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# DRUG METABOLISM AND CHEMOSENSITIZATION

## NITROIMIDAZOLES AS INHIBITORS OF DRUG METABOLISM

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**Abstract**—The nitroimidazole misonidazole (MISO) and related compounds have been shown to enhance the response of tumours to cytotoxic agents, and often to improve their therapeutic indices. Previous experiments suggested inhibition of cytotoxic drug metabolism as a mechanism. We have now investigated the effects of MISO and related compounds on drug metabolism in mice, and the results can be summarised as follows. (1) MISO and related compounds inhibit drug-metabolising enzymes, as measured by pentobarbitone sleep-time and zoxazolamine paralysis-time. (2) Enzyme inhibition is primarily dependent on lipophilicity, with maximum inhibition exhibited by the most active chemosensitizers. (3) MISO significantly slowed the clearance of pentobarbitone, aminopyrine and the cytotoxic agent chlorambucil, but had no effect on renal function or protein binding. These data support the view that inhibition of cytotoxic drug metabolism may be an important factor in chemosensitization.

The ability of the nitroimidazole misonidazole [1-(2-nitroimidazol-1-yl)-3-methoxypropanol (MISO) (Ro 07-0582)] and related compounds to enhance the response of experimental tumours to cytotoxic agents has attracted considerable interest recently [1]. MISO has been shown to enhance tumour response to a variety of agents, particularly alkylating agents and nitrosureas, and in most cases the enhancements are greater than those seen in normal tissues [2, 3]. The improvements in the therapeutic ratio have been considered sufficiently promising to merit clinical evaluation of certain combinations, and several trials are now in progress.

Various mechanisms have been advanced to explain chemosensitization by nitroimidazoles [3–5]. Modification of cytotoxic drug pharmacokinetics was suggested as a possible mechanism in one of the earliest reports of chemosensitization [6]. However most authors have preferred mechanisms operating at the cellular level, including thiol depletion and inhibition of DNA damage repair, and have considered that modifying pharmacokinetics would be unlikely to give rise to a selective enhancement of tumour response [2–4].

Two lines of evidence established in our laboratory have suggested that changes in pharmacokinetics may be important. Firstly, similar *in vivo* chemosensitization to that by MISO can also be obtained with the inhibitor of microsomal drug metabolism, SKF 525A [7–9]. Secondly, structure–activity relationships for the enhancement of CCNU response by MISO analogues showed that whereas electron affinity (redox potential) predominates *in vitro*† lipophilicity is the principal variable *in vivo*, and lipophilicity is known to determine the interaction of these drugs with metabolising enzymes [10, 11].

As recommended by a recent reviewer [5], studies on the pharmacokinetic aspects of chemosensitization have a high priority. We now report on the effects of electron affinic agents and other modifiers on drug-metabolising enzymes *in vivo*, together with observations on renal function and protein binding. Some preliminary data have been published previously [12].

### MATERIALS AND METHODS

*Mice.* Adult male BALB/c mice were obtained from OLAC (Southern) Ltd (Bicester, U.K.), and adult C3H/He mice of both sexes from OLAC and our own breeding colony. Mice were housed in plastic cages on sawdust bedding made from soft white woods, and allowed laboratory chow and water *ad lib*. They were used at 25–35 g body wt.

*Drugs and isotopes.* Some details of the modifying agents used are summarised in Table 1. MISO, desmethylmisonidazole (Ro 05-9963) (DEMIS), benznidazole (Ro 07-1051) (BENZO) and the nitroimidazoles designated Ro were supplied by Roche (Welwyn, U.K.). SR-2508 was supplied by the National Cancer Institute (Bethesda, MD), AM-1 by the Oncology Institute (Gliwice, Poland), metronidazole (METRO) by May & Baker (Dagenham, U.K.), and SKF 525A [β-diethylaminoethyl diphenylpropylacetate hydrochloride (proadifen hydrochloride)] by Smith Kline & French (Welwyn, U.K.). Nitrofurantoin and imidazole were obtained from Sigma (Poole, U.K.), anthraquinone-2-sulphonate (AQS) from British Drug Houses, (Poole, U.K.), and nitrofurazone from Koch-Light (Slough, U.K.). Aminopyrine (4-

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† P. R. Twentyman and P. Workman, unpublished results.

