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Altered pharmacokinetics in the mechanism of chemosensitization: effects of nitroimidazoles and other chemical modifiers on the pharmacokinetics, antitumour activity and acute toxicity of selected nitrogen mustards

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Summary. We have studied the effect of misonidazole (MISO) on the antitumour activity, normal tissue toxicity and pharmacokinetics of four bifunctional nitrogen mustards: chlorambucil (CHL); phenylacetic acid mustard (PAAM), a metabolite of CHL; β,β-difluorochlorambucil (β-F_2CHL), an analogue which is metabolized less efficiently by the β-oxidation pathway; and melphalan (MEL). MISO (2.5 mmol/kg) increased the response of the KHT tumour to CHL, PAAM and β-F_2CHL by dose-modifying factors (DMFs) of 1.55–1.85, 1.35–1.65 and 1.5–1.8, respectively. In contrast, the activity of MEL was not altered. However, with 5.0 mmol/kg MISO an enhanced response to MEL was observed (DMF = 1.35–1.55). Similarly, for CHL and PAAM, but not MEL, acute toxicity was also increased by 2.5 mmol/kg MISO. The increase in toxicity with CHL and PAAM was similar to the increase in antitumour activity, and their therapeutic indices were unchanged. Effective chemosensitizers were shown to be powerful inhibitors of drug clearance. Thus, potent chemosensitizers such as MISO, the lipophilic analogue benznidazole (BENZO), the microsomal enzyme inhibitor SKF 525A, and the parent heterocycle imidazole all reduced the plasma clearance of CHL and its metabolites and therefore increased drug exposure (AUC). Conversely, the hydrophilic MISO metabolite Ro 05-9963 was a poor chemosensitizer and produced only very weak pharmacokinetic effects. As the DMFs for chemosensitization agreed very well with those for increased AUC, it seems likely that pharmacokinetic changes are the major cause of the enhancement of tumour response to CHL. For MEL, chemosensitization also appears to be related to pharmacokinetic changes. MISO at a dose of 2.5 mmol/kg produced no change in MEL pharmacokinetics and no enhancement of tumour response, whereas 5.0 mmol/kg MISO was effective on both counts.

Introduction

Enhancement of the experimental antitumour activity of some cytotoxic drugs by MISO and related nitroimidazoles, a phenomenon known as chemosensitization, is now well established (for review see [14, 20]). Combinations with the most promise involve the nitrosoureas and bifunctional nitrogen mustards, and some of these are currently undergoing clinical investigation.

Although the exact nature of chemosensitization is still not fully understood, there is now considerable evidence in favour of a pharmacokinetic mechanism when the chemosensitizer is administered to mice as a large single dose at more or less the same time as the cytotoxic drug. Pharmacokinetic alterations by MISO have been found for the nitrosoureas [8, 9, 11] and the bifunctional nitrogen mustards melphalan (MEL) [3, 6, 7, 16], cyclophosphamide [6, 23] and chlorambucil (CHL) [27]. However, for most cytotoxic agents the precise relationship between pharmacokinetic changes and chemosensitization remains unclear.

On the basis of very detailed studies with the nitrosourea CCNU we have concluded that reduced drug clearance leading to increased tumour exposure may entirely account for the selective enhancement of tumour response [9]. This conclusion for CCNU, which probably also applies to other lipophilic nitrosoureas [11], is supported by the fact that no clear instance has been reported of chemosensitization occuring independently of pharmacokinetic changes. For MEL, however, it has been argued that in certain circumstances chemosensitization can occur in the absence of pharmacokinetic effects [6]. CHL is a bifunctional nitrogen mustard similar in structure and chemical reactivity to MEL, the antitumour activity of which has also been shown to be enhanced by MISO [24, 26]. It differs from MEL, however, in that its normal tissue toxicity is also increased to the same extent, thus giving no therapeutic benefit. Because of this difference we have now compared the effects of MISO on the pharmacokinetics and activity of administered PAAM, the principal β-oxidation metabolite of CHL, and of β-F_2CHL, a synthetic analogue which is metabolized to PAAM less rapidly and has an improved therapeutic index [12]. Finally, we have compared the changes in CHL pharmacokinetics elicited by MISO with those induced by the nitroimidazole analogues BENZO, a more lipophilic derivative which shows greater chemosensitization [24, 26] and is currently undergoing clinical evaluation with CCNU [17], and the hydrophilic demethylated metabolite of MISO Ro 07-9963, which exhibits very little chemosensitizing activity [24, 26]; also included in the comparison were the parent heterocycle imidazole and the drug metabolism inhibitor SKF 525A, both of which give marked chemosensitization with CHL [24, 26].

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Materials and methods

Mice and tumours. Inbred male C3H/He mice (Olac) weighing 25–35 g were used throughout these studies. The procedures for the transplantation of the KHT fibrosarcoma, the tumour regrowth delay assay, and the acute toxicity testing were as described in the preceding paper [12].

