. *Peak i* is due to metabolite A and *peaks ii and iii* coeluted with temozolomide and metabolite B, respectively. *Au*, absorbance units

for temozolomide, accounting for 52%±7% of the injected radioactivity; 39%±6% of the dose was excreted in the urine as unchanged drug. The percentage of the dose expired as $^{14}CO_2$ was 30%±9%. Only a small amount of radioactivity was detected in the faeces (3%±3%) and the carcass (8%±2%).

Characterisation of urinary metabolites

HPLC analysis of extracts of urine samples from patients who had been treated with temozolomide revealed three peaks (Fig. 3). One of them co-eluted with temozolomide. The retention times of the other two peaks were 2.5 min (metabolite A) and 8 min (metabolite B); these peaks appeared to be due to metabolites of temozolomide, as they were not present in urine samples from healthy humans or from patients either prior to drug administration or after they had received medication other than temozolomide. Furthermore, these peaks were not found in chromatograms of incubates of temozolomide in urine; hence, they were not due to products of the chemical degradation of temozolomide. Metabolite B, but not metabolite A, was also found in extracts of urine from mice that had received temozolomide (not shown).

Figure 4 shows the effect of acidification on the extraction efficiency of metabolites A and B. With increasing pH value, the extraction efficiency of the metabolites was decreased. Metabolite B appeared to be more acidic than metabolite A. After the acidification of urine samples that had been made alkaline, the peaks reappeared, which suggests that their disappearance was not due to chemical degradation. When urine samples were alkalinised with 4 *N* NaOH, the metabolites were removed irreversibly, suggesting that these metabolites behave chemically like temozolomide, which is unstable under alkaline conditions [2, 18].

Metabolite B was isolated and its structure was elucidated by high-field [^{1}H]-NMR and mass spectrometry. HPLC analysis before and after spectral investigation ensured that the material had not undergone decomposition. The [^{1}H]-NMR spectrum of metabolite B (Fig. 5B) was compared with that of temozolomide (not shown). In the NMR spectrum of temozolomide, the *N*-methyl and the imidazole protons yield singlets at 3.9 and 8.8 ppm, respectively, and the carboxamide protons give rise to a doublet at 7.8 ppm. Of these resonance frequencies, only the singlets at 3.9 and 8.8 ppm were present in the spectrum of metabolite B. Comparison of the latter spectrum with that of authentic 3-methyl-2,3-dihydro-4-oxoimidazo[5,1-*d*]tetrazine-8-carboxylic acid (Fig. 5B) suggests identity between the two. The electron-impact mass spectra of both metabolite B (Fig. 6A) and authentic 3-methyl-2,3-dihydro-4-oxoimidazo[5,1-*d*]-tetrazine-8-carboxylic acid (Fig. 6B) display the fragment ion m/z 138 as the base peak, indicating the loss of C_2H_3NO (methyl

Fig. 4 A–E. Effect of pH on the efficiency of extraction of temozolomide metabolites from urine samples adjusted to A pH 1.5, B pH 3, C pH 4, D pH 5; chromatogram E was obtained after acidification of sample D to pH 1.5. *Au*, absorbance units