Table 1. Physico-chemical properties of MISO and other modifiers*

| Compound | Class | Octanol–water partition coefficient (PC)† | One-electron reduction potential ($E_7^1$) (mV) |
|---|---|---|---|
| Imidazole | — | 0.62 | — |
| MISO | 2-Nitroimidazoles | 0.43 | −389 |
| DEMIS | | 0.13 | −389 |
| SR-2508 | | 0.046 | −388 |
| Ro 07-8799 | | 0.4 | −346 |
| Ro 07-1902 | | 2.5 | −391 |
| BENZO | | 8.5 | −380 |
| Ro 07-1127 | | 29 | −390 |
| AM-1 | 4-Nitroimidazole | 0.44 | −564 |
| METRO | 5-Nitroimidazole | 0.96 | −486 |
| Nitrofurantoin | 5-Nitrofurans | 0.39 | −264 |
| Nitrofurazone | | 1.7 | −257 |
| AQS | Quinone | < 0.01 | −375 |

* For fuller details see Ref. 9.
† Corrected to pH 7.4.

dimethylaminoantipyrine) and zoxazolamine (2-amino-5-chlorobenzoxazole) were obtained from Sigma, and chlorambucil (*N,N-p*-di-2-chloroethylphenylbutyric acid) from Sigma & Burroughs Wellcome (Beckenham, U.K.). Sodium pentobarbitone [sodium 5-ethyl-5-(1-methylbutyl)-barbiturate] was supplied by May & Baker, both as the pure compound and as a 60 mg/ml solution for injection (Sagatal).

Isotopes were obtained from Amersham International (Amersham, U.K.). Chromium ($^{51}$Cr)–EDTA injection solution was supplied at a radioactive concn of 100 μCi/ml and a sp. act. of 1–2 mCi/mg chromium. Sodium [$^{125}$I]iodohippurate injection solution (BP) was supplied at a radioactive concn of 450 μCi/ml and a sp. act. of 10–50 μCi/mg sodium iodohippurate.

*Drug administration*. Unless otherwise stated the modifying agents were given 30 min before the second agent. Controls received the appropriate vehicles. Most substances were dissolved in Hanks' balanced salt solution, pH 7.4, and injected at 0.005–0.04 ml/g body wt. All injections were given i.p. except SR-2508 which was given i.v. in 0.01 ml/g Hanks'. The pH of imidazole solutions was corrected to 7.4 with HCl. Nitrofurantoin, nitrofurazone, BENZO, Ro 07-1127 and AQS were suspended in 50% v/v polyethylene glycol [mol. wt 400 (Sigma)] and injected i.p. at 0.005–0.01 ml/g. Zoxazolamine was prepared for injection by dissolving 200 mg in 2.4 ml 1 N HCl and making up to 20 ml with Hanks'. Sodium pentobarbitone solution was diluted to 3 mg/ml in Hanks'. Chlorambucil was dissolved in acidified ethanol and diluted 1:10 with propylene glycol/$K_2HPO_4$ buffer (final pH 7.4). The radioactive isotopes were diluted in Hanks' and injected i.v. at 0.005–0.01 ml/g to give doses of 100–500 μCi/kg.

*Pentobarbitone sleep-time and zoxazolamine paralysis-time*. These were obtained from the time required to regain the righting reflex [13].

*Acute* $LD_{50}$. These values for zoxazolamine and pentobarbitone were obtained from the survival data at 24 hr, using the same mice as in the sleep- and paralysis-time experiments.

*Pharmacokinetics*. Blood samples were collected into heparinised syringes by cardiac puncture under diethyl ether anaesthesia. Plasma was obtained by centrifugation and stored at −20° prior to work-up. For analyses except pentobarbitone, samples (0.1-ml) were mixed thoroughly with 2 vols of methanol [HPLC grade (Rathburns, Walkerburn, U.K.)] and cooled on dry ice. After centrifugation (5000 *g*, 10 min) at −10° in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont, Hitchin, U.K.) the clear supernatant was removed for analysis. Acetonitrile [HPLC low u.v. grade (Rathburns)] was used instead of methanol in the pentobarbitone analysis. Recovery was > 95% in all cases.

*High-performance liquid chromatography (HPLC)*. Pentobarbitone, aminopyrine and chlorambucil were analysed by reversed-phase HPLC. Analysis was performed at ambient temp using Waters modular liquid chromatographs (Water Associates, Hartford, U.K.). The equipment used included Model 6000A chromatography pumps, Model 660 solvent programmer, Model 440 and 441 absorbance detectors, Model 710B automated sample processors (WISP), Data Module, Model 720 system controller and RCM-100 radial compression modules. The analyses were carried out on Waters Radial-PAK reverse-phase bonded silica cartridge columns (8 mm i.d., 5- or 10-μm dia. spherical particles), protected by Waters RCSS Guard-PAK octadecylsilane (C18) guard columns. Flow rates were 2–3 ml/min.

