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Short Communication

Metabolism of two new benzodiazepine-type anti-leishmanial agents in rat hepatocytes and hepatic microsomes and their interaction with glutathione in macrophages

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Abstract

Objectives To measure the metabolism and toxicity of 7-chloro-4-(cyclohexylmethyl)-1-methyl-3,4-dihydro-1*H*-1,4-benzodiazepine-2,5-dione (BNZ-1) and 4-cyclohexylmethyl-1-methyl-3,4-dihydro-1*H*-1,4-benzodiazepine-2,5-dione (BNZ-2), two new benzodiazepine analogues found to be effective against *Leishmania* amastigotes *in vitro*.

Methods The metabolism of BNZ-1 and -2 was investigated in isolated rat hepatocytes and rat liver microsomes. The toxicity of the compounds was assessed in a murine macrophage cell line by determining cell viability and reduced glutathione (GSH) content. The metabolism and toxicity of flurazepam was assessed for comparison.

Key findings BNZ-1 and BNZ-2 underwent similar metabolic transformations by the liver systems, forming *N*-demethylated and hydroxylated metabolites, with subsequent O-glucuronidation. Flurazepam and both analogue compounds depleted macrophage GSH levels without affecting cell viability at the concentrations used (up to 100 μM), but only flurazepam inhibited glutathione reductase activity, indicating that it is acting by a different mechanism.

Conclusions The exact mechanism responsible for GSH depletion is unknown at present. Further experiments are needed to fully understand the effects of BNZs on the parasite GSH analogue, trypanothione, which may be a direct or indirect target for these agents. Pharmacokinetic evaluation of these compounds is required to further progress their development as potential new treatments for leishmaniasis.

Keywords benzodiazepine; glutathione; *Leishmania*; metabolism

Introduction

Leishmaniasis is a tropical disease caused by the parasite *Leishmania,* a genus of trypanosome protozoa. The disease threatens approximately 350 million people in over 80 countries, and resulted in 59 000 deaths in 2002.[1] The existing treatments for leishmaniasis are plagued by issues of toxicity and parasite resistance, and newer drugs are required. The paullones have been identified as a promising class of compounds which inhibit the growth of *L. mexicana* promastigotes *in vitro*;[2] however, these compounds are non-selective. The benzodiazepines, a class of drugs with sedative, anticonvulsant, hypnotic, anxiolytic and muscle-relaxant properties, produce a myriad of therapeutic activities by acting on the gamma-aminobutyric acid $(GABA)_A$ receptor, an abundant inhibitory receptor in the brain. These compounds have been reported to produce inhibitory effects on some parasites via peripheral benzodiazepine receptors.[3] Strong ligands of peripheral benzodiazepine receptors such as flurazepam inhibited the growth of *P. falciparum in vitro* and were active against *Toxoplasma gondii.*

In an attempt to develop new anti-leishmanial agents, we synthesised a series of benzodiazepines structurally related to the paullones.[4] Two new benzodiazepine analogues: 7-chloro-4-(cyclohexylmethyl)-1-methyl-3,4-dihydro-1*H*-1,4-benzodiazepine-2,5-dione (BNZ-1) and 4-cyclohexylmethyl-1-methyl-3,4-dihydro-1*H*-1,4-benzodiazepine-2,5-dione (BNZ-2) (Figure 1) were found to be effective against *L. donovani* amastigotes *in vitro.*[4]

The present study investigates the metabolism of these new chemical entities, together with the prototype benzodiazepine, flurazepam, using rat hepatocytes and hepatic microsomal preparations. These two in-vitro model systems are widely used to profile

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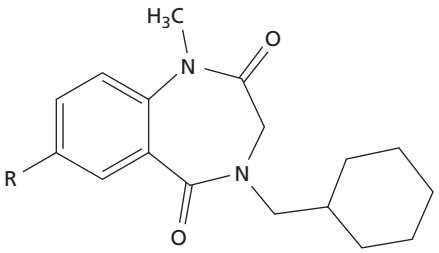

BNZ-1, R = Cl
BNZ-2, R = H

Figure 1 Structures of the benzodiazepine analogues, BNZ-1 and BNZ-2.

the metabolism of novel compounds. Use of both microsomes and hepatocytes helps to identify products of phase I metabolism, their further metabolism by phase II pathways, and metabolites formed by direct phase II metabolism. In this study, the use of the two models, including experiments using microsomes with and without the cofactor for glucuronidation, helped to establish the route of metabolism (i.e. hydroxylation followed by glucuronidation) without ambiguity. The interaction of the compounds with reduced glutathione (GSH) in macrophages was examined to improve our understanding of the mechanism of action of these compounds.