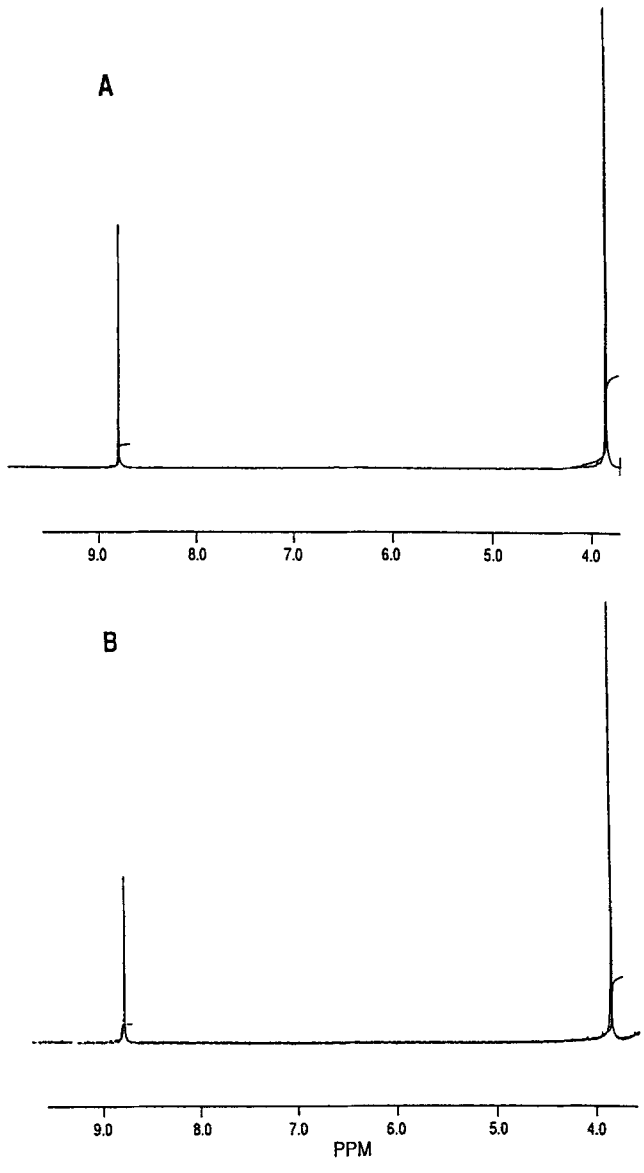

Fig. 5 A, B. [^{1}H]-NMR spectra of A metabolite B after isolation and B authentic 3-methyl-2,3-dihydro-4-oxoimidazo[5,1-*d*]-tetrazine-8-carboxylic acid

isocyanate), probably due to ring cleavage. This fragmentation pattern seems to be analogous with that of temozolomide, which affords m/z 137 as its base peak.

Compounds with a triazene linkage possess a characteristic UV absorption maximum near 320 nm [2, 17, 18]. UV spectral analysis of metabolite B gave a maximum at 328 nm, identical to that of 3-methyl-2,3-dihydro-4-oxoimidazo[5,1-*d*]tetrazine-8-carboxylic acid.

Metabolite A was not amenable to mass spectral analysis in either the chemical ionisation, electron-impact or fast-atom-bombardment modes. Figure 7 shows the [^{1}H]-NMR spectrum of metabolite A. The singlet at 8.8 ppm indicates the presence of the imidazole proton and the doublet at 8.1 ppm might be due to the carboxamide protons. The lack of a resonance frequency near 4 ppm suggests the absence from the molecule of the temozolomide *N*-methyl group. Metabolite A also possesses a number of aliphatic protons that resonate between 1.2 and 2 ppm. The physicochemical properties of metabolite A are reminiscent of those of a conjugate, perhaps a glucuronide, as it is weakly acidic and water-soluble. However, because of the absence in the [^{1}H]-NMR spectrum of the β-anomeric protons of glucuronides, which usually resonate between 5 and 6 ppm [21], and the lack of proton signals corresponding to the β-D-glucuronyl moiety between 3 and 4 ppm [6, 10, 20, 21], it is doubtful that metabolite A is a glucuronide. Furthermore, metabolite A was resistant to hydrolysis by β-glucuronidase or sulphatase. Metabolite A yielded a UV spectrum with absorbance maxima at 228, 250 and 322 nm, typical of imidazotetrazinones and closely resembling that of temozolomide [18].

Cytotoxicity of temozolomide metabolites

Figure 8 A shows the effect of metabolite A on the growth of TLX5 lymphoma cells. The concentration of the metabolite was determined by HPLC analysis using temozolomide as a standard, assuming that the metabolite possesses the same extinction coefficient as temozolomide. Metabolite A lacked cytotoxicity towards TLX5 cells at