Aminopyrine was analysed on octylsilane (C8) columns. Isocratic elution was carried out with a mobile phase of 60–70% methanol containing 0.01 M dibutylamine (BDH) and *ortho*-phosphoric acid to pH 7.4. Absorbance was monitored at 254 nm.

Pentobarbitone was analysed on octadecylsilane

(C18) columns (5-μm) without guard columns. Isocratic elution was carried out with a mobile phase of 45% acetonitrile/water containing 0.01 M $KH_2PO_4$ and HCl to pH 6.7. Absorbance was monitored at 229 nm. The columns were washed briefly with 90% acetonitrile/water after each sample, and the solvents were held under nitrogen.

Chlorambucil and its metabolites were also analysed on octadecylsilane (C18) columns, using two alternative methods. The isocratic elution method employed 65% methanol in phosphate buffer containing 0.005 M *tetra*-butylammonium hydroxide [TBAH (Fisons, Loughborough, U.K.)], pH 7.4. The gradient elution method was based on that described by Newell *et al.* [14]. A linear gradient was run over 5 min from 55 to 80% methanol/phosphate buffer containing 5 mM TBAH, pH 7.4. Absorbance was monitored at 254 nm.

*Protein binding*. Pooled C3H/He mouse plasma containing 30 μg/ml chlorambucil was incubated at 37° for 15 min. Ultrafiltration was carried out at 4° using the Amicon Micropartition System fitted with type YMB membranes [mol. wt cut-off 30,000 (Amicon, Woking , U.K.)] in an MSE Chilspin centrifuge (MSE, Crawley, U.K.) at 2000 g. Plasma and ultrafiltrate were then analysed by HPLC.

Drugs and metabolites were identified by co-chromatography with authentic standards. Quantitation was by peak height with reference to linear calibration curves.

*Pharmacokinetic parameters and statistics*. Lines of best fit were calculated by least-squares regression analysis, yielding half-lives with 95% confidence limits. Values for area under the curve (AUC) were estimated by Simpson's rule. The $LD_{50}$ values and confidence limits were calculated by probit analysis. Dose-modifying factors (DMFs) were calculated from the ratio of isoeffective doses with and without the modifier. Confidence limits and significance levels were calculated using Student's *t*-distribution.

## RESULTS

### *Pentobarbitone sleep-time*

Fig. 1 shows data from several independent experiments in which dose–response curves for pentobarbitone sleep-time were obtained in control BALB/c mice and those treated 30 min previously with MISO or other modifiers. Linear regression lines were fitted to the data and DMFs with 95% confidence limits were calculated from the doses of pentobarbitone required to give sleep-times of 100 min, with and without the modifiers. The classical inhibitor of drug-metabolising enzymes SKF 525A was included as a positive control [13, 15], and a dose of 0.13 mmoles/kg (50 mg/kg) produced a marked enhancement [DMF 2.39 (2.10–2.72)]. The imidazoles were tested at 2.5 mmoles/kg. Increased sleep-times were demonstrated with MISO [DMF 1.38 (1.14–1.68)] but not its desmethyl derivative DEMIS [DMF 1.06 (0.96–1.17)], which is similar in electron affinity but more hydrophilic (Table 1). Prolonged sleep-times were also seen with imidazole [DMF 1.94 (1.65–2.27)] which contains no nitro group but has similar lipophilicity to MISO (Table 1).

