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DRUG METABOLISM INTERACTIONS WITH ANTICANCER AGENTS IN MICE

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Abstract—The effects of seven antineoplastic agents (dacarbazine, procarbazine, hexamethylmelamine, carmustine, lomustine, chlorozotocin, *p*-carbmethoxyphenyldimethyltriazene) on oxidative drug metabolism were studied. With the exception of dacarbazine and chlorozotocin they inhibited *p*-nitroanisol *O*-demethylase *in vitro* in liver 9000 *g* supernatant of CBALac mice. The inhibition mode was mixed for procarbazine and hexamethylmelamine, apparent K_i values for these drugs were 1.9 and 0.8 mM. Carmustine and *p*-carbmethoxyphenyldimethyltriazene (CMPDT) showed uncompetitive inhibition. Procarbazine inhibition is mediated by a metabonate and the inhibition by CMPDT is reduced by non-specific binding. The administration of these agents at tumour curative doses *in vivo* lead only to weak to negligible depression of *O*- and *N*-demethylase activities 10 min, 1 day and 3 days after cessation of drug administration.

Mixed function oxidase enzymes localized in the endoplasmic reticulum (i.e. microsomal fraction) of the hepatocyte mediate the catabolism of many antineoplastic agents. Some of these drugs, like cyclophosphamide, require oxidative metabolism for activity. Also the intensity and duration of their cytotoxicity may be determined by the rate at which the cytotoxic species generated is metabolically detoxified. Many antineoplastic drugs, e.g. cyclophosphamide [1] and 5-fluorouracil [2] have been demonstrated to depress drug biotransformation of the liver by direct or indirect inhibitory actions on cytochrome P-450 related functions. As these drugs are frequently administered repeatedly as single agents or as part of a combination regime with other drugs, their ability to interfere with drug metabolizing enzyme activity may have clinical consequences. If drug induced reduction in metabolizing capacity of the liver occurs, then levels of active metabolites may be too low to cause sufficient tumour cell kill or the sustained presence of cytotoxic species may result in non-selective toxicity to the host.

Unlike in the case of cyclophosphamide and other classical antineoplastic drugs, it is not known whether the cytotoxic drugs which have been introduced into therapy more recently interfere with hepatic drug metabolism. We therefore have investigated the potential of some of them (dacarbazine, hexamethylmelamine and the nitrosoureas carmustine, lomustine and chlorozotocin) to inhibit mixed function oxygenase activity in mouse liver *in vitro*. These drugs are known or suspected to require oxidative activation. In order to detect chronic effects on the metabolizing capacity of the liver by the treatment with these drugs, we have also studied enzyme activity after administration of doses which have been shown to achieve maximal increase in survival time in tumour bearing mice. In addition, procarbazine and *p*-carbmethoxyphenyldimethyltriazene (CMPDT) were included in the study. Procarbazine given as single doses of 100 mg/kg or more has been shown to inhibit drug metabolizing enzymes [3, 4]; in this study we investigated the effect of a lower dose which inhibits tumour growth. The experimental triazene CMPDT is a derivative of dacarbazine and, though more toxic than dacarbazine, is highly active against rodent tumours [5]. We have studied drug metabolism interactions with this agent as it has been used in biochemical and pharmacological investigations into the mode of action of antineoplastic dimethyl-triazenes [6].

MATERIALS AND METHODS

A. *Drugs.* Procarbazine, lomustine (CCNU), carmustine (BCNU) and chlorozotocin were supplied by Dr Harry B. Wood, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, U.S.A. Hexamethylmelamine, CMPDT and its monomethyl and hydroxymethyl derivatives were synthesized by Drs. Stevens and Simmonds in our laboratories according to published methods [7, 8]. Doxepine was provided by Pfizer Ltd. and dacarbazine (DTIC) by Dome Laboratories Ltd. All other drugs and chemicals were commercially available.