Materials and Methods

This study was carried out under UK Home Office Licence number 60/2980. Approval for the animal work was given by the University of Strathclyde Ethics Committee.

Rat hepatocyte incubations

Hepatocytes were isolated from whole livers of male Sprague Dawley rats (200–250 g) by a two-step collagenase perfusion technique.[5] The viability of the cell suspension was determined by Trypan blue exclusion (typically > 87%). For metabolism studies, hepatocytes were incubated in suspensions (2×10^6 viable cells/ml) with the benzodiazepine analogues (100 μM) in Krebs buffer (pH 7.4) containing 2.5 mM Hepes, in rotating round-bottomed flasks under an atmosphere of 95% O_2/5% CO_2 at 37°C for 3 h. The test compounds were dissolved in DMSO; the final concentration of DMSO in incubations was 0.1%. Samples of the incubation mixture were taken over the 3 h period, and extracted twice with an equal volume of ethyl acetate after adjustment of the pH to a value of 10 with 0.1 M carbonate buffer. The ethyl acetate extract was evaporated to dryness under a stream of nitrogen and stored at –20°C until analysis by liquid chromatography–mass spectrometry (LC–MS).

Rat liver microsomal incubations

Rat liver microsomes were prepared by differential centrifugation and the protein concentration determined by the Lowry assay[6] using bovine serum albumin as the standard. The incubation mixture (final volume 1 ml) contained microsomal protein (1.3 mg), substrate (100 μM), $MgCl_2$ (5 mM), NADPH (100 μM), the detergent Brij 58 (0.1 mg) and phosphate buffer (100 mM, pH 7.4). Incubations without NADPH or substrate were used as negative controls. In incubations to investigate glucuronic acid conjugation, 3 mM uridine 5′-diphosphate glucuronic acid (UDPGA) was included as cofactor. After 45 min of incubation in a shaking water bath at 37°C, the substrate and metabolites were extracted twice with an equal volume of ethyl acetate and prepared for LC–MS analysis as described above.

Macrophage incubations

Macrophages (J774.1 cell line; ECACC number 9105151) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum. Twenty-four hours after passaging the cells, the substrate (100 μM) was added to the cells at 50% confluency. After 24 and 48 h incubations at 37°C, samples (1 ml) of the cell suspensions were extracted with ethyl acetate as described above for hepatocytes for analysis by LC–MS. In some experiments the macrophages were activated by adding 1 μg/ml lipopolysaccharide (LPS) to the cells 6 h after they had been passaged. After 18 h of LPS treatment the substrates were added and incubated as for the resting (unactivated) cells.

Determination of reduced glutathione content and glutathione reductase activity

Following 24 and 48 h incubations of substrates with macrophages, the cells were harvested and homogenised in 0.2 M potassium phosphate buffer containing 2 mM EDTA. The GSH content was measured as described by Hissin and Hilf[7] and the activity of glutathione reductase by the method of Carlberg and Mannervik.[8] The viability of the macrophages was measured by the leakage of lactate dehydrogenase[9] and the protein content was determined by the Lowry assay.[6]

HPLC–MS analysis

The substrates and metabolites were resolved on an ACE C18 column (50 mm × 3.0 mm i.d., 5 μm packing (Hichrom, Berkshire, UK), using a solvent gradient consisting of 0.1% formic acid and acetonitrile; the starting composition was 95 : 5 v/v; the composition after 25 min was 65 : 3 (v/v) for flurazepam and 70 : 3 (v/v) for BNZ-1 and -2. The flow rate was delivered at a flow rate of 0.5 ml/min. Analytes were detected by UV (230 nm) and electrospray ionisation mass spectrometry (ESI-MS).

Mass spectrometric data were acquired in both positive- and negative-ion mode with full scanning mode (*m/z* 150–800) using nitrogen gas (30 psi) as the nebulising gas. The conditions were as follows: drying gas temperature 250°C, drying gas flow rate 13 l/min, capillary voltage 4000 V, fragmentation voltage 70 V.

Statistical methods

Where indicated statistical comparisons were made by analysis of variance followed by Dunnett's multiple comparison test.

Results

Exposure to the drugs at the concentration used during the 3 h incubation did not have any significant effect on hepatocyte viability, as determined by Trypan blue exclusion.

