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MISONIDAZOLE AND BENZNIDAZOLE INHIBIT HYDROXYLATION OF CCNU BY MOUSE LIVER MICROSOMAL CYTOCHROME P-450 *IN VITRO*

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Abstract—On the basis of promising experimental studies, the nitroimidazoles misonidazole (MISO) and benznidazole (BENZO) are under clinical investigation as chemosensitizers in combination with the chloroethylnitrosourea CCNU. We have shown previously that MISO and BENZO can alter the pharmacokinetics of CCNU leading to an improved therapeutic index in mice. Here we demonstrate using optical difference spectroscopy that MISO and BENZO are able to bind to cytochrome P-450 of mouse liver microsomes *in vitro*. Binding was type II in nature, indicating co-ordination of the free imidazole nitrogen with the heme moiety of cytochrome P-450. This results in an inhibition of CCNU hydroxylation by the hemoprotein. The kinetics of inhibition were of a mixed competitive–non-competitive type. At a CCNU concentration of 0.05 mM the concentrations causing 50% inhibition (I_{50}) were 5.8 and 0.37 mM for MISO and BENZO respectively. At doses producing a similar improvement in therapeutic index in mice (2.5 mmoles/kg MISO and 0.3 mmoles/kg BENZO) the plasma and tissue concentrations achieved would inhibit CCNU hydroxylation by 30%. For BENZO, but not MISO, similar inhibition would also occur at concentrations which can be achieved safely in man.

The 2-nitroimidajole misonidajole (MISO‖), well known as a radiosensitizer of hypoxic cells [1, 2], is also able to potentiate the toxicity of certain conventional chemotherapeutic agents against both hypoxic cells *in vitro* and experimental tumours *in vivo* [3–5]. This effect, referred to as "chemosensitization", has been observed most frequently with the bifunctional nitrogen mustards cyclophosphamide and melphalan and with the chloroethylnitrosourea CCNU. An essential feature of nitroimidazole chemosensitization, as far as clinical development is concerned, is that toxicity to tumour is increased much more than that towards critical normal tissues, resulting in a clear improvement of therapeutic index. Because of the dose-limiting neurotoxicity of MISO, analogues with greater potency have been sought, one of which is the more lipophilic analogue benznidazole (BENZO) [6]. Lower doses of BENZO in mice give a similar therapeutic gain to higher doses of MISO [7, 8]. Several clinical trials are now in progress or planned, including MISO or BENZO combined with CCNU for the treatment of recurrent glioma [9, 10].

The mechanism of chemosensitization by nitroimidazoles is still not fully understood. Evidence has been obtained for two widely different modes of action. The first type includes such effects as thiol depletion and altered DNA damage or repair, operates at the level of the biochemistry of hypoxic tumour cells, and probably requires nitroreductive bioactivation of the sensitizer [5, 11]. The second type involves modification of the pharmacokinetics of the cytotoxic agent by the sensitizer [5]. These alternative mechanisms are not mutually exclusive and both are likely to be important, though their relative contributions may vary depending on the circumstances. For the combination of a single, relatively high dose of MISO with CCNU in mice, we have shown that the pharmacokinetic mechanism predominates, and that enhancement of tumour response and therapeutic gain can both be obtained by purely pharmacokinetic means [12, 13]. This results from a delayed plasma clearance of CCNU, leading to an elevated peak concentration in tumour but not normal tissues. In addition, under different experimental conditions, reduced peak CCNU concentrations by MISO can give rise to chemoprotection [14]. We have also shown that ability to modify CCNU pharmacokinetics and chemosensitization activity are both functions of the hydrophobic character of the nitroimidazole molecule, lipophilic analogues such as BENZO being particularly effective [6, 15].

In a recent clinical study we have demonstrated significant changes in the plasma pharmacokinetics of CCNU by BENZO in man, including the appearance of parent drug and a reduction in the concentration of hydroxylated metabolites [9]. Metab-

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‖ Abbreviations used: MISO, misonidazole, 1-(2-nitroimidazolyl)-3-methoxypropan-2-ol; BENZO, benznidazole, *N*-benzyl-(2-nitroimidazolyl)acetamide; Ro 07-1902, 1-(2-nitroimidazolyl)-3-allyloxypropan-2-ol; CCNU, lomustine, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; PB, sodium phenobarbitone, phenobarbital; SKF 525A, proadifen hydrochloride, β-diethylaminoethyl diphenylpropylacetate hydrochloride; HPLC, high-performance liquid chromatography.

olism of CCNU to hydroxylated products represents an important *in vivo* elimination pathway [12, 16–18]. This reaction is catalysed by cytochrome P-450 in rodent liver microsomes [17, 19–23]. The hydroxylated metabolites have been shown to possess chemical and biological properties which differ between themselves as well as compared to the parent drug [22]. Effects on CCNU metabolism by liver microsomal cytochrome P-450 were suspected as at least one cause of the pharmacokinetic modifications, and evidence for inhibition of cytochrome P-450-mediated metabolism of other drugs in mice has been obtained in our laboratory [24]. In the present paper we provide direct evidence that at pharmacological concentrations MISO and BENZO are able to bind to the cytochrome P-450 of mouse liver microsomes and thereby inhibit the hydroxylation of CCNU by this hemoprotein.