Drugs. MISO ([1-(2-nitroimidazol-1-yl)-3-methoxypropanol]), Ro 07-9963 (1-(2-nitroimidazol-1-yl)-2,3-propandiol; desmethylmisonidazole) and BENZO (*N*-benzyl-(2-nitroimidazolyl)acetamide) were supplied by Roche Products Ltd; SKF 525A (β-diethylaminoethyl diphenylpropylacetate hydrochloride or proadifen hydrochloride) by Smith Kline and French; and imidazole by Sigma. MEL was obtained from the Chester Beatty Research Institute, the Wellcome Foundation or Sigma, and the other mustards from the sources reported in the preceding paper [12].

Drug administration. All modifying agents were given IP 30 min before the cytotoxic agents, which were also also given IP. MISO, Ro 07-9963, SKF 525A and imidazole were dissolved in Hank's balanced salt solution (HBSS) and injected at 0.04 ml/g. The acidity of imidazole solution was corrected to pH 7.4 with hydrochloric acid. BENZO was suspended in 50% vol/vol polyethylene glycol (Mol. wt. 400) in HBSS and injected at 0.01 ml/g. CHL, PAAM, β-F_2CHL and MEL were dissolved in acidified ethanol, diluted 1:10 with propylene glycol/K_2HPO_4 buffer, pH 7.4, and given at 0.01 ml/g [12].

Sample preparation. Blood was collected by heart puncture into heparinized syringes and was immediately cooled on ice. It was then centrifuged in a refrigerated Du Pont Sorvall RC-5B Superspeed Centrifuge at 4000 *g* for 10 min. For CHL, PAAM and β-F_2-CHL, procedures for the preparation of plasma samples for HPLC analysis can be found in the previous paper [12]. For analysis of MEL, 200 µl aliquots of plasma were deproteinized with 4 vol acidified acetonitrile (1% vol/vol conc. HCl). After centrifugation (4000 *g*, 10 min) at 4°C, 500 µl aliquots of the clear supernatant were removed into glass tubes and evaporated to dryness using a Savant Speed Vac Concentrator coupled to a Model 100A Refrigerated Condensation Trap (Uniscience). The dry residue was then redissolved in 50 µl of running buffer and stored sealed at −20°C. Aliquots (35 µl) of the concentrate were injected into the HPLC apparatus within a week.

High performance liquid chromatography. The HPLC methods for CHL, PAAM, β-F_2CHL and their metabolites were as described earlier [12]. MEL was analysed on a Waters µBondapak C18 Radial-Pak cartridge column (8 mm ID; 10 µm particle size). Two elution methods were used. The first involved isocratic elution and is the preferred method for routine analysis. The mobile phase, adjusted to pH 3 using HCl, consisted of 35% acetonitrile/water containing 5 m*M* pentane sulphonic acid and 10 m*M* dibutylamine. The second method, used for the study of the MEL hydrolysis, involved a linear elution gradient over 8 min from 20% to 45% acetonitrile/water, pH 3, containing 5 m*M* pentane sulphonic acid.

Protein binding. Procedures for the study of reversible protein binding were as described in the preceding paper [12], except that mice were given 2.5 mmol/kg MISO, 0.3 mmol/kg BENZO or the appropriate vehicle IP 30 min before the cytotoxic agents. All cytotoxic agents were given at a dose of 15 mg/kg IP.

Chemical hydrolysis. CHL, PAAM and MEL were incubated at 37°C at a concentration of 10 µg/ml in 0.1 *M* sodium phosphate buffer, pH 7.4, with and without MISO (500 µg/ml) or BENZO (100 µg/ml). Triplicate 150 µl aliquots were removed at various times and each was mixed with an equal volume of ice-cold methanol. Aliquots of the mixture were then stored at −20°C before HPLC analysis within a week.

Pharmacokinetic parameters and statistics. These procedures were carried out as described in the previous paper [12]. Unless stated otherwise, plasma concentrations refer to the sum of free and protein bound material. Dose-modifying factors (DMFs) were calculated from the equation: DMF = Isoeffect dose for cytotoxic drug alone / Isoeffect dose for cytotoxic drug plus modifier.

Results

Effect of MISO on tumour response and acute toxicity

Figure 1 shows the influence of MISO (2.5 mmol/kg) on the response of the KHT tumour to CHL, the administered metabolite PAAM, the analogue β-F_2CHL, and MEL. For the first three drugs the effect of MISO was to shift the dose – response curve to the left, thus increasing the effective dose of the cytotoxic agents. The dose-modifying factors (DMFs) for MISO at a range of isoeffect cytotoxic drug doses were 1.55–1.85 for CHL, 1.35–1.65 for PAAM and 1.50–1.80 for β-F_2CHL. In marked contrast, but in agreement with our previous preliminary findings [26], no enhancement of tumour response was seen with MEL at