B. *Drug administration.* Three to four-week old male CBALac mice were used throughout the study. The cytotoxic agents were administered i.p. in saline or acetone/arachis oil 1:5 either as five daily doses of 40 mg/kg (dacarbazine, procarbazine, hexamethylmelamine, CMPDT) or as a single dose of 40 mg/kg (lomustine, carmustine, chlorozotocin). According to published reports [5, 9] and our results [10] these regimes cause a maximum increase in survival time in mice inoculated with experimental tumours sensitive to these compounds. The livers were excised from untreated animals or 10 min, 1 day or 3 days after cessation of drug administration and mixed function oxidase activities measured immediately.

C. *Incubations.* 9000 *g* liver fractions were prepared by differential centrifugation of a 10% homogenate in 0.25 M sucrose. Microsomes were prepared according to Schenkman and Cinti [11]. Incubations were carried out with 9000 *g* supernatant equivalent to 80 mg of liver in case of the *p*-nitroanisol *O*-demethylations and 100 mg in case of the *N*-demethylations. Substrate concentrations ranged from 0.03 to 0.2 mM for *p*-nitroanisol and were 5 mM for aminopyrine, 1 mM for doxepine and 0.5 mM for CMPDT. Concentrations of cytotoxic drugs ranged from 0.025 to 3 mM. The incubation mixtures in Earl's buffer were fortified with cofactors which generated 1 mM NADPH in a final volume of 2.5 ml. All incubations were performed in duplicate. After 30 min shaking under air the mixtures were deproteinized with 0.6 ml 20% trichloroacetic acid solution or, in the presence of triazenes, 0.6 ml of a 20% $ZnSO_4$ solution followed by 0.6 ml of a saturated $Ba(OH)_2$ solution.

D. *Assay procedures.* *P*-Nitroanisol *O*-demethylation was determined according to Netter and Seidel [12] by measuring *p*-nitrophenol spectrophotometrically. *N*-Demethylations of aminopyrine, doxepine and CMPDT were determined by measuring formaldehyde (and *N*-hydroxymethyl intermediate) with the Nash reagent [13]. The cytotoxic agents used did not themselves interfere with the determination of *p*-nitrophenol and formaldehyde. Rates of *p*-nitrophenol production were linear with time for 30 min at *p*-nitroanisol concentrations <1 mM in the presence and absence of inhibitors. *N*-Demethylation rates were also linear with time for aminopyrine and doxepine but not for CMPDT. In all cases metabolic demethylations were proportional to protein concentration in pilot experiments in which microsomes were used. At the *p*-nitroanisol concentration of 0.03 mM the amount of substrate disappearing during the 30 min incubation period was great enough to alter the initial substrate concentration. However, changed rates were not observed. Apparent K_i values were determined using the method described by Dixon [14]. Intercepts, slopes and points of intersection were calculated from linear regressions for each experiment. The apparent K_i and K_m values given in the results are the mean ± S.E.M. of at least four observations. Due to the heterogeneity of the enzyme source these values are of an indicative rather than a defining nature [15].

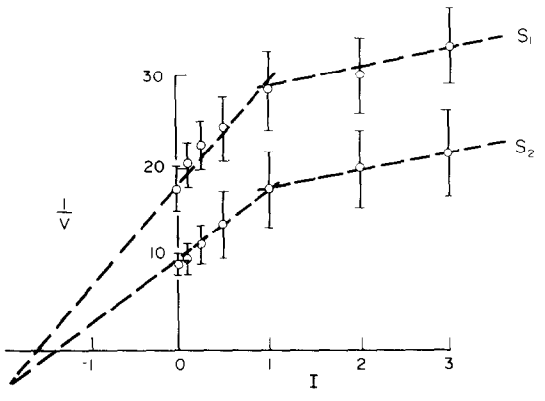

Fig. 1. Inhibition of *p*-nitroanisol *O*-demethylase by procarbazine. S_1 and S_2 represent respectively *p*-nitroanisol substrate concentrations of 0.03 and 0.1 mM. Inhibitor concentrations (I) are given in mM. Velocities (V) are given as nmoles of *p*-nitrophenol formed per 80 mg of liver during a 30 min incubation period. Each point shows the mean of at least four observations. Details of the incubation conditions under Methods.