MATERIALS AND METHODS

Drugs. For *in vitro* work, MISO (Roche Products Ltd, Welwyn Garden City, Herts, U.K.), imidazole (Sigma Chemical Company, Poole, Dorset) and SKF 525A (Smith Kline & French Laboratories Ltd, Welwyn Garden City, Herts, U.K.) were dissolved in 0.1 M potassium phosphate buffer, pH 7.4, while BENZO (Roche) and CCNU (US National Cancer Institute) were dissolved in dimethyl sulphoxide. The volume of solvent did not exceed 0.5% of the incubation volume and had no effect on CCNU hydroxylation or microsomal difference spectra. For induction of mouse liver microsomal cytochrome P-450, sodium phenobarbitone (PB, Evans Medical Ltd, Liverpool, Merseyside, U.K.) was dissolved in Dulbecco "A" phosphate-buffered saline, pH 7.4, and injected i.p. in 0.01 ml/g body weight. Mice received a single dose of 80 mg/kg on the first day, followed by single doses of 100 mg/kg on each of five consecutive days, the last being 24 hr before sacrifice.

Mice. Four- to five-week-old male C3H/He mice (OLAC, Bicester, U.K.) weighing about 20 g were used. They were allowed laboratory chow and water *ad lib* but food was withdrawn 24 hr before use. Mice were killed by cervical dislocation and the liver excised. The gall bladder was discarded and the liver washed with ice-cold 0.15 M KCl.

Preparation of microsomes. Microsomes were prepared and stored based on standard methods [25, 26]. Briefly, pooled, washed livers from several mice were chopped finely and homogenized in 4 vol. of cold 0.25 M sucrose using a Potter S homogenizer (B. Brann Melsungen AG, F.R.G.) fitted with a glass vessel and Teflon pestle (400 r.p.m. with 4 passes). The homogenate was centrifuged at 10,000 g for 20 min at 4°. The supernatant was decanted and centrifuged at 100,000 *g* for 60 min at 4°. The microsomal pellet was then washed by rehomogenization in 0.15 M KCl and recentrifugation as above. For spectral studies, the washed pellet was frozen rapidly and stored at −70°; for metabolic studies, microsomes were stored at −70° as a suspension in 0.15 M KCl with no measurable loss of activity. Protein and cytochrome P-450 content were measured by standard techniques [27, 28].

Microsomal metabolism. Incubation conditions for hydroxylation of CCNU by liver microsomal cytochrome P-450 were very similar to those used previously [19, 20]. The incubation mixture (2.5 ml) contained the following: 0.1 M potassium phosphate buffer, pH 7.4; 3.3 mM NADP; 8.3 mM glucose-6-phosphate; 0.4 Units/ml yeast glucose-6-phosphate dehydrogenase; 0.77–3.1 mg/ml microsomal protein; and 0.01–0.03 mM CCNU. All the biochemical reagents were obtained from Sigma. Modifiers were added before other chemicals. Incubations were carried out at 37° in 25 ml Erlenmeyer flasks with vigorous shaking to ensure full oxygenation. Mixtures were preincubated at 37° for 3 min before the reaction was started by the final addition of CCNU.

Sample preparation and HPLC. At various times after the start of the reaction, 100 μl aliquots of the mixture were removed and placed immediately in glass tubes containing 1 ml cold diethyl ether. Standards were processed at the same time. CCNU and its hydroxylated metabolites were extracted into the organic phase by vigorous shaking, with a recovery of >95%. Aliquots (750 μl) of the ether were removed, and processed and analysed by reversed-phase HPLC as described previously [12, 18].

Enzyme kinetics. Kinetic parameters (K_m and V_{max}) were determined from double-reciprocal Lineweaver–Burk plots [29] by linear regression analysis using the GLIM statistical programs of the Royal Statistical Society of London. Inhibitor constants, K_i and K_i', were determined from direct linear plots of $1/v$ against [i] (modified from ref. 30) and [s]/v against [i] respectively [31].