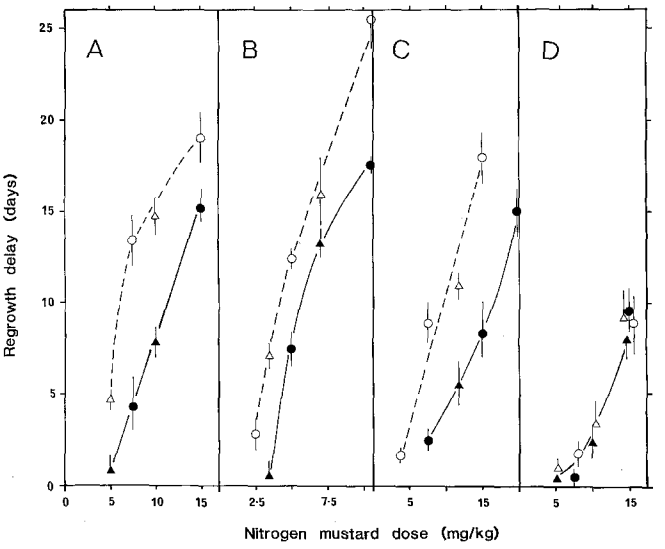


Fig. 1 A–D. The effect of MISO (2.5 mmol/kg) on the in vivo dose – response of the KHT mouse tumour to four nitrogen mustards: CHL **(A)**, PAAM **(B)**, β-F_2-CHL **(C)** and MEL **(D)**. *Closed symbols* represent nitrogen mustard alone; *open symbols* indicate nitrogen mustard plus MISO. Different symbols represent independent experiments. *Error bars* show ± SE

this dose of MISO. Enhancement was, however, obtained with the higher MISO dose of 5 mmol/kg, DMFs being in the range of 1.35–1.55. This is in good agreement with the results of Siemann et al. [21], who obtained a DMF of 1.4 with the same tumour. However, it should be noted that this higher dose, unlike the lower one, does cause a marked drop in body temperature.

The effects of MISO on the acute toxicity of CHL, PAAM and MEL were also studied. For both CHL and PAAM alone, as well as in combination with MISO, deaths occurred within 24 h. For MEL, with and without MISO, the median time of death was 5 days. MISO significantly reduced the LD_{50} of CHL and PAAM from 31.5 (26.2–37.6) mg/kg and 16.3 (13.2–19.8) mg/kg, respectively, to 17.2 (11.3–23.7) mg/kg and 10.6 (7.8–14.7) mg/kg, giving DMFs of 1.83 and 1.54, respectively ($0.05 > P > 0.02$). It is interesting that MISO appears to have a slightly greater effect on CHL than on PAAM activity, in terms of both antitumour effect and toxicity. In contrast, the LD_{50} value of MEL was not significantly altered by 2.5 mmol/kg MISO ($P > 0.1$). With the higher dose of MISO, however, the LD_{50} of MEL was reduced from 13.9 (10.9–17.4) to 8.83 (6.90–10.2) mg/kg ($0.05 > P > 0.02$), giving a DMF of 1.57.

Pharmacokinetic effects of MISO and other chemical modifiers

CHL. We investigated the effects of MISO (2.5 mmol/kg) and its analogues Ro 05-9963 (2.5 mmol/kg) and BENZO (0.3 mmol/kg), together with those of imidazole (2.5 mmol/kg) and SKF 525A (0.13 mmol/kg) on the pharmacokinetics and metabolism of CHL (7.5 mg/kg). The doses chosen were those used previously in chemosensitization experiments [24, 26], and had little or no effect on body temperature. Figure 2 shows the effects on the plasma kinetics of parent CHL, while Figs. 3 and 4 illustrate the respective plasma kinetics for the β-oxidation metabolites 3,4-dehydrochlorambucil (DeHCHL) and PAAM [12], and Fig. 5 shows the data for total bifunctional nitrogen mustards (CHL and metabolites combined). As described in the preceding paper [12], CHL is rapidly absorbed after an IP dose, peak concentrations being reached within 10 min. Post-peak concentrations appeared to decline biphasically. Of the two major CHL metabolites, DeHCHL and PAAM, the former peaked earlier (10–25 min) than the latter (30–50 min), but both showed exponential decline after the peak. Except for Ro 07-9963 all the modifiers gave rise to increased plasma concentrations of CHL, DeHCHL and PAAM, as well as reducing the clearance of total nitrogen mustards. The effects of the modifiers appeared to be greatest on the clearance of PAAM, intermediate for DeHCHL and least for the parent CHL.