The metabolism of flurazepam was used to validate the in-vitro systems, given its similarity in structure to BNZ-1 and BNZ-2. The parent compound (*m/z* 388) is primarily biotransformed to *N*-desethyl flurazepam (*m/z* 360), *N*-didesethyl flurazepam (*m/z* 332), hydroxyethyl flurazepam (*m/z* 333) and *N*-1-desalkyl flurazepam (*m/z* 289) (Figure 2). We confirmed the formation of these metabolites by rat hepatocytes. The most abundant metabolite formed was *N*-desethyl flurazepam (*m/z* 360), followed by *N*-didesethyl flurazepam (*m/z* 332). However, the *N*-1-desalkyl-3-hydroxyflurazepam metabolite was not detected and there was no evidence for either N- or O-glucuronidation of the resulting metabolites.

In microsomes incubated with BNZ-1 and supplemented with NADPH, only a metabolite with *m/z* 337 was formed. In contrast, hepatocytes formed a variety of products from BNZ-1; the *m/z* values and the retention times are shown in Table 1. The product(s) with *m/z* 513 are postulated to be glucuronide conjugates of hydroxylated metabolites; they are also formed by microsomes and require the presence of both UDP-glucuronic acid and NADPH. The identity of these metabolites was further confirmed by negative ion ESI-MS analysis and showed an ion of *m/z* 511 for the putative glucuronide formed in microsomal incubations (Figure 3).

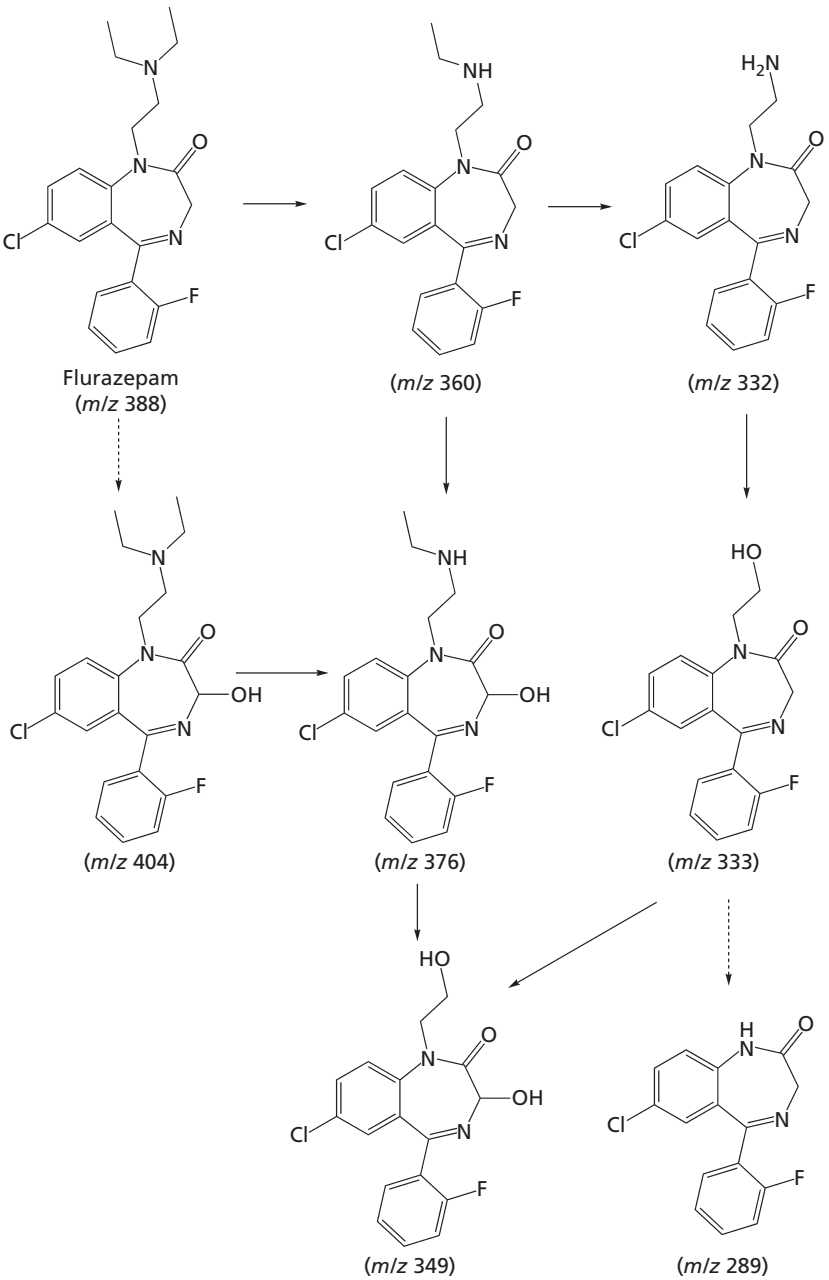

Figure 2 Proposed metabolic pathways for flurazepam in rat hepatocytes. The dashed lines depict minor pathways.