Cytochrome P-450 optical difference spectroscopy. The binding of test substances to PB-induced microsomal cytochrome P-450 was monitored by optical difference spectroscopy as described by Jefcoate [32] and incorporating the split cell modification for the nitroimidazoles. Briefly, microsomes were diluted to approximately 2 mg protein/ml in 0.05 M potassium phosphate buffer (pH 7.4) and spectra were recorded between 360 and 480 nm using a Cary 219 twin-beam scanning spectrophotometer with automatic baseline subtraction capability.

RESULTS

Hydroxylation of CCNU by mouse liver microsomes

The hydroxylation of CCNU is catalysed by cytochrome P-450 in rodent liver microsomes [17, 19–23]. Our work was carried out with microsomes from the livers of untreated male C3H/He mice, since these were used for our previous *in vivo* studies [12–15]. The characteristics of the reaction were similar to those observed for other rodent microsomal preparations, and a brief summary is given below.

We confirmed the finding [22] that mouse liver microsomes metabolise CCNU to form five of the possible six isomeric monohydroxylated derivatives; in our system the proportions were typically *cis*-4-hydroxy CCNU, 64%; *trans*-4, 15%; *cis*-3, 9%; *trans*-3, 12%; and *trans*-2, trace. Progress curves for the formation of the metabolites were linear up to 8 min but significantly non-linear thereafter. Initial velocity was determined from the linear region, six time points normally being used. Less than 20% of the

CCNU substrate was converted to products during the 8 min incubation period. Previous studies with a very similar assay system have shown the chemical half-life of CCNU and its monohydroxylated metabolites to be 51 min [17]; thus the maximum loss of nitrosoureas could not exceed 10% in 8 min. Other studies, also with a similar system, have shown that the rate of the competing denitrosation pathway was <5% of the hydroxylation reaction [21]. No correction was made for these losses. No hydroxylation was seen in the absence of microsomes or with boiled microsomes. The individual components of the NADPH generating system were required for activity, and their concentrations were shown to be not rate-limiting. The rate of formation of metabolites was proportional to microsomal protein in the range 0.77–3.1 mg/ml assay mixture.

The production of hydroxylated metabolites followed Michaelis–Menten kinetics and double-reciprocal plots were linear (Fig. 1), although some substrate inhibition was seen above 0.3 mM CCNU (not shown). Values of K_m and V_{max} for the formation of 3 most abundant metabolites are given in Table 1. The K_m values were generally similar while considerable differences were seen in V_{max}. The sum of the individual V_{max} values was close to a previous estimate for non-induced rat liver microsomes [21], as well as to rates of hydroxylation by the latter at 0.6 mM CCNU substrate concentration [19, 20]. The K_m is much lower than an earlier value for non-induced rat liver microsomes [21], but very close to the spectral binding constant (K_s) for PB-induced rat liver microsomes [19].

Effects of MISO and BENZO on CCNU hydroxylation by mouse liver microsomes

Having established the characteristics of the reaction for microsomes from our mice, we studied the effect of MISO and BENZO on CCNU hydroxylation by cytochrome P-450. For the first series of experiments the CCNU substrate concentration was fixed at 0.05 mM (11 μg/ml), a value similar to both the K_m (Table 1) and the peak concentration in mouse plasma [12], while the nitroimidazole concentration was varied over a wide range. Both MISO and BENZO were shown to inhibit the reaction in a concentration-dependent manner (Fig. 2). (For MISO, preliminary experiments showed that the extent of inhibition was independent of the hydroxylated product measured, and the principal *cis*-4-hydroxy metabolite was chosen for quantitation. The different HPLC conditions required to resolve BENZO from the hydroxylated nitrosoureas did not allow resolution of individual metabolites from each other, and the formation of total hydroxylated products was therefore used.) The concentrations of MISO and BENZO causing 50% inhibition (I_{50}) were 5.8 mM and 0.37 mM respectively: thus BENZO was a 15-fold more potent inhibitor of CCNU hydroxylation.

In the next series the CCNU substrate was varied in the presence or absence of the nitroimidazoles, and Fig. 1 shows some typical double-reciprocal plots. Experiments were also carried out in which both substrate and inhibitor concentrations were varied. The results were compatible with mixed competitive–non-competitive inhibition. Using direct linear plots [31], the inhibition constants K_i (competitive component) and K_i' (non-competitive component) were found to be 6.2 mM and 15.8 mM respectively for MISO, compared to 0.044 and 0.174 mM respectively for BENZO. Both constants were about 100-fold lower for BENZO than for MISO, again reflecting the greater inhibitory potency of BENZO. It can also be seen that for both compounds K_i is much lower than K_i', indicating that the competitive component is predominant.