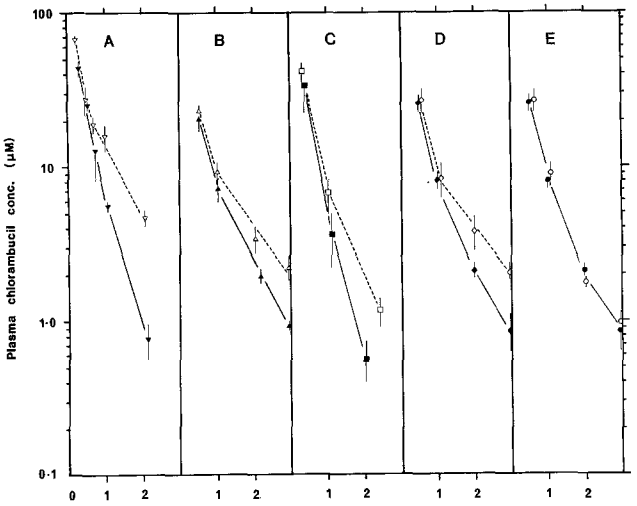
The parent CHL data are in good agreement with those obtained in our preliminary investigation [27]. The quantitative effects of the modifiers on the area under the concentration – time curves (AUC) and apparent elimination $t_{1/2}$ values are illustrated in Table 1, which summarizes data from four independent experiments. Because of the apparently biphasic nature of the clearance curves for CHL, which was more marked in some experiments, particularly with the modifiers, $t_{1/2}$ values are not given for this compound. The pharmacokinetics of CHL were generally very reproducible, all four experiments giving similar results for the controls. In two experiments (A and C) the pharmacokinetics were also determined at the higher dose of 15 mg/kg CHL given on its own, to provide an approximate estimate of DMF for drug exposure. The AUC for total nitrogen mustards was increased by a factor of 2.90 for SKF 525A, 2.72 for imidazole, 2.52 for BENZO, 2.02 for MISO and 1.11 for Ro 05-9963, most of these increases being due to elevated PAAM concentrations. It can also be seen that the DMF for plasma exposure was around 2 for MISO, > 2 for SKF 525A, BENZO and imidazole and << 2 for Ro 05-9963.

The effect of MISO on the formation of monodechlorethylated PAAM (DeC-PAAM) [12] was also studied (Fig. 6). In complete contrast to the β-oxidation metabolites of CHL, which were elevated by MISO, plasma concentrations of DeC-PAAM were markedly reduced. This occurred despite the presence of increased concentrations of PAAM, which is likely to be the principal substrate for dechlorethylation.

Additional experiments were carried out to assess whether the slowing of CHL clearance by MISO was due to end-product "feedback" inhibition as a result of raised PAAM concentrations. PAAM (2.5 mg/kg) was administered to mice 1 h before and 1 h after CHL treatment (7.5 mg/kg), to simulate the increase in PAAM concentration produced by MISO. It was found that a very similar increase in PAAM concentration to that produced by MISO had no effect on the rate of clearance of CHL.

PAAM and β-F_2-CHL. We also carried out limited studies to determine the effect of 2.5 mmol/kg MISO on the clearance of injected PAAM and β-F_2CHL. Similar reductions in the clearance rates of these compounds were observed. For example, at 2 h the concentration of PAAM remaining in the plasma of mice given 15 mg/kg PAAM was 52.0 ±5.9 (2 SE) μ*M* for the control, compared with 85.5 ± 7.0 (2 SE) μ*M* after MISO. For β-F_2CHL, the 2 h control value was 56.5 ± 5.7 (2 SE) μ*M*, compared with 129 ± 16.0 (2 SE) μ*M* with MISO. Both differences were significant ($0.01 > P > 0.002$). For β-F_2CHL the β-oxidation metabolite concentrations were increased as with CHL. In contrast concentrations of DeC-PAAM and one of the two new metabolites [12] were reduced by factors of 2.6 and 1.8, respectively. (The second metabolite could not be quantitated in the presence of MISO.)

MEL. The effect of MISO on MEL pharmacokinetics was studied using sensitizer doses of 2.5 and 5.0 mmol/kg (Fig. 7). MEL was absorbed rapidly following an IP dose of 7.5 mg/kg and reached peak levels in the plasma within 5–10 min. After the peak MEL concentrations declined exponentially, with an apparent $t_{1/2}$ of 26.1 (22.2–31.8) min and an AUC of 0.876 m*M*. min. MISO at a dose of 2.5 mmol/kg had only minimal effect on the pharmacokinetics of MEL. Neither $t_{1/2}$ nor AUC was significantly altered, the treated values being 31.2 (25.4–28.8) min ($P > 0.1$) and 0.924 m*M*. min, respectively. At a dose of 5 mmol/kg, however, MISO caused considerable alteration of MEL pharmacokinetics. The AUC was increased to 1.58 m*M*. min and the $t_{1/2}$ lengthened to 54.4 (49.8–60.0) min ($0.01 > P > 0.02$). The peak concentrations were also increased slightly. As mentioned earlier, this higher dose of MISO produced an appreciable drop in mouse core temperature.



Figs. 2–5. The effect of various modifiers on the plasma pharmacokinetics of 7.5 mg/kg CHL in mice. **Fig. 2.** Parent CHL. **Fig. 3.** Metabolite PAAM. **Fig. 4.** Metabolite DeHCHL. **Fig. 5.** Total nitrogen mustards. Panels **A–E** of each figure show the effects of 2.5 mmol/kg MISO (A), 0.3 mmol/kg BENZO **(B),** 0.13 mmol/kg SKF 525A **(C),** 2.5 mmol/kg imidazole (D) and 2.5 mmol/kg Ro 05-9963 **(E).** Results are from typical experiments. Different symbols represent independent experiments. Each *datum point* is for three mice. *Error bars* show ± SD