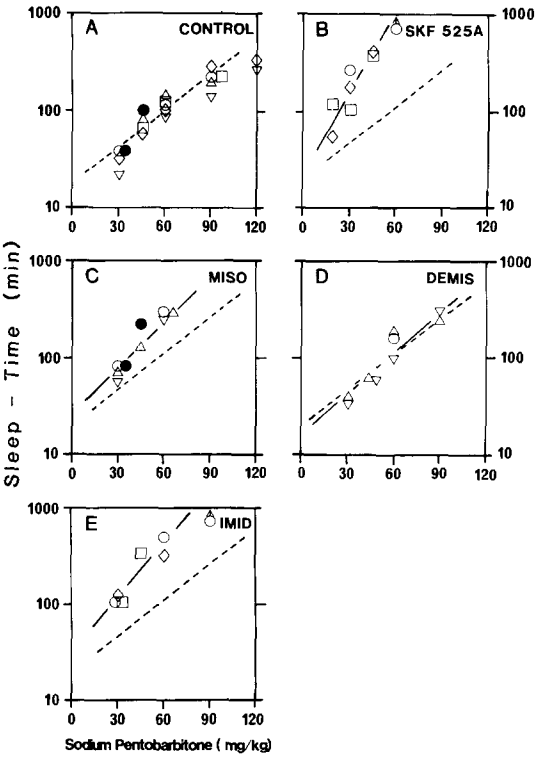


Fig. 1. The effect of various modifiers on sodium pentobarbitone sleep-time in BALB/c mice. The dose of each modifier was 2.5 mmoles/kg, except SKF 525A (0.13 mmoles/kg). Different symbols indicate independent experiments. Data points are geometric means for groups of four or five mice. Lines were fitted by least-squares linear regression analysis. The dotted line from the control dose–response curve in panel A is reproduced in the other panels for comparison.

Similar studies were carried out with other electron-affinic agents, and two typical experiments are shown in Table 2. Of the neutral 2-nitroimidazoles similar in electron affinity to MISO, considerably greater enhancement was obtained with the more lipophilic BENZO and Ro 07-1902 (both gave DMFs > 2), but none was seen with the most lipophilic Ro 07-1127 or the very hydrophilic SR-2508. The less electron-affinic but reasonably lipophilic 5-nitroimidazoles AM-1 and METRO gave enhancements similar to MISO, as did the basic 2-nitroimidazole Ro 03-8799 which is also lipophilic. Enhancements superior to MISO were seen with the quinone AQS and the nitrofuran nitrofurantoin (Table 2). Another nitrofuran, nitrofurazone, was lethal at the dose tested when combined with pentobarbitone.

In another series of experiments the doses of the modifying agents were varied to establish potency with respect to SKF 525A. Some dose–response data for BENZO are included in Table 2. Summarising, MISO, METRO, AM-1 and imidazole were quite active at 1.25 mmoles/kg but gave little or no enhancement at 0.625 mmoles/kg. Greater potency was observed with Ro 07-1902 and BENZO. At the lowest dose of Ro 07-1902 tested, 0.16 mmoles/kg, the enhancement was superior to that with 2.5 mmoles/kg MISO. BENZO gave enhancement at doses as low as 0.033 mmoles/kg but was almost

Table 2. Effect of various modifiers on sodium pentobarbitone sleep-time in BALB/c mice

| Expt. | Modifier | Modifier dose (mmoles/kg) | Pentobarbitone dose (mg/kg) | Sleep-time* (min) |
|---|---|---|---|---|
| 1† | Vehicle | — | 30 | 37 (30–45) |
| | Vehicle‡ | — | 45 | 41 (33–50) |
| | Vehicle | — | 60 | 77 (58–96) |
| | Vehicle | — | 90 | 176 (137–225) |
| | MISO | 2.5 | 30 | 80 (46–138) |
| | AM-1 | 2.5 | 30 | 77 (74–81) |
| | SR-2508‡ | 2.5 | 45 | 45 (39–53) |
| | Ro 07-1902 | 0.63 | 30 | 169 (128–222) |
| 2§ | Vehicle | — | 30 | 50 (39–59) |
| | Vehicle | — | 45 | 99 (81–121) |
| | Vehicle | — | 60 | 144 (124–167) |
| | Vehicle | — | 75 | > 400 |
| | BENZO | 2.5 | 30 | > 400 |
| | BENZO | 0.3 | 30 | 103 (73–147) |
| | BENZO | 0.03 | 30 | 76 (63–92) |
| | BENZO | 0.003 | 30 | 63 (35–109) |
| | Ro 07-1127 | 2.5 | 30 | 53 (45–64) |
| | AQS | 2.5 | 30 | 214 (154–299) |
| | Nitrofurantoin | 0.21 | 30 | 305 (291–320) |
| | Nitrofurazone | 0.63 | 30 | Toxic‖ |

Four mice were used in each treatment group.
* Geometric means with 2 S.E. limits in parentheses.
† All injections were made in Hanks' solution (0.04 ml/g i.p.) except ‡ (0.01 ml/g i.v.).
§ All injections were made in 50% polyethylene glycol/Hanks' solution (0.01 ml/g i.p.).
‖ All mice died.