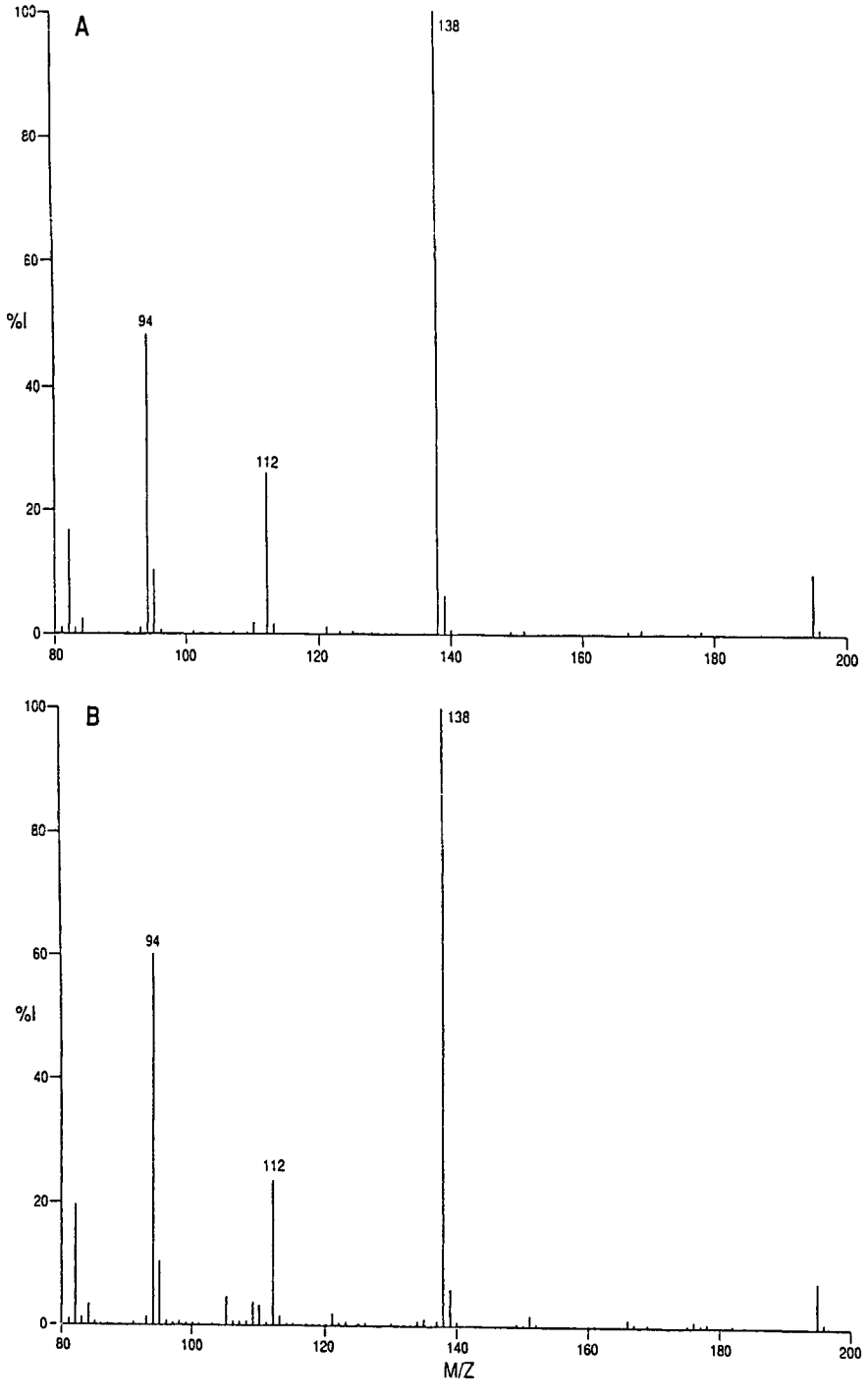

Fig. 6 A, B. Electron-impact mass spectra of **A** metabolite B after isolation and **B** authentic 3-methyl-2,3-dihydro-4-oxoimidazo[5,1-*d*]tetrazine 8-carboxylic acid

concentrations of up to 20 mg temozolomide equivalents/l. In contrast, the IC_{50} of metabolite B was almost identical to that of temozolomide (Fig. 8 B) and that of authentic 3-methyl-2,3-dihydro-4-oxoimidazo[5,1-*d*]-tetrazine-8-carboxylic acid (not shown).