Drug-cytochrome P-450 binding characteristics

Since the nitroimidazoles were found to elicit fairly weak optical difference spectra for cytochrome P-450 in liver microsomes from untreated C3H/He male mice, PB-induced microsomes were used in subsequent studies to give a stronger signal. However, the spectra obtained from the two types of microsomes were demonstrated to be quantitatively identical. The intrinsic absorption of the nitroimidazole ligands necessitated the use of split cells [32] to record acceptable spectra. Figure 3 shows the difference spectra resulting from the addition of either MISO or BENZO to PB-induced mouse microsomes. Evidence of binding to cytochrome P-450 was obtained in each case. Both compounds gave spectra with a broad trough at ~390 nm, a peak at 425 nm, and a zero-crossing (isosbestic) point at 410–415 nm. These are characteristic of type II difference spectra [32–34]. It is noteworthy that BENZO gave a significant difference spectrum at a concentration as low as 0.1 mM, whereas for MISO a difference spectrum could not be demonstrated below 1 mM. This suggests that BENZO is a more potent ligand than MISO.

K_s values were not obtained for MISO or BENZO because of relatively low affinity and limited solubility respectively. However, with the close analogue Ro 07-1902,* which has intermediate inhibitory potency between MISO and BENZO but is more soluble than the latter, a K_s value of 5.4 mM was obtained.

Several positive controls were included. As found previously (34, 35), the parent heterocycle imidazole gave a typical type II difference spectrum (peak 430 nm; trough 395 nm; isosbestic point 420 nm). The classical microsomal enzyme inhibitor SKF 525A and the substrate CCNU both gave the expected type I binding spectra [19, 34, 35].

DISCUSSION

MISO and BENZO are currently undergoing clinical trial in man as chemosensitizers with CCNU [9, 10, 36]. These trials are based on extensive experimental studies in mice which show that each is able to enhance the response of solid tumours to CCNU, whereas the response of critical normal tissues are relatively unaffected, resulting in clear therapeutic gain [6–8, 37–39]. We have previously shown that with single high doses of MISO in mice the above effects can be explained by changes in CCNU pharmacokinetics [12, 13]. MISO delayed the systemic

* 1-(2-nitroimidazolyl)-3-allyloxypropan-2-ol.