Duration of pentobarbital anaesthesia (sleeping time) was measured as the interval between the loss and the voluntary recovery of the righting reflex. Pentobarbital sodium was injected i.p. at the dosage of 60 mg/kg and mice were placed in dorsal recumbancy during the period they were anaesthetised. The mean duration of anaesthesia in 30 untreated mice was 66 ± 12 min.

RESULTS

In vitro *interactions*

The *O*-demethylation of *p*-nitroanisol is a well investigated model reaction of oxidative metabolism [12]. We determined the influence of different concentrations of cytotoxic agents on the rate of *p*-nitroanisol biotransformation in 9000 *g* fractions of liver preparations. Dacarbazine and chlorozotocin did not inhibit the reaction at concentrations <3 mM. The other agents interfered with mixed function oxygenase activity. This was supported by pilot experiments in which they inhibited aniline *p*-hydroxylase to a similar extent. On the assumption that the drugs undergo metabolic oxidation similarly

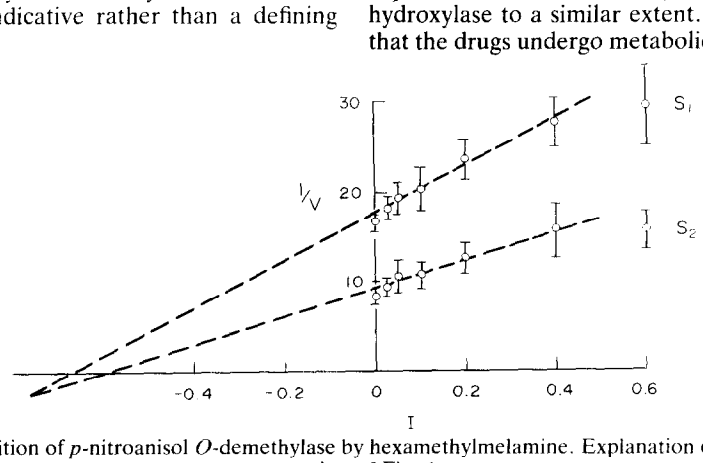

Fig. 2. Inhibition of *p*-nitroanisol *O*-demethylase by hexamethylmelamine. Explanation of symbols, see caption of Fig. 1.

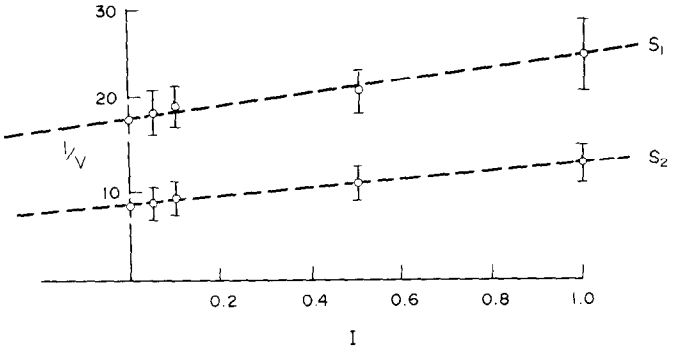

Fig. 3. Inhibition of *p*-nitroanisol *O*-demethylase by carmustine. Explanation of symbols, see caption of Fig. 1.