Fig. 2. Parent CHL

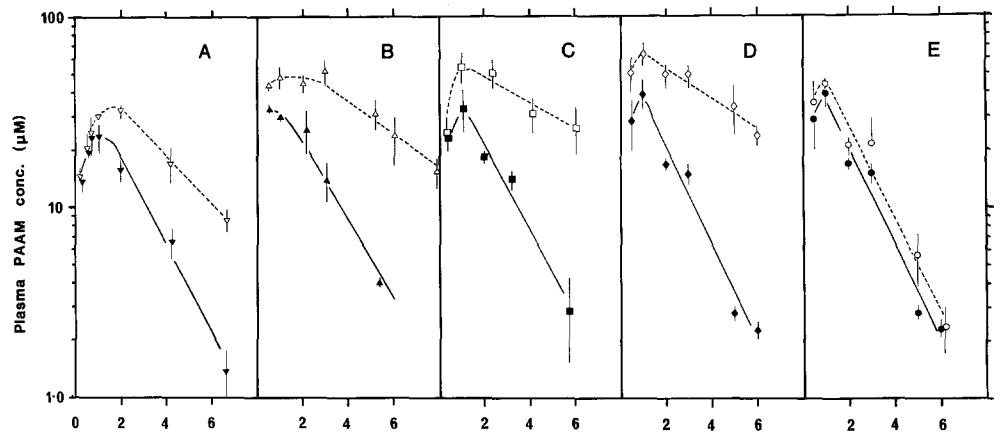


Fig. 3. Metabolite PAAM

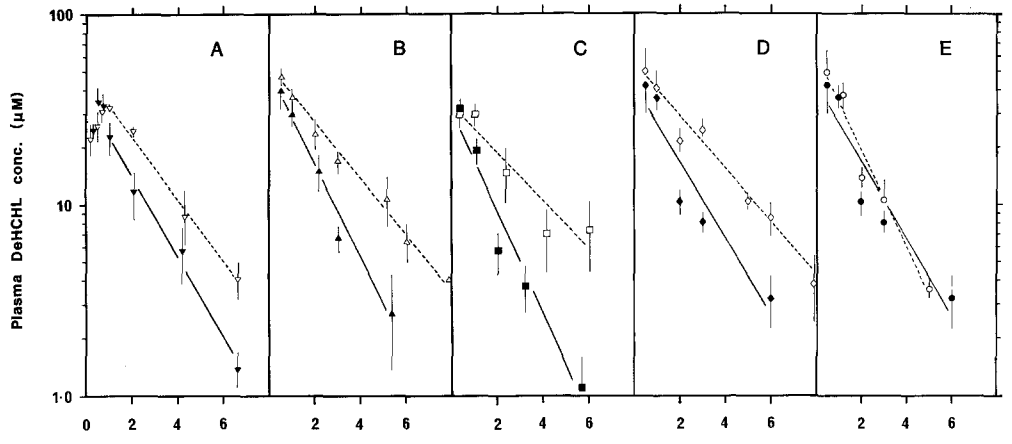


Fig. 4. Metabolite DeHCHL

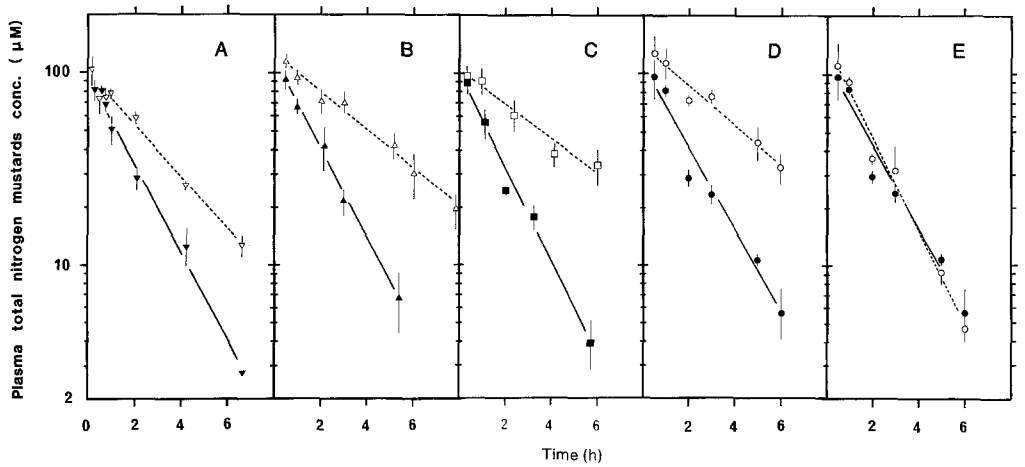
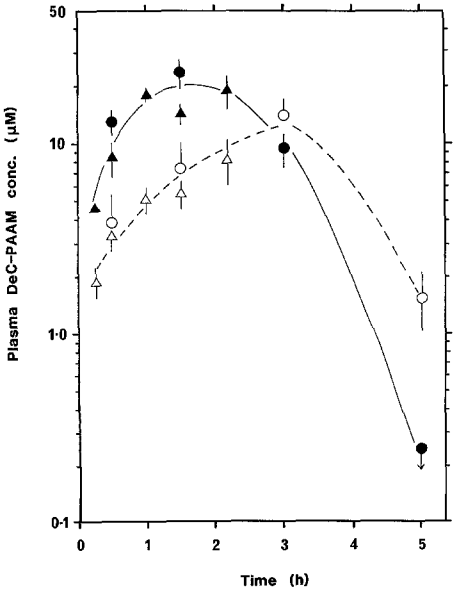