Table 3. Effect of various modifiers on sodium pentobarbitone $LD_{50}$ in BALB/c mice

| Modifier | Modifier dose (mmoles/kg) | Pentobarbitone $LD_{50}$ (mg/kg)* | DMF* |
|---|---|---|---|
| Vehicle | — | 117 (111–123) | — |
| MISO | 2.5 | 69 (60–78) | 1.70 (1.46–1.97) |
| DEMIS | 2.5 | 118 (105–133) | 0.99 (0.87–1.13) |
| Imidazole | 2.5 | 65 (56–76) | 1.80 (1.54–2.11) |
| SKF 525A | 0.13 | 73 (63–85) | 1.60 (1.37–1.88) |

* 95% confidence limits in parentheses.
Data were pooled from three independent experiments. Six to eight different pentobarbitone dose levels were used with each modifier, and the total number of mice per dose level was 10–25.

Table 4. Effect of MISO and SKF 525A on zoxazolamine paralysis-time and toxicity in BALB/c mice

| Modifier | Modifier dose (mmoles/kg) | Zoxazolamine dose (mg/kg) | Paralysis-time* (min) | Survivors/ total |
|---|---|---|---|---|
| Vehicle | — | 75 | 17 (13–22) | 5/5 |
| Vehicle | — | 100 | 30 (29–31) | 5/5 |
| Vehicle | — | 200 | 203 (177–232) | 5/5 |
| MISO | 2.5 | 75 | 52 (46–58) | 5/5 |
| MISO | 2.5 | 100 | 112 (95–132) | 5/5 |
| MISO | 2.5 | 200 | > 420 | 1/5 |
| SKF 525A | 0.026 | 75 | 78 (69–88) | 5/5 |
| SKF 525A | 0.026 | 100 | 102 (90–116) | 5/5 |
| SKF 525A | 0.026 | 200 | > 420 | 4/5 |

Five mice were used in each treatment group.
* Geometric means with 2 S.E. limits in parentheses.

ineffective at 0.0033 mmoles/kg (Table 2). However, SFK 525A was by far the most potent, giving demonstrable enhancement in the range 1.3–12 nmoles/kg.

Some of the sleep-time experiments were also repeated in C3H/He mice. Both MISO (2.5 mmoles/kg) and BENZO (0.3 mmoles/kg) gave similar enhancements to those in BALB/c mice. A pretreatment regimen where BENZO was given daily at 0.3 mmoles/kg for 6 days, followed by a 48-hr recovery period, gave no evidence of enzyme induction.

*Pentobarbitone toxicity*

The modifiers which prolonged pentobarbitone sleep-times also increased the drug's toxicity. For example, Table 3 shows that 2.5 mmoles/kg MISO or imidazole reduced the acute $LD_{50}$ of pentobarbitone by an amount comparable to that by 0.13 mmoles/kg SKF 525A, whereas 2.5 mmoles/kg DEMIS had no effect.

*Zoxazolamine paralysis-time and toxicity*

We compared the effects of MISO (2.5 mmoles/kg) and SKF 525A (0.026 mmoles/kg) on the paralysis-time and toxicity of zoxazolamine in two experiments. Similar results were obtained in these, and data from one are shown in Table 4. Prolonged paralysis-times were observed with both agents, and the DMF for doses giving paralysis-times of 100 min was about 1.5 in each case. There was also evidence of enhanced acute toxicity (Table 4).

*Renal function*

Clearances of $^{51}$Cr–EDTA and [$^{125}$I]iodohippurate were used to measure the glomerular filtration rate and effective renal plasma flow, respectively [16], in BALB/c mice. Doses of 2.5–5 mmoles/kg MISO had no effect on renal function (Fig. 2). The $t_{\frac{1}{2}}$ for $^{51}$Cr–EDTA clearance was 17.1 (14.0–22.1) min in the controls and 17.1 (15.1–19.7) min after MISO ($P > 0.1$). Corresponding values for [$^{125}$I]iodohippurate clearance were 12.6 (9.9–17.5) min in the controls and 10.2 (8.6–12.5) min after MISO ($P > 0.1$).