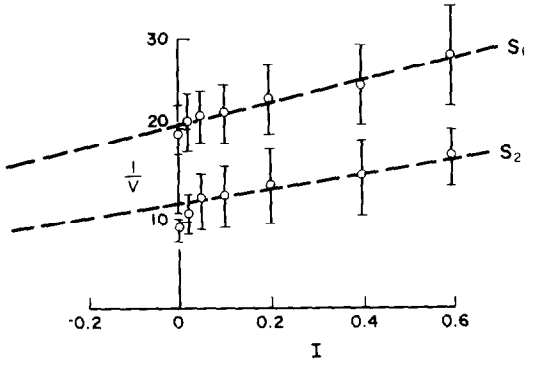

Fig. 4. Inhibition of *p*-nitroanisol *O*-demethylase by *p*-carbmethoxyphenyldimethyltriazene (CMPDT). Explanation of symbols, see caption of Fig. 1.

to *p*-nitroanisol competing for the same binding sites on the enzymes the reciprocal of the rate of *p*-nitroanisol *O*-demethylation at two substrate concentrations was plotted against cytotoxic drug concentration [14]. Different inhibition profiles were obtained. Procarbazine exhibits a biphasic profile with an app. K_i value for the inhibition data below 1 mM of $1.9 \pm 1.1 \times 10^{-3}$M (Fig. 1). The profile for hexamethylmelamine (Fig. 2) at concentrations >0.5 mM also levels off, indicating a change in the nature of the type of inhibition. The app. K_i for hexamethylmelamine is $7.6 \pm 2.8 \times 10^{-4}$M. From these plots one cannot ascertain whether the mode of inhibition is competitive or of a mixed type. Cornish-Bowden plots (s/v vs I) used in conjunction with the Dixon plots [16] revealed that the mode of

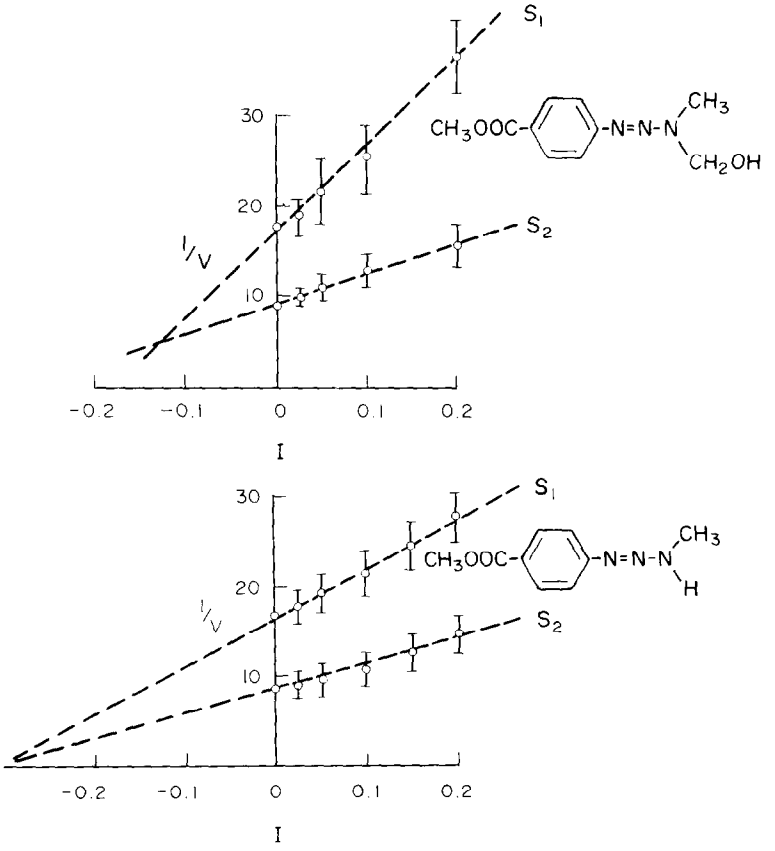

Fig. 5. Inhibition of *p*-nitroanisol *O*-demethylase by two metabolites of CMPDT. Explanation of symbols, see caption of Fig. 1.