Fig. 5. Total nitrogen mustards

Table 1. Effects of various modifiers on the pharmacokinetics of CHL in mice

Experiment	Treatment (CHL dose) (mg/kg)	CHL Total AUC (mMmin)	PAAM Total AUC (mMmin)	PAAM Plasma $t_{1/2}$[a] (min)	DeHCHL Total AUC (mMmin)	DeHCHL Plasma $t_{1/2}$[a] (min)	Total bifunctional mustards Total AUC (mMmin)	Total bifunctional mustards Plasma $t_{1/2}$[a] (min)
A	Control (7.5)	1.26	4.44	76.5 (60.8–103)	4.24	86.2 (73.1–103)	9.94	78.9 (73.3–85.3)
	Control (15.0)	4.54	8.93	89.2 (67.6–119)	5.31	88.7 (69.4–117)	18.8	72.7 (64.1–84.2)
	MISO[b] (7.5)	2.04	9.96	143 (138–149)	6.96	109 (95.0–127)	19.0	134 (120–151)
B	Control (7.5)	1.28	6.42	86.3 (67.5–120)	4.90	73.8 (63.4–88.0)	12.4	77.2 (70.9–84.6)
	BENZO[c] (7.5)	1.59	21.5	212 (161–307)	8.46	131 (117–148)	31.4	183 (165–206)
C	Control (7.5)	1.50	5.52	80.2 (65.9–114)	3.0	67.5 (53.4–91.8)	10.3	73.4 (64.3–85.4)
	Control (15.0)	3.02	8.90	72.3 (52.9–114)	7.19	54.6 (48.5–62.4)	19.1	63.0 (54.9–73.8)
	SKF 525A[d] (7.5)	2.05	21.9	259 (191–401)	6.30	205 (164–274)	29.9	205 (164–274)
D	Control (7.5)	1.56	5.46	71.3 (58.8–90.7)	5.13	88.9 (63.1–150)	12.7	83.2 (70.4–101)
	Imidazole[b] (7.5)	1.73	22.9	228 (183–204)	9.60	131 (106–171)	34.6	117 (150–218)
	Ro 07-9963[b] (7.5)	1.44	6.84	74.7 (60.9–97.6)	5.71	72.0 (60.3–89.3)	14.1	74.0 (66.2–83.9)

[a] 95% confidence limits in parenthesis
Modifier doses: [b] 2.5 mmol/kg; [c] 0.3 mmol/kg; [d] 0.13 mmol/kg
Pharmacokinetic parameters were calculated from typical experiments. Replicate experiments gave similar results



Plasma protein binding

As described in the previous paper [12], CHL and its metabolites are extensively bound to plasma protein, and this is also true for MEL. We have argued that plasma free drug concentration or exposure may be particularly important in determining biological effects [12]. Table 2 shows that MISO and BENZO had no effect on the reversible plasma protein binding of CHL and MEL, and this was also true for the CHL metabolites, DeHCHL and PAAM. Combination of these protein binding results with the data in Table 1 allows the exposure to plasma free mustards to be calculated. With CHL, BENZO (0.3 mmol/kg) increased this by a factor of 2.9, compared with 2.05 for MISO (2.5 mmol/kg). This dose of MISO had no significant effect on the plasma free melphalan concentrations.

Fig. 6. The effect of MISO (2.5 mmol/kg) on the plasma pharmacokinetics of DeC-PAAM in mice following treatment with 7.5 mg/kg CHL. *Closed symbols* indicate results with CHL alone; *open symbols*, results with CHL + MISO. Data are from two repeated experiments represented by different symbols. Each datum point represents three mice. *Error bars* show ± SD

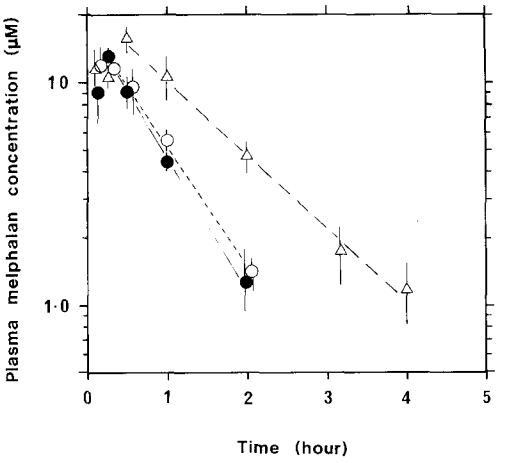


Fig. 7. The effect of MISO on the pharmacokinetics of MEL (7.5 mg/kg). ●———●, MEL alone; O·····O, MEL + 2.5 mmol/kg MISO; △– – –△, MEL + 5.0 mmol/kg MISO. Data are from a typical experiment. Each *datum point* is for three mice. *Error bars* represent ± SD

Chemical hydrolysis

MISO and BENZO had no significant effect on the rates of hydrolysis of the various bifunctional nitrogen mustards in aqueous solution ($P > 0.1$) (Table 3).