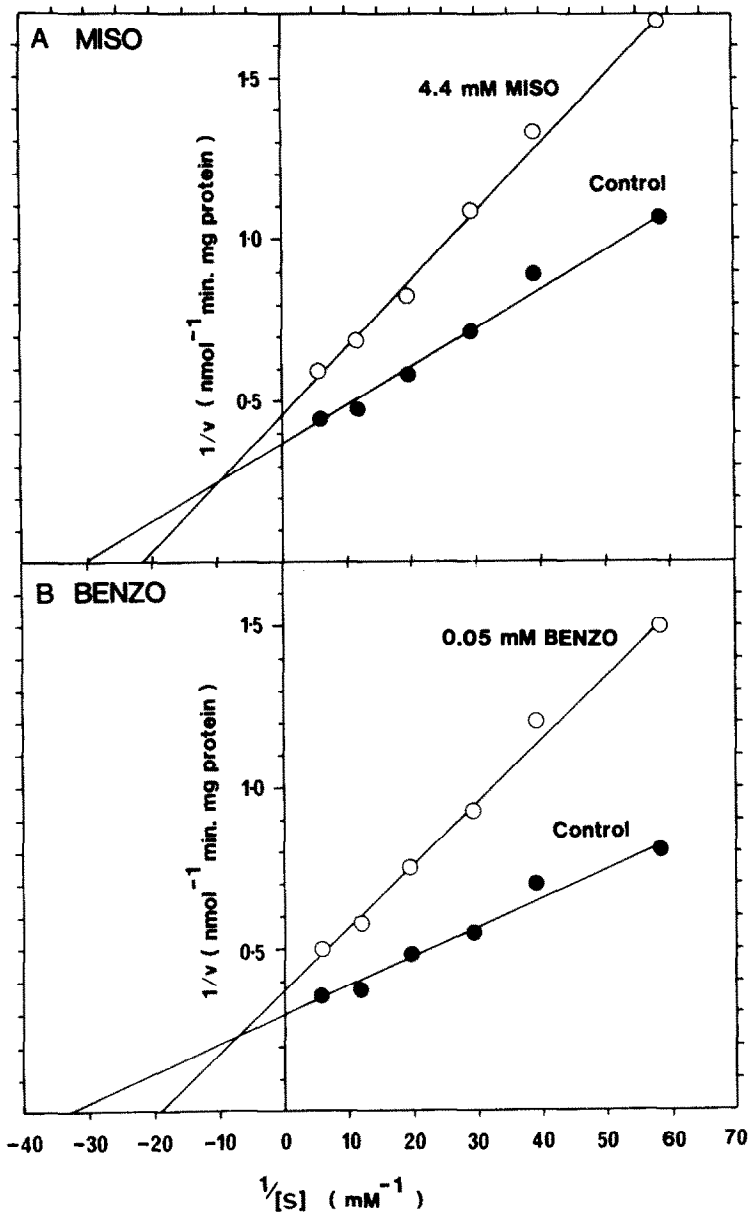

Fig. 1. Double-reciprocal plots of the effects of MISO and BENZO on the *in vitro* hydroxylation of CCNU by liver microsomes from untreated male C3H/He mice. Reaction mixtures (2.5 ml) contained: 0.1 M potassium phosphate buffer, pH 7.4; 3.3 mM NADP; 8.3 mM glucose-6-phosphate; 0.4 Units/ml yeast glucose-6-phosphate dehydrogenase; 1–2 mg/ml microsomal protein; 0.01–0.3 mM CCNU; and MISO, BENZO or nitroimidazole vehicle alone. (A) Effect of 4.4 mM MISO on the formation kinetics of the major *cis*-4 hydroxy metabolite. (B) Effect of 0.05 mM BENZO on the formation kinetics of total hydroxylated metabolites. Closed circles, without modifier; open circles, with modifier. Results shown are for typical experiments.

(plasma) clearance of CCNU leading to elevated peak CCNU concentrations in tumour but not normal tissues. BENZO was more active than MISO in reducing CCNU clearance in mice [15]. Moreover in human studies BENZO was shown to cause the appearance of parent CCNU in the plasma after oral administration, whereas only hydroxylated metabolites were seen without BENZO [9, 18]. These results suggested that the nitroimidazole chemosensitizers were reducing CCNU clearance by inhibiting its rate of hydroxylation. In this study we sought to establish that MISO and BENZO do indeed inhibit CCNU metabolism by hepatic microsomal cytochrome P-450.

The optical difference spectra data showed that, as with other nitrogen containing heterocyclic compounds [40], MISO and BENZO act as ligands of cytochrome P-450 yielding typical type II binding spectra. This type of spectrum is thought to be indicative of the formation of nitrogenous ligands with the

Table 1. Values of K_m and V_{max} for the formation *in vitro* of monohydroxylated metabolites of CCNU by liver microsomes from untreated male C3H/He mice

Metabolite	K_m (mM)	V_{max} (nmol·min^{-1}·mg protein^{-1})
cis-4-OH CCNU	0.026 (0.019–0.032)	1.92 (1.70–2.22)
trans-4-OH CCNU	0.030 (0.017–0.043)	0.470 (0.371–0.668)
trans-3-OH CCNU	0.043 (0.029–0.058)	0.083 (0.067–0.108)

Values were computed from a typical experiment using the GLIM program. Repeat experiments gave similar results. 95% confidence limits are shown in parentheses. For reaction conditions see legend to Fig. 1.

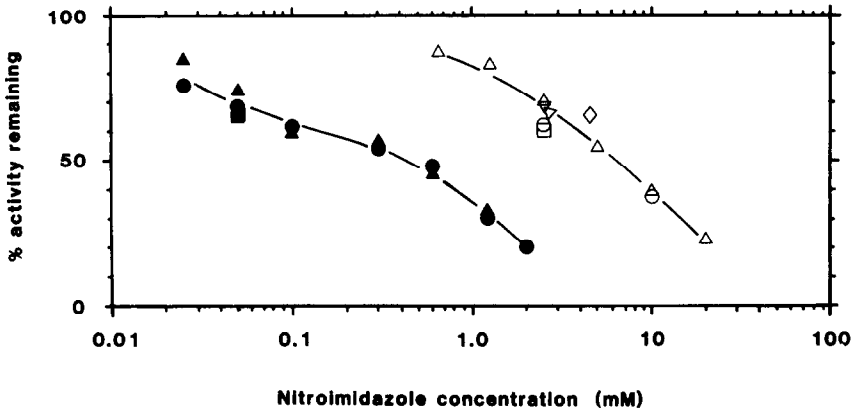

Fig. 2. Effect of MISO (open symbols) and BENZO (closed symbols) on the *in vitro* hydroxylation of CCNU by liver microsomes for untreated male C3H/He mice. Reaction mixtures (2.5 ml) contained: 0.1 M potassium phosphate buffer, pH 7.4; 3.3 mM NADP; 8.3 mM glucose-6-phosphate dehydrogenase; 1–2 mg/ml microsomal protein; 0.05 mM CCNU, and 0.6–20 mM MISO, 0.025–2 mM BENZO or nitroimidazole vehicle alone. Results are pooled for several independent experiments, indicated by different symbols. Control rates were typically 2 nmol min^{-1} mg protein^{-1}.