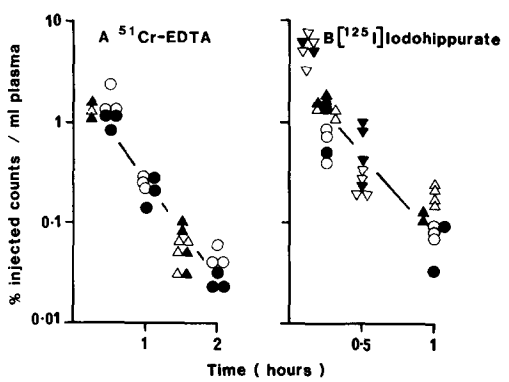


Fig. 2. The effect of MISO on renal function. The clearance of $^{51}$Cr–EDTA (A) and [$^{125}$I]iodohippurate (B) was determined in control BALB/c mice (open symbols) or mice pretreated with MISO (closed symbols). The MISO dose was 5 mmoles/kg except for the experiment depicted by the circles in panel A (2.5 mmoles/kg). Each datum point is for an individual mouse. Different symbols indicate independent experiments.

*Pharmacokinetics of pentobarbitone and aminopyrine*

Fig. 3 shows that 2.5 mmoles/kg MISO inhibited the clearance of both pentobarbitone and aminopyrine in BALB/c mice. The $t_{\frac{1}{2}}$ for pentobarbitone (60 mg/kg) was increased from 1.56 (0.2–2.08) hr to 8.3 (4.6–43) hr ($P < 0.001$). (In the same experiment shown in Fig. 3A, the sleep-time for the MISO group was > 5 hr compared to 2.2 hr in the controls.) The $t_{\frac{1}{2}}$ for aminopyrine (100 mg/kg) was increased from 8.9 (8.0–10.0) min to 14.9 (13.2–17.0) min ($P < 0.001$) (Fig. 3B).

*Pharmacokinetics and protein binding of chlorambucil*

Fig. 4 shows data from a typical experiment to determine the effects of 2.5 mmoles/kg MISO on the pharmacokinetics of chlorambucil in C3H/He mice. In the absence of MISO chlorambucil showed dose-dependent pharmacokinetics, with an elimination $t_{\frac{1}{2}}$ of 19.7 (17.4–22.7) min at 7.5 mg/kg compared to 40.0 (37.8–42.5) min at 15 mg/kg ($P <$

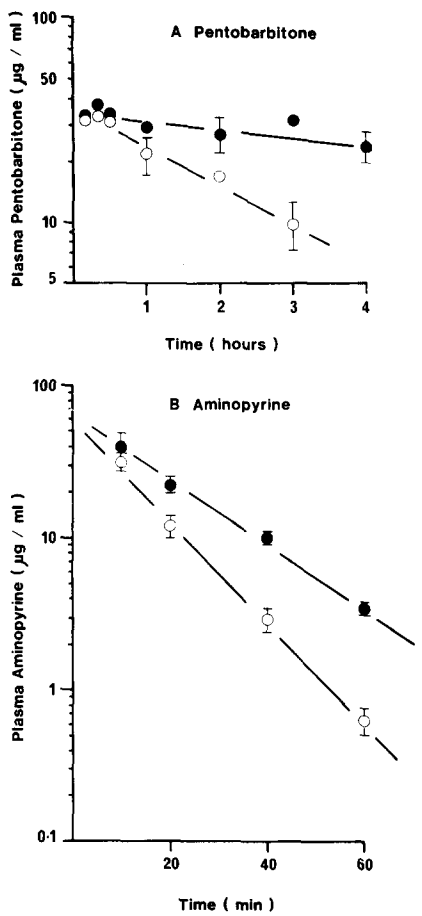


Fig. 3. The effect of 2.5 mmoles/kg MISO on the pharmacokinetics of sodium pentobarbitone (60 mg/kg) (A) and aminopyrine (100 mg/kg) (B) in BALB/c mice. (○) Drug alone, (●) drug after MISO. Results are means (± S.E.) of three (A) or five (B) mice.

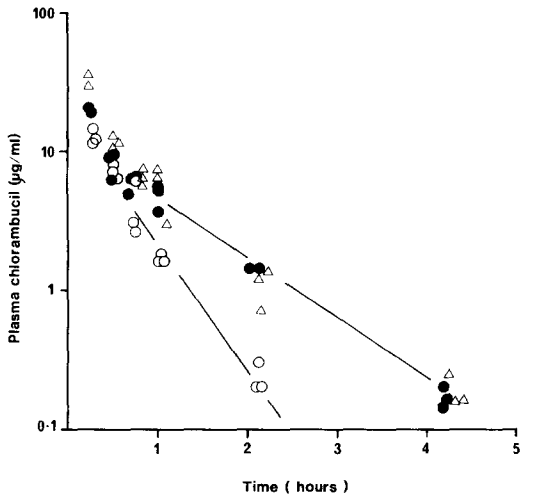


Fig. 4. The effect of 2.5 mmoles/kg MISO on the pharmacokinetics of chlorambucil in C3H/He mice. (○) 7.5 mg/kg chlorambucil alone, (△) 15 mg/kg chlorambucil alone, (●) 7.5 mg/kg chlorambucil after MISO. Each datum point is for an individual mouse.

