Biochemical Pharmacology, Vol. 35, No. 1, pp. 117–119, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

Benznidazole: nitroreduction and inhibition of cytochrome P-450 in chemosensitization of tumour response to cytotoxic drugs

Benznidazole (*N*-benzyl-(2-nitroimidazolyl)-acetamide; Ro 07-1051, Radanil, Roche; BENZO) is widely used in South America to treat the human trypanosomal infection Chagas' Disease [1–3] and has also been used in American mucotaneous leishmaniasis [4]. Studies in this laboratory have demonstrated the ability of relatively low doses of BENZO to enhance the response of mouse tumours to the nitrosourea CCNU [5] and to produce a similar therapeutic gain to that seen with much higher doses of the more familiar analogue misonidazole (MISO) [6–7]. Chemosensitization of mouse tumours by BENZO has also been observed with melphalan [6, 8, 9] and cyclophosphamide [10], but there is no clear evidence of therapeutic gain.

Because of the potential for therapeutic gain with CCNU in combination with low, non-toxic doses of BENZO, clinical studies were initiated in this Unit. In a Phase I study it was shown that sensitizing concentrations could be achieved in man with no toxicity due to BENZO and no enhancement of the haematological or gastrointestinal toxicity of CCNU [11]. A local Phase 2 study in melanoma has demonstrated some tumour responses* and a multi-centre Phase 3 study in recurrent glioma is now in progress under the auspices of the U.K. Medical Research Council.

A number of mechanisms have been proposed to account for the chemosensitization and therapeutic gain with nitroimidazoles (for recent review see [12]). These can broadly be classified into two types. The first are those operating at the level of biochemistry of the hypoxic tumour cell, including thiol depletion and altered DNA damage or repair. The dependence on hypoxia implicates a role for nitroreduction which generates reactive species thought to be responsible for nitroimidazole cytotoxicity [13].

The second type involves alterations in the pharmacokinetics of the cytotoxic agent by the sensitizer. We have shown that, under certain circumstances at least, the reduction in CCNU clearance by MISO can result in a preferential increase in tumour CCNU concentrations which accounts for the chemosensitization and therapeutic gain produced in mice [14]. On the other hand, when CCNU is administered orally to mice the resulting decrease in nitrosourea concentrations induced by MISO results in chemoprotection [15]. Modified nitrosourea pharmacokinetics were also seen with low doses of BENZO in mice [16] and in the human Phase I study [11]. All these findings are consistent with a role for inhibition of cytochrome P-450 by MISO and BENZO for which strong, though indirect, support was published previously [17].

In view of the two alternative mechanisms of chemosensitization by BENZO we have studied the nitroreduction of the drug to the amine metabolite by mouse tissues *in vitro* and *in vivo*, and the inhibition of CCNU hydroxylation, the principal metabolic pathway, by mouse liver microsomal cytochrome P-450 *in vitro*.

Materials and methods

Drugs. BENZO and its amine metabolite (*N*-benzyl-(2-aminoimidazolyl) acetamide, hydrochloride; Ro 11-1721) were obtained from Roche, Welwyn, U.K. and Basle, Switzerland, respectively. CCNU (1-(2-chloroethyl)-*N*-nitrosourea) was obtained from Lundbeck and the U.S. National Cancer Institute.

Mice. Mice (BALB/c and C3H/He) were obtained from OLAC (Bicester, U.K.) and our own breeding colony and used at 25–35 g. They were allowed laboratory chow and water *ad lib.* KHT and EMT6 tumours were grown in the gastrocnemius muscle of the hind leg [18]. For enzymological studies, microsomal and cytosolic fractions were prepared and stored using standard methods [19–21].

BENZO metabolism. For *in vivo* studies, BENZO (2.5 mmoles/kg) was injected i.p. in 0.01 ml/g of 50% polyethylene glycol/Hanks' salt solution. Plasma samples were obtained after cardiac puncture and tissues were immediately frozen at −70°. Urine was collected frozen over 24 hr.

Incubation conditions for the *in vitro* nitroreduction of BENZO were very similar to those of McManus *et al.* [22]. Flasks (25 ml conical) contained the following in volume of 3 ml: 83 mM phosphate buffer (pH 7.4), 0.9 mM NADPH and NADH, enzyme preparation (200–800 μl of 33–50% whole homogenate, 6 mg of microsomal protein or 21–24 mg cytosolic protein). Incubation mixtures were pregassed at 37° in a shaking water-bath for 7 min with humidified grade 0 nitrogen passed through a 15% w/v alkaline solution of pyragallol at a flow rate of 50 ml/min. The reaction was started by the addition of BENZO (0.083–1 mM). At intervals over 15 min, samples (100 μl) were removed into methanol prior to analysis.