heme-iron of the cytochrome P-450 molecule [32–34]. In the case of MISO and BENZO binding would be through the single free imidazole nitrogen. BENZO appeared to be a more potent ligand than MISO: this probably results, at least in part, from the greater hydrophobic character of BENZO, a property which increases the binding affinity of other classes of substituted imidazoles [40].

Although MISO and BENZO both bind to cytochrome P-450 as characteristic type II ligands, both can also act as substrates, MISO for *O*-demethylation [41] and BENZO for nitroreduction [42]. Thus there may be a hidden type I binding component, as has been demonstrated for the lipophilic 1-geranylimidazole [43]. The kinetics of inhibition of CCNU hydroxylation by MISO and BENZO are therefore likely to be quite complex.

As might be predicted from the binding experiments BENZO proved to be a more potent inhibitor than MISO. Following the model of Wilkinson *et al.* [44] it is tempting to speculate that the molecular mechanism of inhibition involves two components: (1) co-ordination of the imidazole nitrogen to the heme-iron of cytochrome P-450, thereby interfering with the binding of molecular oxygen; (2) attachment of the hydrophobic side-chain to the substrate binding site, thus reducing the accessibility of CCNU.

With I_{50} values of 0.37 and 5.8 mM respectively, BENZO and MISO cannot be regarded as highly potent inhibitors of cytochrome P-450. This was to be predicted from previous structure–activity relationships which showed 2-substituted imidazoles to exhibit weaker binding and inhibitory activity compared to mono-1 and mono-4(5)-substituted derivatives [44, 45]. These studies identified the requirement for at least one sterically unhindered imidazole nitrogen. MISO and BENZO are suboptimal ligands and inhibitors because of substitution at N-1 together with steric hindrance at N-3 from the nitro group in the 2-position.

Nevertheless it is important to emphasize that MISO and BENZO both are able markedly to inhibit hydroxylation of CCNU at concentrations similar to those found in the plasma and liver of mice given doses producing effective chemosensitization. For example, at 2.5 mmoles/kg the MISO concentration achieved (2.5 mM [46]) would inhibit CCNU hydroxylation by 32% (see Fig. 2). At the lower but equally effective BENZO dose of 0.3 mmoles/kg the achieved concentration (0.12 mM [47]) would inhibit to an identical extent. Both these regimens yield a similarly improved therapeutic index. On the other