0.001). Apart from the earliest data points, the data from the mice pretreated with MISO prior to 7.5 mg/kg chlorambucil were superimposable on those for 15 mg/kg chlorambucil alone (Fig. 3) and the $t_{\frac{1}{2}}$ was 43.2 (36.3–53.2) ($P < 0.001$ compared to control). Peak plasma chlorambucil levels after the low dose plus MISO were intermediate between those for the low and high doses alone. Values for the plasma AUC (in μg/ml hr) were 7.9 for 7.5 mg/kg chlorambucil, 19.7 for 15 mg/kg chlorambucil and 14.9 for the low dose plus MISO. Thus the DMF for drug exposure was about 2. Circulating concns of the metabolites dehydrochlorambucil and phenyl acetic mustard [14, 17] were also elevated in mice pretreated with MISO (data not shown).

Chlorambucil exhibits considerable binding to plasma proteins. *In vitro* studies were carried out to assess the effect of 2.5 mM MISO on the protein binding of chlorambucil (30 μg/ml) in pooled C3H/He mouse plasma. In a typical experiment 93.1 ± 0.3% (2 S.E., N = 4) was bound to control plasma (7.0 ± 0.4% free), and this was similar to the 95.1 ± 0.1% bound (4.9 ± 0.1% free) in the presence of MISO. Similar results were obtained for *in vivo* binding (data not shown).

## DISCUSSION

The results reported here provide clear evidence that inhibition of drug-metabolising enzymes occurs with doses of MISO which have been used in mice to demonstrate enhancement of cytotoxic drug response and, in many cases, improved therapeutic indices [1]. MISO prolonged sleep-times of the barbiturate pentobarbitone and paralysis-times of the muscle relaxant zoxazolamine. These assays have been used widely [13, 15] as indirect measures of hepatic microsomal P-450-mediated oxidations, and in particular aliphatic (pentobarbitone) and aromatic (zoxazolamine) hydroxylation. Enhancement of the toxicity of these two agents was also seen. Direct HPLC analysis confirmed that the increased pentobarbitone responses resulted from an inhibition of the drug's clearance. Likewise, MISO impaired the clearance of aminopyrine, which is metabolised by P-450-mediated *N*-demethylation [18].

Our demonstration of the ability of MISO and related compounds to inhibit drug metabolism is not surprising in view of the well-documented ability of imidazole derivatives to inhibit reactions mediated by cytochrome P-450 [19]. Our data on the enhancement of pentobarbitone sleep-time by the parent heterocycle imidazole are very similar to those previously reported for hexobarbitone [20]. Structure–activity relationships with substituted imidazoles have established that ligand binding to cytochrome P-450 is a major determinant of inhibitory activity, and strong binding requires at least one imidazole nitrogen to be sterically unhindered [19]. Steric hindrance may account for the relatively weak inhibitory activity of 2-substituted imidazoles compared to 1- or 4(5)-substituted imidazoles.

Our studies have shown that enzyme inhibition by MISO analogues is highly dependent on lipophilicity. The most hydrophilic and the most lipophilic showed little inhibitory activity, while the maximum inhibition was observed with those somewhat more lipophilic than MISO (e.g. BENZO and Ro 07-1902). A good example of the predominance of lipophilicity over electron affinity is afforded by comparing the enzyme inhibitory activity of the nitroimidazoles MISO, METRO and AM-1. METRO and AM-1 are much less electron-affinic than MISO, yet they exhibit comparable inhibitory activity because their lipophilicities are similar to MISO. On the other hand, BENZO and Ro 07-1902, though similar in electron affinity to MISO, show superior inhibitory activity because of their greater lipophilicity. The sole non-lipophilic modifier to exhibit enzyme inhibition in our study was anthraquinone sulphonate, and this is almost certainly metabolised to the lipophilic free quinone [21]. Our findings on the relationship between inhibitory activity and lipophilicity are in keeping with those of Wilkinson *et al.* [22] who demonstrated optimum binding and inhibition of microsomal aldrin epoxidation by 1-alkylimidazoles of intermediate lipophilicity.