CCNU metabolism. Incubation conditions for CCNU hydroxylation by liver microsomal cytochrome P-450 were very similar to those described previously [23, 24]. Flasks (as above) contained the following in 2.5 ml: 100 mM phosphate buffer (pH 7.4), 3.3 mM NADP, 8.3 mM glucose-6-phosphate, 0.4 Units/ml glucose-6-phosphate dehydrogenase and 2.5–5 mg microsomal protein. These were preincubated at 37° for 3 min, with or without MISO (0.65–20 mM) or BENZO (0.025–2 mM). The reaction was started by the addition of CCNU (0.01–0.6 mM) and the flasks were shaken vigorously to ensure full oxygenation. At intervals over 8 min, samples were removed into cold ether prior to analysis.

High-performance liquid chromatography (HPLC). Techniques for the analysis of CCNU and its hydroxylated metabolites and of BENZO and its amine metabolite were as described in detail elsewhere [14, 25].

Enzyme kinetics. Progress curves were linear in all cases and reaction rates were linear with protein concentration. Heat denatured preparations gave no activity. Cofactor concentrations were shown to be optimal and < 20% substrate was consumed. Kinetic data were analysed by standard techniques [26].

Results

Inhibition of CCNU hydroxylation by MISO and BENZO. In the initial studies the CCNU concentration (0.0485 mM) was approximately equal to the K_m and similar to peak plasma levels in mice [14]. Both MISO and BENZO were able markedly to inhibit the formation of hydroxylated CCNU metabolites by mouse liver cytochrome P-450 in a dose-dependent fashion (Fig. 1). It can be seen that the dose–response curve for BENZO was less steep than that for MISO, and that the former was a considerably more potent inhibitor over the entire concentration range tested. The I_{50} values were 0.37 mM and 5.8 mM for BENZO and MISO respectively. Significant inhibition occurred at pharmacological concentrations. For example, doses of 2.5 and 0.3 mmoles/kg MISO and BENZO respectively are commonly used in chemosensitization experiments in mice [6, 7]. These produce peak plasma concentrations of 2.5 mM and 0.12 mM respectively [27, 28], which would, in turn, inhibit CCNU hydroxylation by 30 and 32% respectively. BENZO amine was tested for inhibitory activity at 0.19 mM, the concentration achieved in mouse liver after

* Bleehen *et al.*, unpublished data.

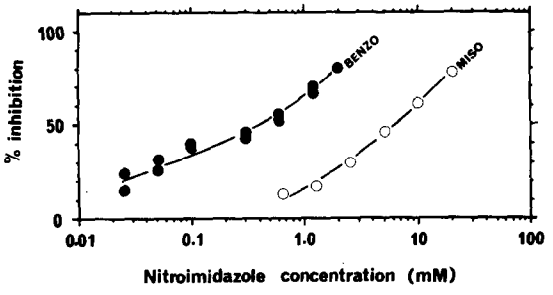

Fig. 1. Dose–response curve for the inhibition by BENZO (●) and MISO (○) of the hydroxylation of CCNU by mouse liver microsomal cytochrome P-450 *in vitro*. Pooled data from independent experiments.

a high dose of BENZO *in vivo* (see below). No inhibition was seen.

Detailed kinetic studies were carried out to determine the nature of the inhibition of CCNU hydroxylation by the nitroimidazoles. In experiments where the CCNU concentration was varied at fixed inhibitor concentrations, double-reciprocal (Lineweaver–Burk) plots revealed mixed competitive–non-competitive kinetics. For other experiments in which both CCNU and the inhibitor concentration were varied, the inhibitor constants K_i (competitive component) and K_i' were obtained from direct linear and Hunter-Downs plots. For BENZO, K_i (0.04 mM) was considerably lower than K' (0.174 mM) indicating that the competitive component predominates. The results for MISO were similar, but the values were about tenfold higher, consistent with less potent inhibition.

Nitroreduction of BENZO. In the *in vivo* metabolism studies there were no significant differences in results between C3H/He and BALB/c mice. Three hours after a single, large i.p. dose of BENZO (2.5 mmoles/kg), the concentration of parent drug in the plasma was 0.392–0.408 μmoles/ml. For all tissues studied, liver, kidney and the three solid tumours, the concentrations were virtually identical to the plasma at 0.385 μmoles/g. Concentrations of BENZO amine in mouse plasma were very low, being about 0.5% of the parent drug. Amine concentrations were also low in the KHT and RIF-1 tumours (0.6 and 1.1% of parent drug respectively). Increasing levels of the metabolite were seen in the EMT6 tumour (3.6%), kidney (14%) and particularly liver (50–55%). No amine could be detected in the brain. Urinary recoveries for BENZO and its amine metabolite were 3.5–5% and 2.7–3.3% respectively.