Discussion

CHL has been shown to undergo β-oxidation, presumably by mitochondrial enzymes, to form the intermediate DeHCHL and the end-product PAAM (see [13] and refs. therein). In addition, the monodechloroethylated derivative of PAAM, DeC-PAAM, was found as a urinary metabolite in the rat [13], and we have now identified it in quite large amounts in mouse plasma. Although the most likely pathway for its production involves monodechloroethylation of PAAM, some may also be formed via an initial monodechloroethylation of CHL and or DeHCHL, subsequently followed by β-oxidation.

MISO and the other modifiers studied here, with the exception of the hydrophilic MISO metabolite Ro 07-9963, profoundly reduced the rate of plasma clearance of PAAM and also, though to a lesser degree, the clearance of DeHCHL and CHL. As a consequence the drug exposure (AUC) to total bifunctional nitrogen mustards was significantly increased. This increase was mainly due to higher levels of PAAM, particularly when plasma free drug concentrations were considered. This is due not only to the fact that PAAM clearance was the most affected by modifiers, but also that it is proportionally the least bound to plasma protein when compared with the other circulating bifunctional nitrogen mustards [12]. However, the extent of plasma protein binding of the various mustards was not affected by MISO or BENZO, nor were their rates of hydrolysis. In significant contrast to the increase in plasma concentrations of CHL, DeHCHL and PAAM induced by MISO, the formation of DeC-PAAM was markedly impaired. This effect was also demonstrated with BENZO and SKF-525A, though not studied with the remaining modifiers.

The pharmacokinetic effects of MISO and other active modifiers are thus consistent with a model in which the modifiers inhibit the microsomal monodechloroethylation of PAAM, leading to increased plasma concentrations and reduced clearance. We have shown that the reduced clearance of CHL and DeHCHL is not due to feedback inhibition as a result of increased PAAM concentrations, and it is also unlikely for MISO and the other modifiers to act directly on β-oxidation, although this cannot be ruled out. Further, the clearance of β-F_2CHL was greatly reduced even though β-oxidation of this compound has been shown to proceed at a much slower rate than that of CHL [12].

A possible mechanism is that CHL and DeHCHL are indeed metabolized by hepatic cytochrome P-450, and that this reaction is inhibited by the modifiers. The most likely possibility is that CHL and DeHCHL undergo cytochrome P-450-catalysed monodechloroethylation, to intermediates which are then β-oxidised to DeC-PAAM. None of these intermediates have so far been detected, but this may be because the rate of β-oxidation is much faster than the rate of hepatic dechloroethylation [12], so that the equilibrium is shifted greatly in favour of the production of DeC-PAAM. If so, we might expect to find these intermediates in a situation where the β-oxidation process is inhibited. Indeed, two new, unidentified metabolites were found in the plasma of mice receiving β-F_2-CHL, and we were able to show that one of these was markedly reduced in the presence of MISO. Thus, the new metabolites may be dechlorethylation products. On the basis of their chromatographic behaviour and in view of the fact that DeHCHL concentrations are very low, we feel these may be the mono- and didechlorethylation products of β-F_2CHL. In support of our hypothesis that CHL may be dechlorethylated we have shown that it exhibits a type I optical difference binding spectrum with mouse liver microsomes (Lee et al.,

Table 2. The effects of MISO and BENZO on mouse plasma protein binding of various bifunctional nitrogen mustards

Bifunctional nitrogen mustard	% Protein bound[a]		
	Control	MISO	BENZO
MEL	3.78 ± 0.23	3.95 ± 0.33	3.86 ± 0.33
CHL	0.95 ± 0.02	0.88 ± 0.05	0.91 ± 0.04
DeHCHL	1.22 ± 0.04	1.26 ± 0.04	1.25 ± 0.04
PAAM	3.16 ± 0.08	3.21 ± 0.13	3.19 ± 0.07
β-F_2CHL	0.46 ± 0.01	0.48 ± 0.02	0.49 ± 0.02

[a] Values show ± 1 SD

Table 3. The effects of MISO and BENZO on the hydrolysis of CHL, PAAM and MEL in phosphate buffer, pH 7.4, at 37° C

	Half-life (min)[a]		
	Control	MISO	BENZO
CHL	14.1 (11.6–17.1)	15.2 (12.2–18.7)	15.7 (13.1–18.8)
PAAM	16.9 (14.7–19.7)	16.4 (13.1–19.8)	15.9 (13.6–18.6)
MEL	49.2 (46.1–53.0)	56.1 (49.7–61.9)	51.4 (48.3–55.1)

[a] 95% confidence limits in parentheses

unpublished results), indicating that it may be a substrate for cytochrome P-450. Inhibition of dechlorethylation by MISO and other modifiers is not surprising, since we have previously shown them to be effective inhibitors of other liver microsomal P-450-mediated reactions [27] including hydroxylation of the nitrosourea CCNU [10]. Against our hypothesis, however, is the finding by McLean et al. [13] that rat liver microsomes appeared to be unable to metabolize CHL.