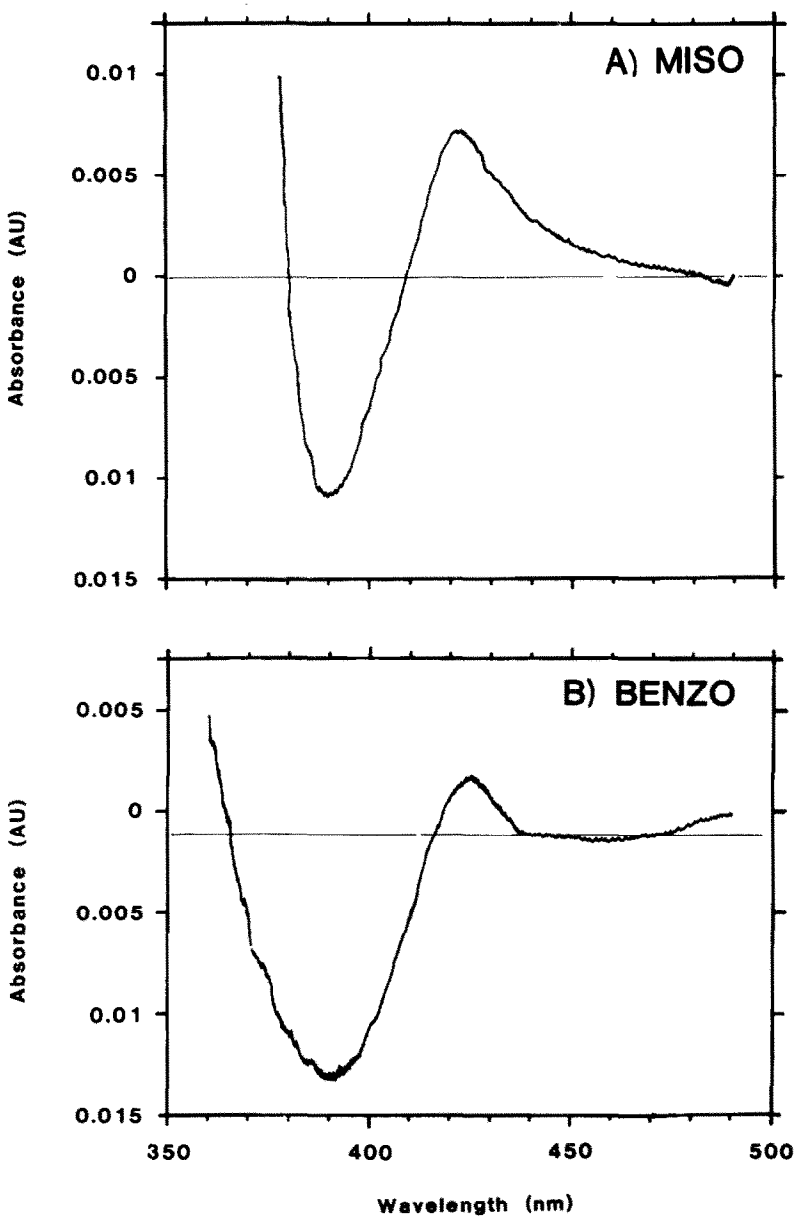

Fig. 3. Optical difference spectra for the *in vitro* interaction of liver microsomes from PB-induced male C3H/He mice with (A) 2.5 mM MISO or (B) 0.1 mM BENZO. The cytochrome P-450 concentration was 9.82 nmol/ml in 0.05 M potassium phosphate buffer, pH 7.4. Results shown are for typical experiments. Spectra are of type II [32–34]. The steep rise at low wavelengths is caused by a reduction in energy due to the intrinsic absorption of the nitroimidazole ligands.

hand, with a higher dose BENZO regimen which results in a reduced therapeutic gain [48] the achieved concentration of 0.38 mM would inhibit CCNU hydroxylation by as much as 52%. We propose, therefore, that optimal enhancement of CCNU response through the pharmacokinetic mechanism requires nitroimidazole concentrations which inhibit CCNU metabolism by approximately 30%.

In the Cambridge Phase I study of BENZO plus CCNU, oral doses of 8–40 mg/kg BENZO produced plasma BENZO concentrations of 0.04–0.24 mM [9]. Extrapolating from the *in vitro* mouse data, these would be expected to inhibit CCNU metabolism by 20–43%. Thus concentrations of BENZO achieved in man are sufficient to inhibit CCNU metabolism to an extent which results in an improved therapeutic index in mice. In contrast, with the normal maximum oral dose of 3 g/m^2 MISO, the human plasma concentration of 0.5 mM [49] would produce less than 10% inhibition.

In summary, direct evidence has been obtained that the nitroimidazole chemosensitizers, MISO and BENZO, are able to bind to cytochrome P-450 of mouse liver microsomes, leading to an inhibition of CCNU hydroxylation. The kinetics of inhibition have been partially characterised, and an estimate made

of the extent of inhibition required to produce a therapeutic gain in mice through altered pharmacokinetics. It is envisaged that these findings will facilitate the interpretation of current clinical investigations of nitroimidazole–CCNU combinations. In addition, we hope that further structure–activity relationship studies for inhibition of CCNU hydroxylation by nitroimidazoles will provide guidelines for the optimization or elimination of this property in the design of future sensitizers.