Table 1 Metabolism of BNZ-1 in rat hepatocytes and liver microsomes

m/z values	Retention time (min)	Formed by hepatocytes	Formed by microsomes	Characteristics
321 (BNZ-1)	23.1			Parent
337	12.3; 14.0	Yes	Yes	Major metabolite: the only phase I metabolite in microsomes
307	22.0	Yes	No	Major metabolite in hepatocytes
323	12.0; 14.2	Yes	No	Minor
353	15.5; 16.2	Yes	No	Minor
513	10.3; 12.0	Yes	Yes, with added UDPGA	Two glucuronides formed

UDPGA, uridine 5′-diphosphate glucuronic acid.

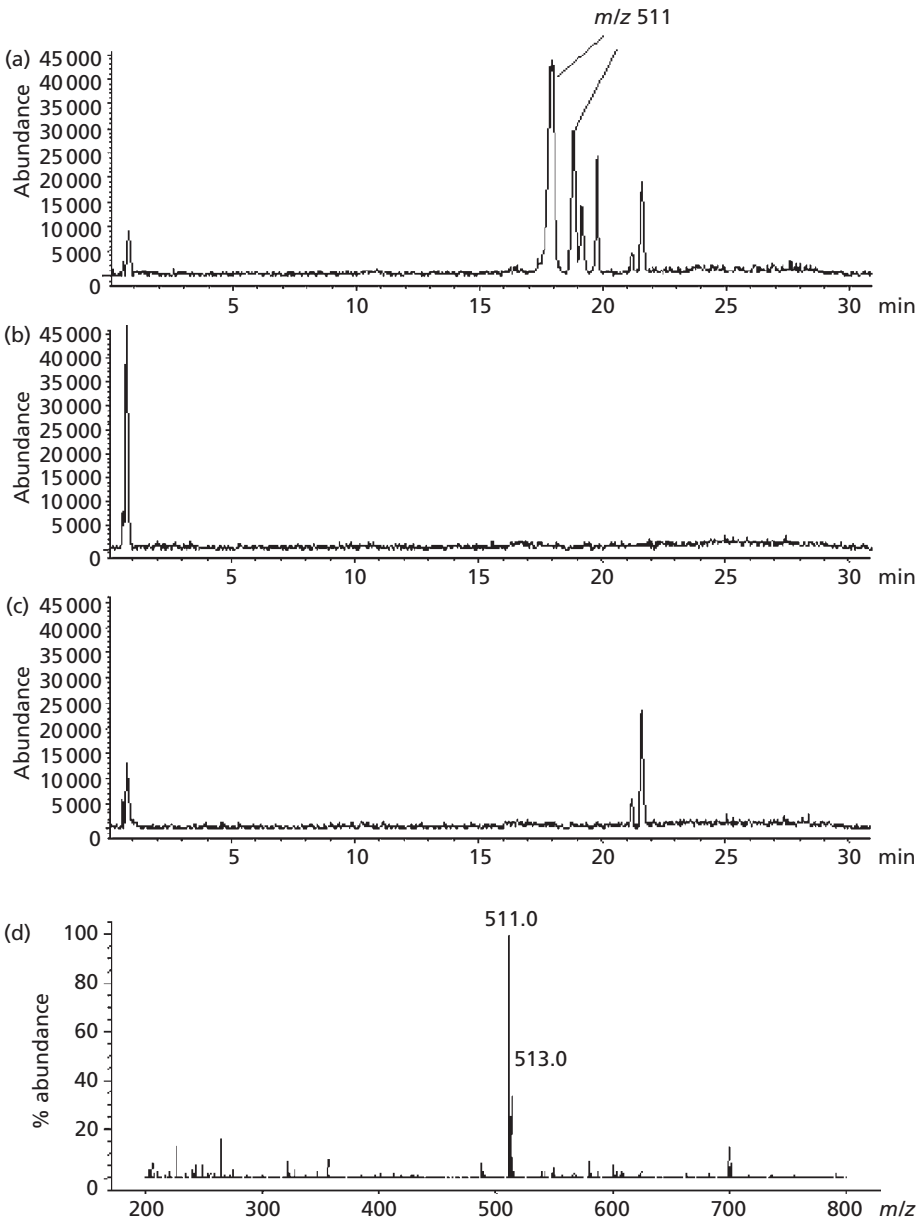

Figure 3 Chromatograms for compound with *m/z* = 511 from incubations of BNZ-1 (100 μM) with (a) microsomes, NADPH and uridine 5′diphosphate glucuronic acid; (b) microsomes only, no cofactors; (c) microsomes and NADPH. (d) Mass spectrum of the compound with *m/z* = 511 in negative scanning mode.

Table 2 Metabolism of BNZ-2 in rat hepatocytes and liver microsomes

m/z values	Retention time (min)	Formed in hepatocytes	Formed in microsomes	Characteristics
287 (BNZ-2)	23.0			Parent
303	7.0; 9.0	Yes	Yes	Dominant metabolites in hepatocytes after 30 min
273	20.2	Yes	Yes	Minor, formed slowly
289	5.0; 6.8	Yes	No	Appeared after 120 min in hepatocytes
479	5.8; 8.0	Yes	Yes, with added UDPGA	Two glucuronides formed

UDPGA, uridine 5′-diphosphate glucuronic acid.

BNZ-2 was also extensively metabolised: three phase I metabolites were formed in microsomal incubations, with further compounds formed in hepatocytes. Table 2 shows the *m/z* and retention times of the metabolites formed, and indicates whether they were formed in hepatocyte or microsomal incubations.

Flurazepam, BNZ-1 and BNZ-2 did not form any detectable metabolites when incubated for up to 48 h with mouse macrophages, either in the resting state or after activation with LPS.

Figure 4a shows that all the compounds significantly decreased intracellular GSH levels within 24 h in the macrophage cell line. Only flurazepam caused significant inhibition of glutathione reductase activity in the macrophages (Figure 4b). A decrease in GSH is often symptomatic of an adverse affect on mammalian cell viability, but there was no evidence of an increase in the leakage of lactate dehydrogenase from the macrophages within 48 h at the concentration used. The effect of the drugs on hepatocyte GSH levels is not known.

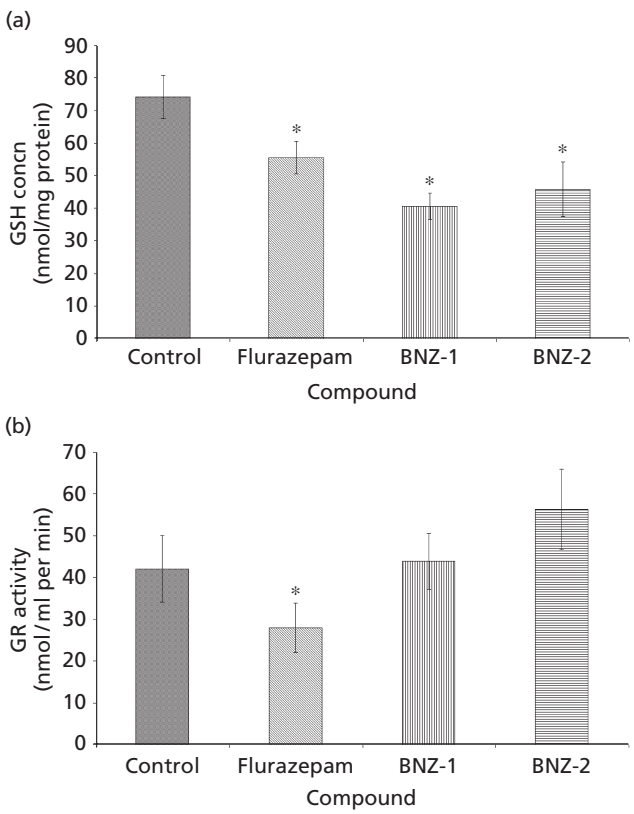

Figure 4 (a) Reduced glutathione (GSH) content and (b) glutathione reductase (GR) activity measured after macrophages were exposed to the compounds for 24 h. Results are means ± SEM, $n = 3$. In (a) all test incubations were significantly different from the control; in (b) only flurazepam had a significant effect on glutathione reductase activity. $P < 0.05$ (analysis of variance followed by Dunnett's multiple comparison test).