Previously we have shown that MISO and other chemical modifiers could enhance the antitumour activity of CHL against the KHT sarcoma in mice [26]. We have confirmed and extended the results for MISO in the present study. Furthermore, MISO also increased the activity of PAAM, a major metabolite of CHL, and β-F_2CHL, an analogue of CHL for which β-oxidation proceeds more slowly and which exhibits an improved therapeutic index [12]. Besides MISO, other modifiers of diverse chemical structures and physiochemical properties, including the lipophilic MISO analogue BENZO, the parent heterocycle imidazole, and the inhibitor of microsomal enzymes SKF 525A, were also effective chemosensitizers of higher potency than MISO [24, 26]. In contrast, the hydrophilic metabolite of MISO, Ro 05-9963, was ineffective.

We have now shown that, without exception, the effective chemosensitizers are also powerful modifiers of CHL pharmacokinetics, and there is no doubt that the two are closely related. DMF values for enhancement of tumour response agreed very well with those for increases in AUC. Thus, the DMFs for chemosensitization were approximately 2 for MISO, $<<2$ for Ro 05-9963 and >2 for SKF 525A, BENZO and imidazole [24, 26], values identical to those reported here for exposure to total free bifunctional nitrogen mustards. Also relevant is the fact that imidazole and SKF 525A lack the nitro moiety but are nevertheless potent chemosenisitizers. The relative potencies of the various modifiers as chemosenisitizers also correlates well with those for pharmacokinetic modification. For example, BENZO is active at a considerably lower dose than MISO. Overall, for CHL as for CCNU [8, 9], we feel the evidence is strong that increased drug exposure is an important factor in MISO enhancement of tumour response. More specifically for CHL, the most important factor is the increase in PAAM exposure, since this is the most predominant circulating bifunctional nitrogen mustard and the least protein bound [12].

In contrast to the results with CHL and its analogues, we have shown that 2.5 mmol/kg MISO has no effect on the acute toxicity or KHT tumour response to the closely related bifunctional nitrogen mustard MEL. We have previously obtained similar negative results in both the KHT and RIF-1 tumours [24, 26]. Also in contrast to the situation with CHL, MEL pharmacokinetics were unchanged by this dose of MISO. On the other hand, however, the higher dose of 5 mmol/kg did increase plasma MEL exposure, by lengthening the elimination $t_{1/2}$ and increasing the peak concentration. This dose also enhanced the acute toxicity and antitumour activity of the drug, the DMFs being respectively 1.6 and 1.35–1.55. A number of other studies have been carried out with this combination (e.g., 2, 3, 6, 7, 15, 16, 18, 19, 21, 22). In general, DMFs for tumour response in the range 1.4–2.7 have been reported for MISO doses of 3.75–5 mmol/kg. Although there has been a report of significant chemosensitization (DMF 1.7) by 2.5 mmol/kg MISO [19], our results and those of others [15, 16] indicate the existence of a threshold dose of >2.5 mmol/kg.

It is significant, therefore, that a similar threshold dose is also required for pharmacokinetic modification with MEL. Our results with 5 mmol/kg closely resemble those obtained by others with 3.75–5 mmol/kg [3, 6, 7, 16, 22]. It should also be pointed out that although these high doses do induce considerable hypothermia, reduced drug clearance was still seen in mice whose body temperatures were maintained at 37°C, albeit to a lesser extent [7, 15, 16, 22]. The mechanism is unknown, although the present results with CHL suggest that inhibition of MEL dechlorethylation might be involved. There is, however, little evidence that MEL is metabolized by microsomal enzymes [15].

There is one important line of evidence suggesting that chemosensitization with MEL does not result solely from modified pharmacokinetics. Using multiple doses of MISO to simulate human pharmacokinetcs, McNally et al. [6, 15] were able to demonstrate enhancement of tumour response to MEL in the absence of pharmacokinetic changes. However, although a similar positive effect was noted by Brown and Hirst [2], other experiments have demonstrated little or no chemosensitization with this regime [15, 16, 25].

In summary, for single large doses of sensitizers, chemosensitization with CHL and MEL by MISO and other chemical modifiers is always accompanied by alterations in the pharmacokinetics of the alkylating agent. Increased exposure to cytotoxic species is a major factor in the mechanism. Other mechanisms may also contribute with MEL [1, 7, 16], particularly in chronic low-dose sensitizer protocols which attempt to mimic clinical pharmacokinetics. In no case was the therapeutic index for the combination with MISO better than with the bifunctional nitrogen mustard given alone, although improved therapeutic indices for MEL have been reported previously (see [14, 20]). With CHL, however, the detailed pharmacokinetic data have shed further light on the relationship between pharmacokinetics, toxicity and antitumour activity, as well as on the pathways of drug metabolism.

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