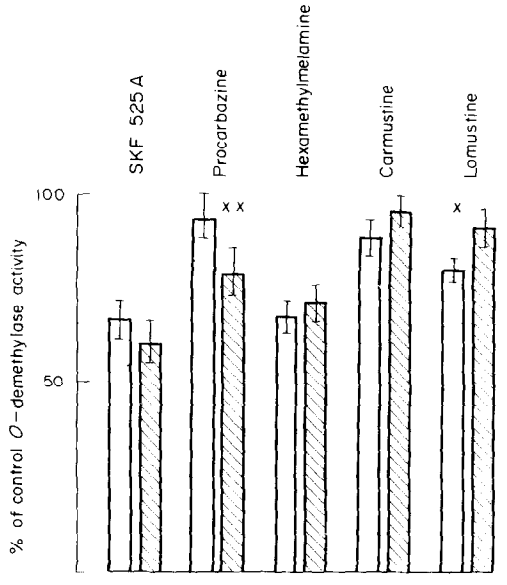

Fig. 6. Influence of preincubation of antineoplastic agents with 9000 *g* liver supernatant on their ability to inhibit *p*-nitroanisol *O*-demethylation *in vitro*.

Drugs (0.4 mM SKF 525-A and procarbazine, 0.2 mM lomustine, carmustine and hexamethylmelamine) were incubated with 9000 *g* supernatant and cofactors 15 min prior to the addition of *p*-nitroanisol (0.1 mM). *p*-Nitrophenol formation was determined after 30 min incubation according to the details outlined under Methods. Results are expressed as per cent of control *p*-nitroanisol *O*-demethylase activity which was 118 ± 17 nmoles *p*-nitrophenol/80 mg liver/30 min in 46 experiments. Open bars represent enzyme activities in the presence of drugs without preincubation, hatched bars after preincubation. They are the mean ± S.E.M. of at least six experiments. A significant difference in degree of inhbition between the two incubation modes is denoted by × ($p < 0.05$) and ×× ($p < 0.01$).

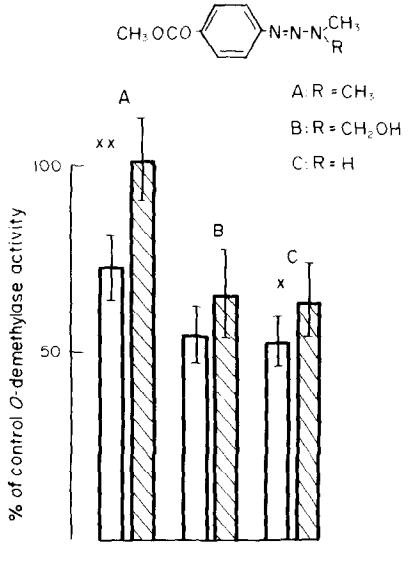

Fig. 7. Influence of preincubation of triazenes (0.2 mM) with 9000 *g* mouse liver supernatant on their ability to inhibit *p*-nitroanisol *O*-demethylation. Details, see caption of Fig. 6.

inhibition by both drugs is mixed. Carmustine (Fig. 3), lomustine, and CMPDT (Fig. 4) showed uncompetitive inhibition. Two active metabolites of CMPDT, the monomethyltriazene and the *N*-hydroxymethyltriazene (Fig. 5) inhibited *p*-nitroanisol *O*-demethylation more strongly than CMPDT and by a different mode, namely of a mixed type. The app. K_i values for the metabolites are $2.8 \pm 0.9 \times 10^{-4}$M for the monomethyl- and $1.2 \pm 0.8 \times 10^{-4}$M for the hydroxymethyltriazene. By comparison the app. K_m for *p*-nitroanisol *O*-demethylation was determined as $7.0 \pm 1.9 \times 10^{-5}$M. The marked inhibition shown by the triazene metabolites is in accordance with the finding that the rate of dimethyltriazene *N*-demethylation *in vitro* is linear only for several minutes after which it decreases dramatically [17].