Microsomal metabolism of temozolomide and 3-methylbenzotriazinone

Liver homogenate, 10,000-*g* post-mitochondrial supernatant and microsomes were used to investigate the in vitro metabolism of temozolomide. The rate of degradation of temozolomide observed in either suspensions of liver homogenate or its subcellular fractions was identical to that seen in phosphate buffer only (result not shown). Likewise, species generated from temozolomide by catalysis of hepatic enzymes could not be detected by HPLC. In contrast, incubation of 3-methylbenzotriazinone (for structure, see Fig. 1) with liver microsomes under conditions identical to those used with temozolomide afforded a metabolite with a retention time identical to that of authentic benzotriazinone. The identity of this metabolite was confirmed by comparison of the respective chemical ionisation mass spectra (Fig. 9).

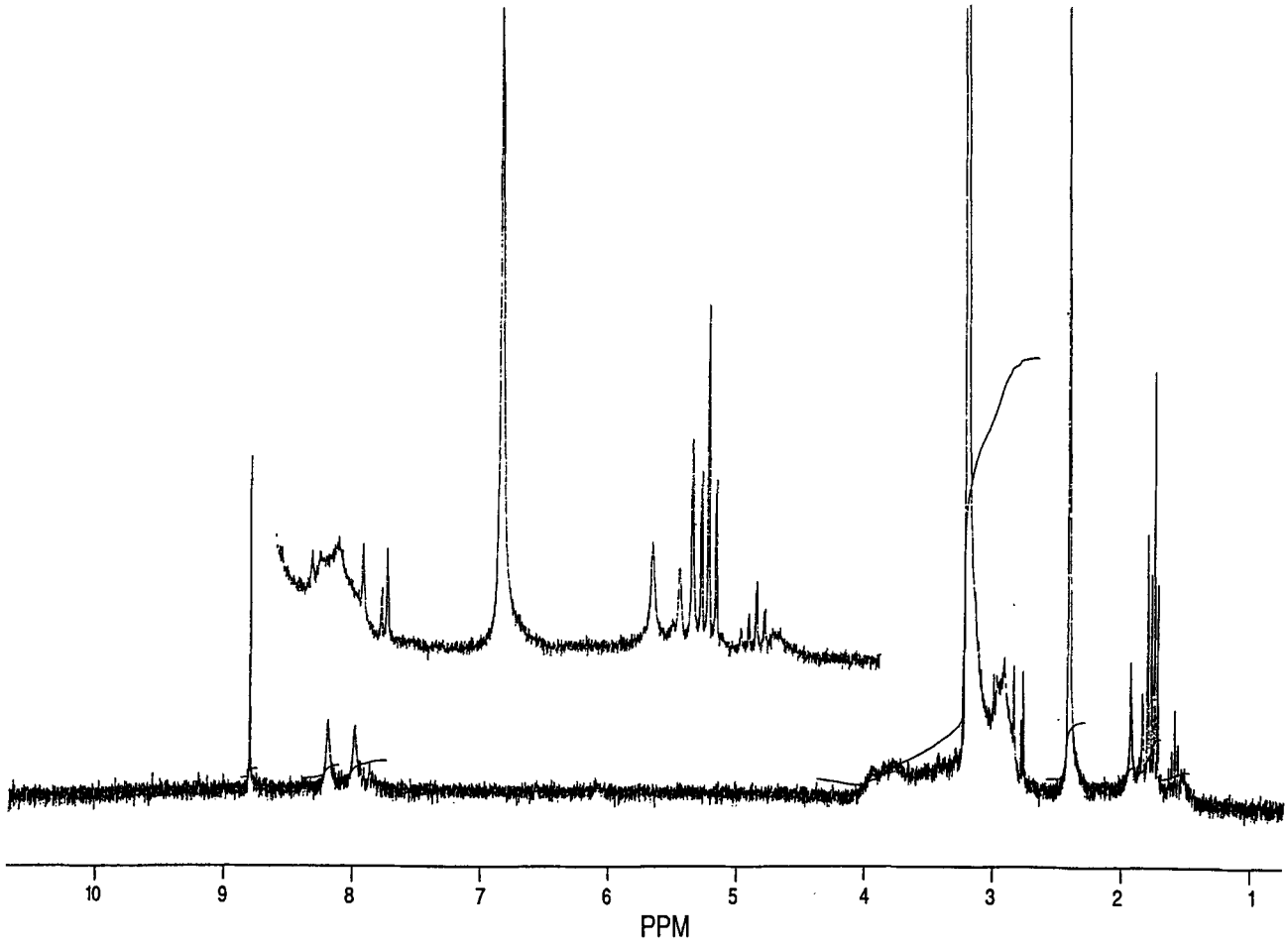

Fig. 7. [^{1}H]-NMR spectrum of metabolite A after isolation

Discussion

Temozolomide degrades chemically in physiological solution to yield measurable quantities of MTIC (Tsang et al., unpublished). It is therefore possible that temozolomide exerts its antineoplastic activity via this monomethyltriazene, which is also thought to be the major cytotoxic species generated by the metabolism of dacarbazine. We investigated the question as to whether temozolomide is biotransformed in addition to its degradation to MTIC. Unlike its structural analogue *N*-methylbenzotriazinone, temozolomide does not undergo metabolic *N*-demethylation by mouse liver microsomes. This result demonstrates that when the substituent in position 1 of the triazene linkage is situated in a molecule as part of a ring, its chemical nature determines its susceptibility towards hepatic metabolism. 3-Methylbenzotriazinone is devoid of antineoplastic activity in mice (Chubb et al., unpublished results), and one might speculate that this difference is related, perhaps indirectly, to the observed difference in metabolism.