Discussion

The metabolism of flurazepam has been described previously in man, dog and rat.[10,11] The compound was used to validate the in-vitro systems, given its similarity in structure to BNZ-1 and BNZ-2. The metabolic pathways observed with flurazepam are shown in Figure 2. The *N*-1-desalkyl-3-hydroxyflurazepam metabolite, initially reported by Schwartz and Postma in man and dogs,[10] but not subsequently in rat serum,[11] was not detected. There was no evidence for either N- or O-glucuronidation of the resulting metabolites.

Schemes showing the proposed metabolic pathways of BNZ-1 and BNZ-2 in rat liver are shown in Figures 5 and 6, respectively. Both BNZ-1 and BNZ-2 demonstrated similar metabolism routes in liver: N-demethylation, hydroxylation and O-glucuronidation. With both BNZ-1 and BNZ-2, a greater range of metabolites was formed in hepatocytes than in microsomes. The former are a closer representation of the situation *in vivo* in the liver of an animal. In hepatocytes, the phase I metabolites are secreted from the cell, often following phase II metabolism, and they are therefore unable to exert significant inhibitory/modulatory effects on the enzymes. This mimics the situation in the liver, and thus more closely represents the balance of pathways occurring *in vivo* than is the case in microsomes.

The lack of metabolism of flurazepam, BNZ-1 and BNZ-2 in macrophages was not unexpected, given that macrophages, even when activated, have been reported to have low cytochrome P450 expression.[12,13] Species differences in the metabolism of the drugs may contribute to the differences observed between liver tissue and macrophages, as they are derived from rat and mouse, respectively. To establish for certain the potential for metabolism of these drugs in macrophages, further studies in cells freshly isolated from blood would be necessary, as the macrophage cell line used may have lost metabolic characteristics in culture.

Only flurazepam caused significant inhibition of glutathione reductase activity in the macrophages (Figure 4b).