In order to elucidate whether metabolites or the unchanged compounds were involved in the inhibition of demethylase activity the agents were exposed to liver homogenate at a concentration of 0.2 mM or 0.4 mM 15 min prior to the addition of 0.1 mM *p*-nitroanisol (Fig. 6). Procarbazine showed marked inhibition only after preincubation, like SKF 525-A, 2 diethylaminoethyl 2,2-diphenylvalerate, a standard inhibitor of mixed function oxygenases [18]. Preincubation of the chemically unstable procarbazine [19] with buffer alone also lead to an increase in inhibition by 24 per cent. This indicates that the inhibition is mediated by a procarbazine metabonate.

Carmustine and lomustine exhibited decreased inhibition after preincubation with liver homogenate, so did CMPDT and its two metabolites (Fig. 7). Preincubation of CMPDT with microsome free liver cytosol or with a 0.3% bovine serum albumin solution 15 min before the addition of microsomes, cofactors and substrate also resulted in decreased inhibition (Table 1). Apparently, this inhibition is partly abolished by non-specific binding of triazene rather than by biotransformation to non-inhibitory metabolites.

Enzyme activity after drug administration

Body and liver weight of the animals were not affected by the administration of the drugs at tumour inhibitory doses. Three days after cessation of cytotoxic drug administration hepatic mixed function

Table 1. Influence of different preincubations on the inhibition of microsomal *p*-nitroanisol *O*-demethylation by CMPDT

Conditions of CMPDT preincubation	% of control *O*-demethylase activity
No preincubation	69.4 ± 11.0
Buffer	71.7 ± 12.2
Liver cytosol	98.1 ± 6.7
BSA 0.3%	94.2 ± 7.3

CMPDT (0.2 mM) was incubated with 2 ml of buffer, 20% liver homogenate microsomal supernatant or 0.3% buffered bovine serum albumin solution prior to the addition of microsomes, cofactors and 0.1 mM *p*-nitroanisol. Incubation details under Methods. *O*-Demethylase activities are expressed as per cent of enzyme activity in the absence of CMPDT, which was 123 ± 12 nmoles *p*-nitrophenol/80 mg liver/30 min ($n = 8$), and are the mean ± S.E.M. of at least 4 experiments.

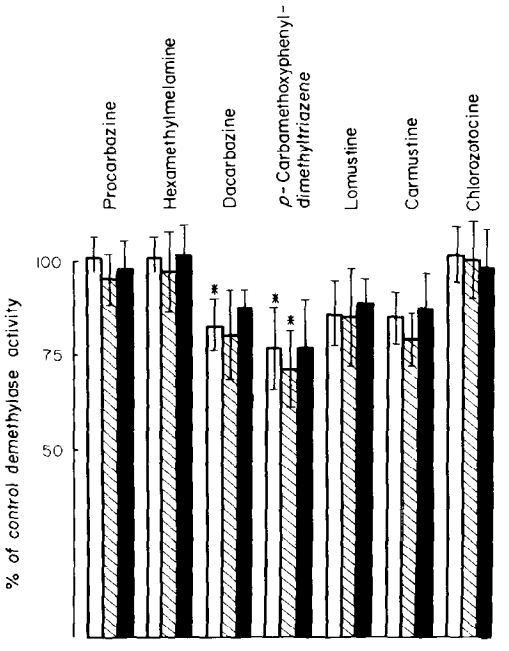

Fig. 8. Mouse hepatic *N*-demethylase activities 3 days after the administration of antineoplastic agents.

N-Demethylations were measured with aminopyrine (open bars) doxepine (hatched bars) and CMPDT (black bars) as substrates. Incubation conditions and doses of antineoplastic agents are given under Methods. Results are the mean of at least six animals and are expressed as per cent of *N*-demethylations in control animals, which were 311 ± 51 nmoles HCHO for aminopyrine, 81 ± 18 nmoles HCHO for doxepine and 385 ± 62 nmoles HCHO for CMPDT per 100 mg liver formed during 30 min incubation in 18 experiments. Stars indicate that *N*-demethylations were significantly different ($P < 0.05$) from 100 per cent values.