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REFERENCES

1. G. E. Adams, I. R. Flockhart, C. E. Smithen, I-J. Stratford, P. Wardman and M. E. Watts, *Radiat. Res.* **67**, 9 (1976).
2. J. F. Fowler and J. Denekamp, *Pharmac. Ther.* **7**, 413 (1979).
3. N. J. McNally, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 593 (1982).
4. D. W. Siemann, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 1029 (1982).
5. D. W. Siemann, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1585 (1984).
6. P. Workman and P. R. Twentyman, *Br. J. Cancer* **46**, 249 (1982).
7. P. R. Twentyman and P. Workman, *Br. J. Cancer* **48**, 17 (1983).
8. D. W. Siemann, S. Morrisey and K. Wolf, *Cancer Res.* **43**, 1010 (1983).
9. J. T. Roberts, N. M. Bleehen, F. Y. F. Lee, P. Workman and M. I. Walton, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 1745 (1984).
10. D. S. Fulton and R. C. Urtasun, *Int. J. Radiat. Oncol. Biol. Phys.* in press.
11. J. M. Brown, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 675 (1982).
12. F. Y. F. Lee and P. Workman, *Br. J. Cancer* **47**, 659 (1983).
13. F. Y. F. Lee and P. Workman, *Br. J. Cancer* **49**, 579 (1984).
14. F. Y. F. Lee and P. Workman, *Br. J. Cancer* **51**, 85 (1985).
15. F. Y. F. Lee and P. Workman, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1627 (1984).
16. M. D. Walker and J. Hilton, *Cancer Treatment Rep.* **60**, 725 (1976).
17. J. Hilton and M. D. Walker, *Biochem. Pharmac.* **24**, 2153 (1975).
18. F. Y. F. Lee, P. Workman, J. T. Roberts and N. M. Bleehen, *Cancer Chemother. Pharmac.* **14**, 125 (1985).
19. H. E. May, R. Boose and D. J. Reed, *Biochem. biophys. Res. Comm.* **57**, 426 (1974).
20. H. E. May, R. Boose and D. J. Reed, *Biochemistry* **14**, 4723 (1975).
21. D. L. Hill, M. C. Kirk and R. F. Struck, *Cancer Res.* **35**, 296 (1975).
22. G. P. Wheeler, T. P. Johnston, B. J. Bourdon, G. S. McCalen, D. L. Hill and J. A. Montgomery, *Biochem. Pharmac.* **26**, 2331 (1977).
23. D. W. Potter, W. Levin, D. E. Ryan, P. E. Thomas and D. J. Reed, *Biochem. Pharmac.* **33**, 609 (1984).
24. P. Workman, P. R. Twentyman, F. Y. F. Lee and M. I. Walton, *Biochem. Pharmac.* **32**, 857 (1983).
25. L. C. Eriksson, J. W. De Pierre and G. Dallner, *Pharmac. Ther.* **2**, 281 (1974).
26. P. Borton, R. Carson and D. J. Reed, *Biochem. Pharmac.* **23**, 2332 (1974).
27. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
28. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
29. J. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56**, 658 (1934).
30. M. Dixon, *Biochem. J.* **55**, 170 (1953).
31. A. Cornish-Bowden, *Biochem. J.* **137**, 143 (1974).
32. C. R. Jefcoate, in *Methods in Enzymology*, Vol. 52, *Biomembranes Part C: Biological Oxidations, Microsomal, Cytochrome P-450 and Other Hemoprotein Systems* (Eds. S. Fleischer and L. Packer), pp. 258–279. Academic Press, New York (1978).
33. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharac.* **3**, 113 (1967).
34. J. B. Schenkman, S. G. Sliger and D. L. Cinti, *Pharmac. Ther.* **12**, 43 (1981).
35. K. K. Hajek, N. I. Cook and R. F. Novak, *J. Pharmac. exp. Ther.* **223**, 97 (1982).
36. N. M. Bleehen, J. T. Roberts and H. F. V. Newman, *Int. J. Radiat. Oncol. Phys.* **112**, 1401 (1986).
37. D. W. Siemann, *Br. J. Cancer* **43**, 367 (1981).
38. D. W. Siemann, *Br. J. Cancer* **45**, 272 (1982).
39. D. G. Hirst, J. M. Brown and J. L. Hazlehurst, *Br. J. Cancer* **46**, 109 (1982).
40. B. Testa and P. Jenner, *Drug Metab. Rev.* **12**, 1 (1981).
41. D. D. Schoemaker, M. E. McManus and J. M. Strong, *Cancer Treat. Rep.* **66**, 1343 (1982).
42. M. I. Walton and P. Workman, *Biochem. Pharmac.* **36**, 887 (1987).
43. E. D. Palmer and M. A. Cawthorne, *Xenobiotica* **4**, 209 (1974).
44. C. F. Wilkinson, K. Hetnarski and L. J. Hicks, *Pesticide Biochem. Physiol.* **4**, 299 (1974).
45. T. D. Rogerson, C. F. Wilkinson and K. Hetnarski, *Biochem. Pharmac.* **26**, 1039 (1977).
46. P. Workman, *Cancer Chemother. Pharmac.* **5**, 27 (1980).
47. P. Workman, R. A. S. White, M. I. Walton, L. N. Owen and P. R. Twentyman, *Br. J. Cancer* **50**, 291 (1984).
48. D. G. Hirst, J. M. Brown and J. L. Hazlehurst, *Cancer Res.* **43**, 1961 (1983).
49. P. Workman, in *Pharmacokinetics of Anticancer Agents*, (Eds. M. M. Ames, G. Powis and J. S. Kovach), pp. 291–361. Elsevier, Amsterdam (1983).