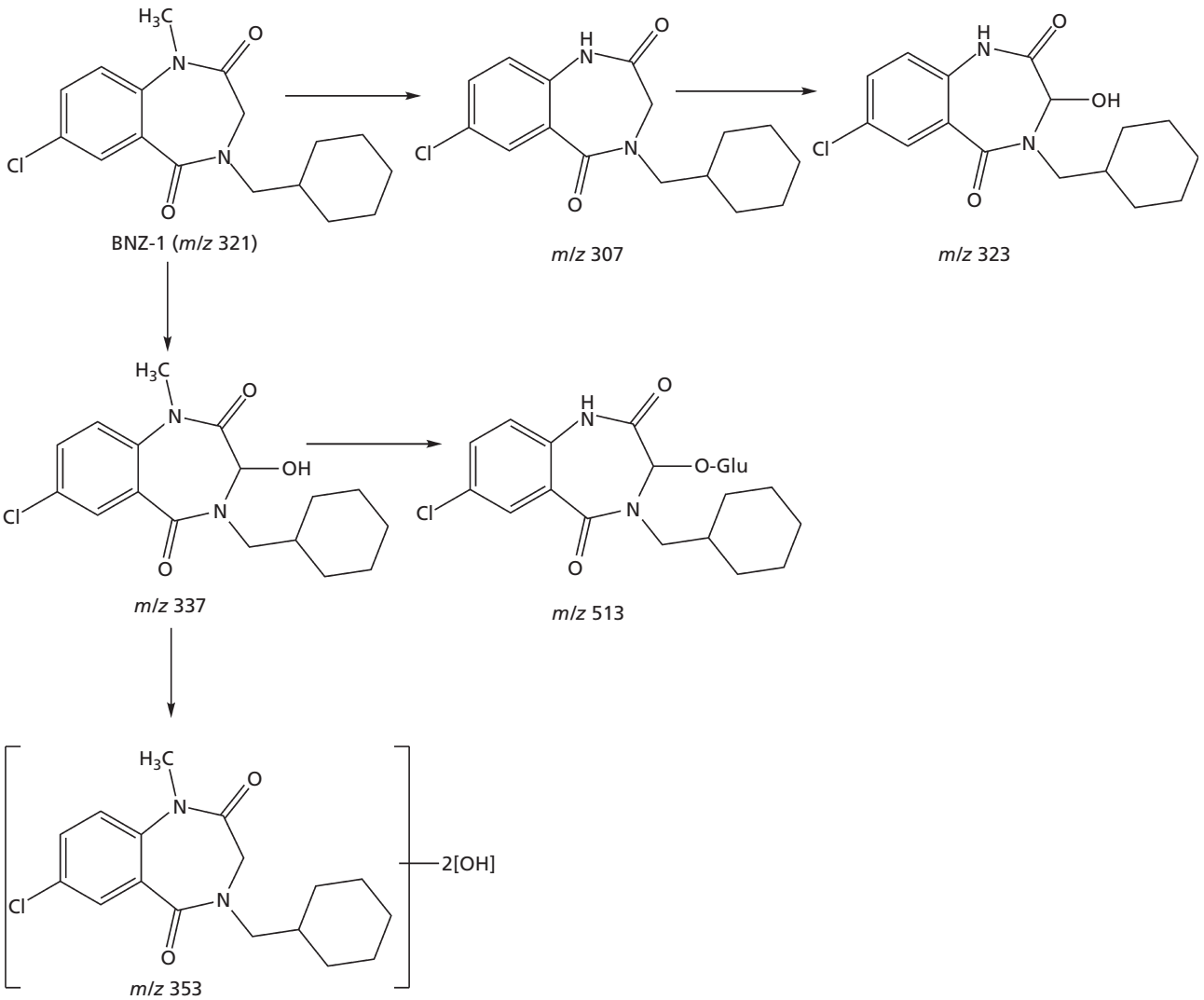

Figure 5 Proposed metabolic pathways of BNZ-1 in rat liver.

This may have contributed to the observed decrease in GSH levels, and flurazepam may therefore be acting by a different mechanism than the other two compounds. Further experiments on the mechanism of GSH depletion with these compounds may increase understanding of their effects in parasites, where a thiol, trypanothione, is essential for maintaining redox control required for virulence and survival.[14,15]

At present we do not have evidence of GSH oxidation, and further experiments are required to measure glutathione disulfide. Mechanistic studies are thus ongoing