oxygenase activity was assessed by measuring aminopyrine, doxepine and CMPDT *N*-demethylase (Fig. 8) and *p*-nitroanisol *O*-demethylase activities (not shown) in 9000 *g* liver fractions. Enzyme activities were significantly depressed only by pretreatment with dacarbazine and CMPDT. Dacarbazine administration caused a decrease in *N*-demethylase activities of 17% (aminopyrine), 20% (doxepine), and 12% (CMPDT); treatment with CMPDT lead to a depression of 24% (aminopyrine), 29% (doxepine), and 23% (CMPDT). The administration of hexamethylmelamine, procarbazine and chlorozotocin did not show an effect and lomustine and carmustine exhibited weak depression of 11–20 per cent which was not significant. The pattern of enzyme activities observed 10 min and 1 day after administration and the values obtained with *p*-nitroanisol as substrate were similar to those shown in Fig. 8.

None of the drugs caused a significant prolongation of pentobarbital induced sleeping time in these mice at several time intervals after cessation of drug administration.

DISCUSSION

Zubrod recently put the view forward that combination chemotherapy in the treatment of malignancies has a number of serious drawbacks, among them are complexity, expense, induction of secondary tumours and other toxicities [20]. Therefore, he questioned if cure rates can be increased by empirical combinations of current drugs. However, combinations based on cell kinetics and pharmacological data may represent a more promising approach and may lead to decreased toxicity. Pharmacologically rationalized drug combinations should take drug metabolism interactions of the components into account, especially when drugs requiring activation are used. The results reported here show that hexamethylmelamine, procarbazine, lomustine, carmustine and the experimental triazene CMPDT and its active metabolites are capable of inhibiting hepatic mixed function oxygenases and thus oxidative bioactivation or detoxification reactions *in vitro*.

Compounds that are effective inhibitors of metabolism *in vitro* often fail to act as inhibitors *in vivo*. A drug has to possess several pharmacokinetic properties to render it capable of inhibiting the metabolism of another drug *in vivo* [21]. The inhibitor must exert its effects at therapeutic levels. Absorption and distribution of the inhibitor should favour its accumulation in sufficient quantity to compete with the substrate in the liver. The metabolism of the inhibitor should be sufficiently slow so as to maintain its presence at the metabolic site. Non microsomal and extrahepatic pathways of metabolism should play a minor role in the disposition of the inhibitor. The information available on the clinical pharmacokinetics of most of the drugs tested here do not suggest that they accumulate in the liver, that their metabolism is particularly slow or that metabolic pathways other than oxidative microsomal ones contribute considerably to their activation or detoxification. The apparent inhibitor constants of four of these agents for *p*-nitroanisol-*O*-demethylase measured *in vitro* are in the order of 10^{-4} to 2×10^{-3} M and appreciably above the apparent K_m of *p*-nitroanisol *O*-demethylation. This already indicates that mixed function oxidase activities are probably not affected by the inhibitory protential of these cytotoxic agents when they are present at therapeutic levels. Of all the drugs tested here hexamethylmelamine has the longest biological half life, between 5 and 10 hr as measured in patients with ovarian malignancies [22]. A long half life may be a predisposing factor for drug metabolism interactions in the liver. However, when given in tumour inhibitory doses to mice in this study hexamethylmelamine and the other antineoplastic agents did not notably influence oxidative metabolism measured shortly after administration.

The chronic administration of the dimethyltriazenes dacarbazine and CMPDT lead to a significant, even though not dramatic depression of mixed function oxidase activities after three days. Marinello *et al.* [23] recently showed that the depression of metabolism by cyclophosphamide is due to the denaturation of microsomal cytochrome P-450 by cyclophosphamide metabolites. It remains to be seen if other cytotoxic drugs have a similar effect on the hepatocyte. This study indicates however that inhibitory drug metabolism interactions involving hexamethylmelamine, dacarbazine, procarbazine, carmustine, lomustine and chlorozotocin may not be of major

importance in the chemotherapy of patients with malignancies.

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