Although we could not identify metabolites of temozolomide in liver preparations in vitro, biotransformation does occur in vivo in humans and in mice. Nevertheless, metabolism is not the major mechanism of clearance of temozolomide from the murine organism: most of the urinary radioactivity consisted of unchanged drug. The major metabolite of temozolomide in human urine was 3-methyl-2,3-dihydro-4-oxoimidazo[5,1-*d*]tetrazine-8-carboxylic acid, the carboxylic acid analogue of temozolomide. The absence of this metabolite from the urine of mice that had received temozolomide indicates a species difference in metabolism. Nevertheless, we cannot exclude the possibility that this difference is related to the difference in the route of administration, which was i.p. in mice and p.o. in patients. It is likely that the metabolite is generated, possibly extrahepatically, by hydrolysis of the carboxamide group. Substrates that undergo this type of metabolic hydrolysis seem to be somewhat rare; only a few examples have been reported in the literature, among them, metopimazine [1], rilmazafone [9] and LY195448 [5, 7]. Unlike the carboxylic-acid analogue of mitozolomide [8], metabolite B is cytotoxic and is not a detoxification product, having an IC_{50} similar to that of its metabolic progenitor.

The structure of another in vivo metabolite of temozolomide, which was found in the urine of both humans and mice, could not be fully solved. Spectral analysis suggests that this metabolite possesses the intact NNN-linkage and the imidazocarboxamide moiety of temozolomide but not its *N*-methyl moiety, which is consistent with the possibility that the formation of this metabolite involves the *N*-methyl moiety as the locus of primary metabolic attack. Since the metabolite contained radiolabel when found in

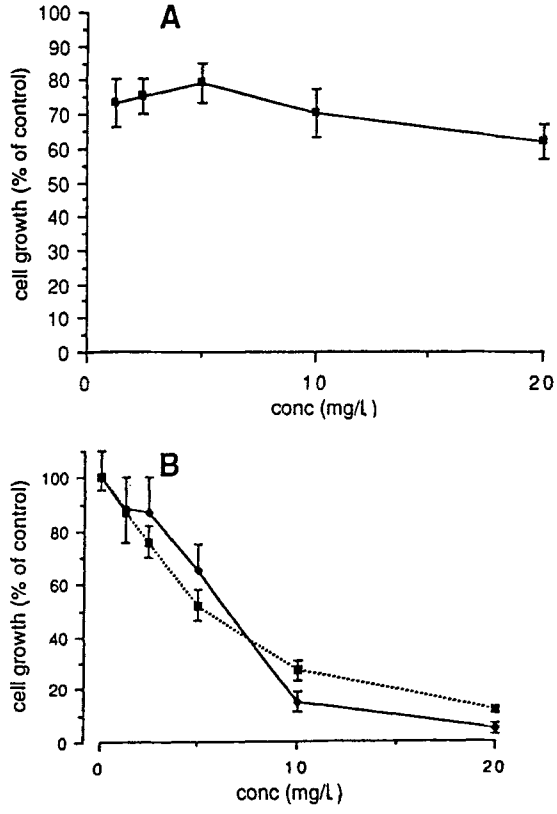

Fig. 8 A, B. Cytotoxicity against TLX5 lymphoma cells of A metabolite A and B temozolomide (*squares*) and metabolite B (*rhombi*). Values represent the mean ± SD of 4 experiments