Using whole liver homogenates *in vitro* the stoichiometry for the BENZO loss : amine formation was 1 : 0.35. The K_m for amine formation was 0.2 mM and the V_{max} 2 nmoles/min/mg protein. The reaction was inhibited by about 90% in air but only 20% in carbon monoxide, and exhibited a requirement for NADPH. The activity was located predominantly in the microsomal fraction. These characteristics suggest a major involvement of NADPH : cytochrome P-450 (cytochrome *c*) reductase. Evidence with inhibitors was consistent with a role for both aldehyde oxidase and xanthine oxidase in the liver cytosol activity.

Studies with whole homogenates of KHT and EMT6 tumours *in vitro* confirmed their low reductase activity compared to liver. However, the order of activity was KHT > EMT6 which is in contrast with the concentrations of the amine present in these tumours *in vivo*.

Discussion

Hypoxia-mediated nitroreduction of nitroimidazoles in tumour cells leading to increased cellular sensitivity, and altered pharmacokinetics of cytotoxic agents by nitroimidazoles are alternative, though not mutually exclusive, mechanisms for chemosensitization. Both may operate *in vivo*, to an extent depending on the particular circumstances. The present paper describes results pertinent to both mechanisms, with particular reference to BENZO which is currently being evaluated clinically in combination with the nitrosourea CCNU.

Although BENZO pharmacokinetics have been reported previously in animals [28] and in man [11, 29, 30] only brief mention had been made of its metabolism [31]. We have shown that the amine nitroreduction product is present in large amounts in mouse liver and to a lesser extent kidney, with much lower levels in tumours and none in brain. This distribution is presumed to reflect the capacity to reduce BENZO *in vivo*. *In vitro* studies confirmed the high BENZO nitroreductase activity of the liver compared to the tumours but the individual tumour reductase activities did not predict correctly for the amine distribution *in vivo*. The latter is likely to be a function of (1) the intrinsic nitroreductase activity of the tissue and (2) the ambient oxygen tension, the latter being a potent inhibitor of nitroreduction [13].

Evidence was obtained that the main mouse liver enzyme responsible for BENZO reduction is cytochrome P-450 (cytochrome *c*) reductase. This is in contrast to previous findings [32] but in agreement with results for MISO [22]. About 35% of BENZO consumed in the nitroreduction reaction was detected as the terminal amine reduction product. Previous work has shown that reduction of both MISO and BENZO results in covalent reaction of putative reactive intermediates with protein [22, 32]. In addition, the imidazole ring may fragment on nitroreduction, as occurs with MISO (see [13]).

From the point of view of the mechanism of chemosensitization, the most important finding is that nitroreduction cannot be assumed to occur more abundantly in tumour compared to normal tissues. The toxocological consequences remain to be established.

In contrast to BENZO amine, pharmacological concentrations of BENZO and MISO were able to inhibit the oxidative hydroxylation of CCNU by mouse liver cytochrome P-450 *in vivo*. This provides clear confirmation that inhibition of CCNU metabolism is a major mechanism of the altered pharmacokinetics of CCNU, and in turn of chemosensitization. BENZO was 15 times more potent an inhibitor than MISO. At optimal doses for chemosensitization [6, 7] the plasma concentrations of both agents [27, 28] would inhibit microsomal hydroxylation of CCNU by about 30%. Similar concentrations of BENZO (0.115 mM) were achieved in the Phase I study in man [11]. This leads to inhibition of first-pass metabolism of oral CCNU, a breakthrough of CCNU in the plasma and an increased exposure to total nitrosoureas. Neither this increased exposure nor the inevitable nitroreduction of BENZO appear to result in enhanced normal tissue toxicity. It remains to be seen whether enhancement of antitumour activity will ensue.

MRC Unit and University Department of Clinical Oncology and Radiotherapeutics Medical School Hills Road Cambridge CB2 2QH, U.K.

PAUL WORKMAN*
MICHAEL I. WALTON
FRANCIS Y. F. LEE†

* To whom correspondence should be addressed.

† Present address: Experimental Therapeutics Division and Division of Radiation Oncology, University of Rochester Cancer Center, 601 Elmwood Avenue, Box 704, Rochester, NY 14642.