We are currently carrying out experiments with *in vitro* enzyme preparations to establish the nature of the inhibition mechanism. Since the inhibitory analogues of MISO are also those which are metabolised *in vivo* [10, 11] there may be a component of competitive inhibition involved. A recent review [5] justifiably pointed out that the hypothermic effect of high-dose MISO may impair drug clearance. For this reason, as in our chemosensitization studies, we have restricted the MISO dose to 2.5 mmoles/kg which causes a minimal temp decrease. Thus with MISO and its lipophilic analogues marked inhibition of enzyme activity was observed with no effects on body temp.

These findings lend support to our earlier suggestion that modification of cytotoxic drug metabolism may be an important aspect of the mechanism of chemosensitization by nitroimidazoles *in vivo* [7–9]. We showed previously that for enhancement of CCNU cytotoxicity lipophilicity is the principal var-

iable *in vivo*, whereas electron affinity predominants *in vitro*, and that chemosensitization can be obtained with non-electron-affinic inhibitors of drug metabolism [7–9]. From the work reported here we can add that inhibition of drug metabolism is seen over a similar dose range to that for chemosensitization, and that the structure–activity relationships for the two effects are very similar. Particularly striking is the enzyme inhibition shown by lipophilic MISO analogues such as BENZO and Ro 07-1902, and these are known to exhibit superior sensitization to CCNU [8, 9], chlorambucil [7, 8] and melphalan [23].* Some anomalies do exist, however; notably that imidazole inhibits drug metabolism and sensitizes to chlorambucil but not CCNU [7–9]. This is perhaps not too surprising in view of the different enzymes and metabolic routes involved. Moreover, certain substituted imidazoles are known to be highly potent inhibitors of microsomal epoxidase and hydroxylase but considerably less active against *N*-demethylase [24]. In agreement with this we have shown here that MISO inhibits pentobarbitone hydroxylation more effectively than aminopyrine *N*-demethylation. Thus although the assays described earlier are useful for initial screening, it will be necessary to determine the effects of chemosensitizers on the pharmacokinetics and metabolism of the individual cytotoxic agents concerned.

For this reason we present here some preliminary data on chlorambucil pharmacokinetics, which confirm directly that MISO can reduce the clearance of cytotoxic drugs. MISO increased the plasma AUC for chlorambucil by a DMF of 2, which is closely similar to the DMFs obtained for enhancement of its *in vivo* cytotoxicity by MISO [7, 8, 25]. Chlorambucil is metabolised by mitochondrial $\beta$-oxidation to phenyl acetic mustard, with dehydrochlorambucil as an intermediate [14, 17]. We have also found the circulating concns of these two metabolites to be elevated by MISO, suggesting that the subsequent dechlorethylation of phenyl acetic mustard by microsomal enzymes is also inhibited.† These data will be reported in detail elsewhere.

In considering the mechanism of *in vivo* chemosensitization by nitroimidazoles, most authors have felt that simple modifications of cytotoxic drug pharmacokinetics would be unlikely to enhance tumour response selectively, but would increase cytotoxicity equally in tumour and normal tissues [2, 4]. With chlorambucil this does indeed appear to be so. However, using HPLC analysis for melphalan and CCNU we have recently shown that an inhibition of the metabolism of these agents may be responsible for their improved therapeutic indices in combination with MISO.† Reduced clearance of melphalan by MISO has also been observed in other laboratories, using both [$^{14}$C]melphalan [26] and HPLC.‡ Studies with *in vitro* bioassays have suggested that MISO may also inhibit the clearance of the metabolites of cyclophosphamide [27]§ and BCNU [27].

In summary, we have shown that MISO and other electron-affinic agents can inhibit drug-metabolising enzymes in mice. Changes in cytotoxic drug pharmacokinetics are seen in experimental protocols for which chemosensitization has been reported with single doses of sensitizer. Moreover, we are not aware of any studies in which single doses of nitroimidazoles give chemosensitization in the absence of pharmacokinetic changes. However, this is not to deny the potential importance of mechanisms which operate *in vitro*, such as thiol depletion and DNA damage repair [3–5]. Indeed, these cellular mechanisms may be responsible for *in vivo* chemosensitization in protocols where multiple low doses of sensitizer are given to simulate human pharmacokinetics [28, 29] and where altered cytotoxic drug metabolism is less likely. We are now investigating this possibility.

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* R. D. Clutterbuck and J. L. Millar, personal communication.

† F. Lee and P. Workman, unpublished results.

‡ D. Newell *et al.*, and M. R. L. Stratford *et al.*, personal communications.

§ A. C. Begg *et al.*, personal communication.

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