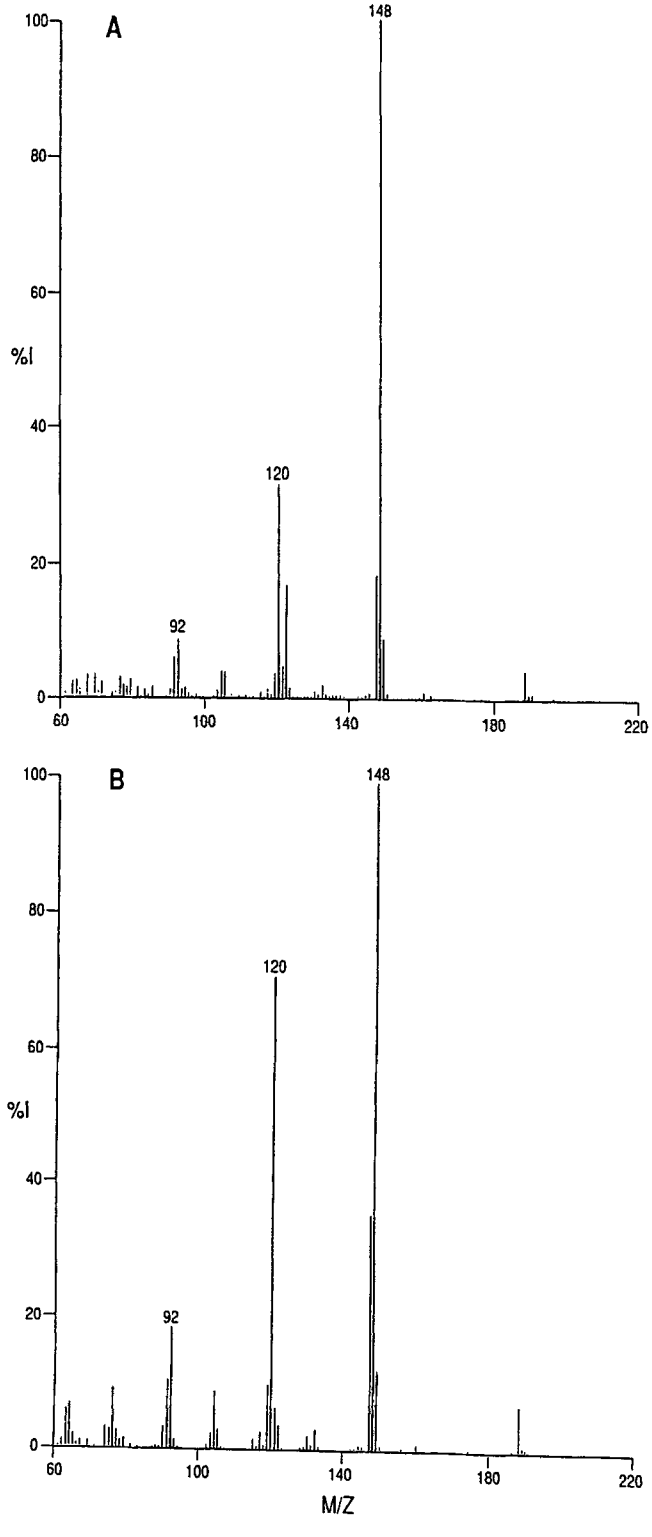

Fig. 9 A, B. Chemical ionisation mass spectra of A a metabolite isolated from the microsomal incubate with 3-methylbenzotriazinone (0.58 mM) and B authentic benzotriazinone

the urine of mice that had received [methyl-^{14}C]-temozolomide (result not shown), it is likely that it retains the methyl carbon, conceivably as -NCH_2-. Thus, it is possible that this metabolite is a conjugate linked to the imidazotetrazinone ring via an -NCH_2- moiety. The nature of the endogenous conjugation partner is unresolved. Unlike the carboxylic-acid derivative of temozolomide, this metabolite was not cytotoxic in vitro, which suggests that it is a detoxification product of the drug.

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