to elucidate the molecular pathways involved in the depletion/oxidation of GSH. In addition, determination of the pharmacokinetic profile of the drugs is essential for their future development as new options for treatment of leishmaniasis.

Conclusions

Despite their biological activity against leishmaniasis *in vitro,* the extensive metabolism of these novel benzodiazepine analogues may limit their therapeutic value, and it may be necessary to optimise these lead structures to reduce the rate and extent of metabolism.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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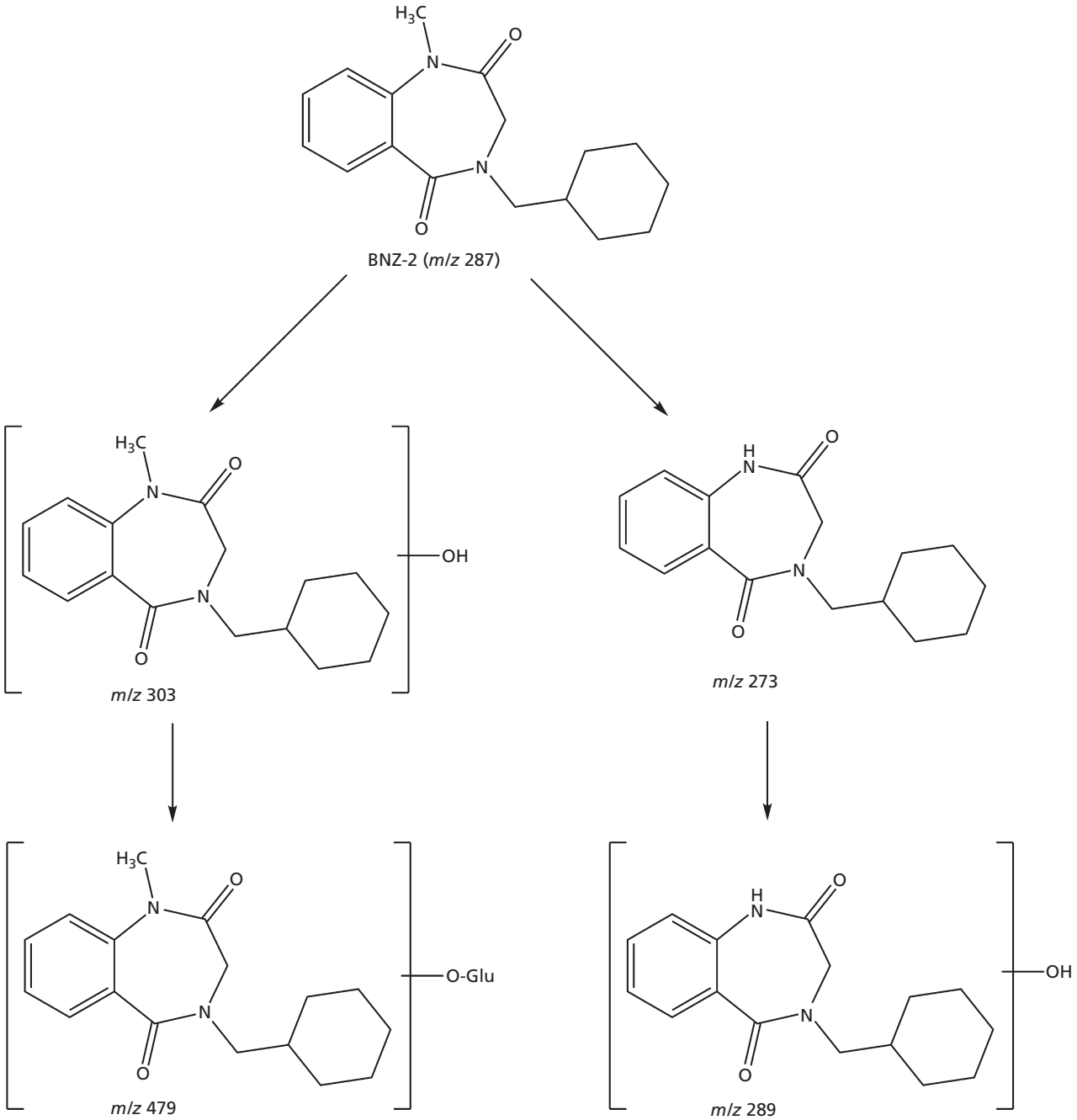