REFERENCES

1. C. A. Barclay, J. A. Cerisola, H. Lugones and O. Ledesma, in *Current Chemotherapy, Proceedings of the 10th International Congress of Chemotherapy,* Vol. 1 (Eds. W. Siegenthaler and R. Luthy), p. 158. American Society for Microbiology, Washington (1978).
2. J. A.Cerisola, C. A. Barclay, J. L. Silva and G. Mouzo, in *Current Chemotherapy, Proceedings of the 10th International Congress of Chemotherapy,* Vol. 1 (Eds. W. Siegenthalen and R. Luthy), p. 159. American Society for Microbiology, Washington (1978).
3. J. R. Coura, P. J. Brindeiro and I. Ferreira, in *Current Chemotherapy, Proceedings of the 10th International Congress of Chemotherapy,* Vol. 1 (Eds. W. Siegenthaler and R. Luthy), p. 161. American Society for Microbiology, Washington (1978).
4. S. Di C. Fava, V. A. Zamith, L. C. Luce and S. A. P. Sampaio, in *Current Chemotherapy, Proceedings of the 10th International Congress of Chemotherapy,* Vol. 1. (Eds. W. Siegenthaler and R. Luthy), p. 63. American Society for Microbiology, Washington (1978).
5. P. Workman and P. R. Twentyman, *Br. J. Cancer* **46**, 249 (1982).
6. P. R. Twentyman and P. Workman, *Br. J. Cancer* **48**, 17 (1983).
7. D. W. Siemann, S. Morrisey and K. Wolf, *Cancer Res.* **43**, 1010 (1983).
8. P. W. Sheldon and E. L. Batten, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 635 (1982).
9. D. W. Siemann, K. Maddison and K. Wolf, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1657 (1984).
10. D. J. Chaplin, P. W. Sheldon, I. Ahmed and G. E. Adams, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1647 (1984).
11. J. T. Roberts, N. M. Bleehen, F. Y. F. Lee, P. Workman and M. I. Walton, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1745 (1984).
12. D. W. Sieman, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1585 (1984).
13. A. M. Rauth, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1293 (1984).
14. F. Y. F. Lee and P. Workman, *Br. J. Cancer* **49**, 579 (1984).
15. F. Y. F. Lee and P. Workman, *Br. J. Cancer* **51**, 85 (1984).
16. F. Y. F. Lee and P. Workman, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1627 (1984).
17. P. Workman, P. R. Twentyman, F. Y. F. Lee and M. I. Walton, *Biochem. Pharmac,* **32**, 857 (1983).
18. P. R. Twentyman, R. F. Kallman and J. M. Brown, *Int. J. Radiat. Oncol. Biol. Phys.* **5**, 1255 (1979).
19. M. K. Wolpert, J. R. Althaus and D. G. Johns, *J. Pharmac. exp. Ther.* **185**, 202 (1973).
20. L. C. Eriksson, J. W. De Pierre and G. Dallner, *Pharmac. Ther.* **2**, 281 (1978).
21. P. Borton, R. Carson and D. J. Reed, *Biochem. Pharmac.* **23**, 2332 (1974).
22. M. E. McManus, M. A. Lang, K. Stuart and J. Strong, *Biochem. Pharmac.* **31**, 547 (1982).
23. H. E. May, R. Boose and D. J. Reed, *Biochem. biophys. Res. Commun.* **57**, 426 (1974).
24. H. E. May, S. J. Kohlhepp, R. B. Boose and D. J. Reed, *Cancer Res.* **39**, 762 (1979).
25. M. I. Walton and P. Workman, submitted to *J. Chromatog.*
26. A. Cornish-Bowden, *Principles of Enzyme Kinetics.* Butterworth, London (1976).
27. P. Workman and J. M. Brown, *Cancer Chemother. Pharmac.* **6**, 39 (1981).
28. P. Workman, R. A. S. White, M. I. Walton, L. N. Owen and P. R. Twentyman, *Br. J. Cancer* **50**, 291 (1984).
29. J. Raaflub and W. H. Ziegler, *Arzneimittel-Forsch.* **29**, 1611 (1979).
30. J. Raaflub, *Arzneimittel-Forsch.* **30**, 2192 (1980).
31. D. E. Schwartz and W. Hofheinz, in *Nitroimidazoles: Chemistry, Pharmacology and Clinical Application* (Eds. A. Breccia, C. Rimondi and G. E. Adams), *Nato Advanced Study Institute Series, Series A: Life Sciences,* p. 91. Plenum, New York (1978).
32. H. Masana, E. G. D. de Toranzo and J. A. Castro, *Biochem. Pharmac.* **33**, 1041 (1984).