Figure 6 Proposed metabolic pathways of BNZ-2 in rat liver.

References

1. World Health Organization. Leishmaniasis: magnitude of the problem. Geneva: WHO. www.who.int/leishmaniasis/burden/magnitude/burden_magnitude/en/index.html (accessed 2008).
2. Knockaert M *et al.* Intracellular targets of paullones. Identification following affinity purification on immobilized inhibitor. *J Biol Chem* 2002; 277: 25493–25501.
3. Dzierszinski F *et al.* Ligands of the peripheral benzodiazepine receptor are potent inhibitors of *Plasmodium falciparum* and *Toxoplasma gondii in vitro*. *Antimicrob Agents Chemother* 2002; 46: 3197–3207.
4. Clark RL *et al.* Identification of the benzodiazepines as a new class of antileishmanial agent. *Bioorg Med Chem Lett* 2007; 17: 624–627.
5. Moldeus P *et al.* Isolation and use of liver cells. *Methods Enzymol* 1978; 52: 60–71.
6. Lowry OH *et al.* Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265–275.
7. Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* 1976; 74: 214–226.
8. Carlberg I, Mannervik B. Glutathione reductase. *Methods Enzymol* 1985; 113: 484–490.

9. Anuforo DC *et al.* Hepatotoxicity studies with primary cultures of rat liver cells. *In Vitro* 1978; 14: 981–988.
10. Schwartz MA, Postma E. Metabolism of flurazepam, a benzodiazepine, in man and dog. *J Pharm Sci* 1970; 59: 1800–1806.
11. Lau CE *et al.* Simultaneous determination of flurazepam and five metabolites in serum by high-performance liquid chromatography and its application to pharmacokinetic studies in rats. *J Chromatogr* 1987; 423: 251–259.
12. Peterson TC. Drug-metabolizing enzymes in rat, mouse, pig and human macrophages and the effect of phagocytic activation. *Biochem Pharmacol* 1987; 36: 3911–3916.
13. Germolec DR *et al.* Comparative assessment of metabolic enzyme levels in macrophage populations of the F344 rat. *Biochem Pharmacol* 1995; 50: 1495–1504.
14. Krauth-Siegel RL *et al.* The parasite-specific trypanothione metabolism of *Trypanosoma* and *Leishmania*. *Biol Chem* 2003; 384: 539–549.
15. Ramao PR *et al.* Glutathione and the redox control system trypanothione/trypanothione reductase are involved in the protection of *Leishmania* spp. against nitrosothiol-induced cytotoxicity. *Braz J Med Biol Res* 2006; 39: 355–363.