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CYCLOPHOSPHAMIDE CYSTITIS—IDENTIFICATION OF ACROLEIN AS THE CAUSATIVE AGENT*

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Abstract—Haemorrhagic cystitis of the bladder caused by the antitumour agents cyclophosphamide {2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide} and ifosfamide [3-(2-chloroethyl)-2-(2-chloroethylamino)tetrahydro-1,3,2-oxazaphosphorine 2-oxide] was studied in the rat. Optimum conditions in this model for protection from toxicity by *N*-acetyl-L-cysteine were found. Phosphoramide mustard, the ultimate alkylating metabolite of cyclophosphamide, and 5,5-dimethylcyclophosphamide, which is metabolised but forms no cytotoxic products, had minimal effects on the bladder. However, diethylcyclophosphamide [2-(diethylamino)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide] caused severe cystitis in the male rat, and significant, but less extensive damage, in the female rat; *N*-acetyl-L-cysteine protection against this toxicity was demonstrated. As acrolein is the only reactive and cytotoxic metabolite of diethylcyclophosphamide, the role of acrolein as the causative agent in cyclophosphamide cystitis was proven.

Cyclophosphamide (CP)† was introduced as an antitumour agent in 1958 [1] and since 1959 numerous reports [see ref. 2] have been published concerning cystitis, a side effect not observed with other alkylating agents. The first experimental study of bladder toxicity caused by CP was that of Phillips *et al.* [3], who concluded that it was due to contact between the bladder wall and a CP metabolite in the urine. Primack [4] studied the use of *N*-acetyl-L-cysteine (NAC) instilled into the bladder as a protective regimen, on the grounds that alkylating agents may be rapidly inactivated by reaction with sulphydryl groups. Since that time various reports of the protective effect of NAC against the toxic effects of CP [5–7] and its congener, ifosfamide (IP) [8] have been made. All have assumed that protection against bladder toxicity with NAC is due to removal of reactive alkylating agents. Indeed dose-limiting toxicity of IP is urinary tract toxicity [9] and this has been tentatively related to the urinary excretion of more alkylating metabolites than with CP [10]. However, a recent report [11] cleverly differentiated between CP metabolites causing cystitis and lethality, and those alkylating products with immunosuppressive properties. However, direct evidence that antitumour efficacy was not adversely affected was not provided. The authors concluded that the metabolite causing haemorrhagic cystitis was possibly acrolein.

From this report [11] it was apparent that acrolein could be positively implicated as the reactive metabolite responsible for CP cystitis by using carefully chosen metabolites and analogues of CP. These are shown in Fig. 1, together with their principal cytotoxic metabolites. CP yields phosphoramide mustard (PAM) and acrolein, IP yields iphosphoramide mustard and acrolein, 5,5-dimethylcyclophosphamide (5,5-diMeCP) yields neither PAM nor an acrolein analogue and, most importantly, diethylcyclophosphamide (diethyl CP) yields acrolein alone. PAM is the ultimate cytotoxic

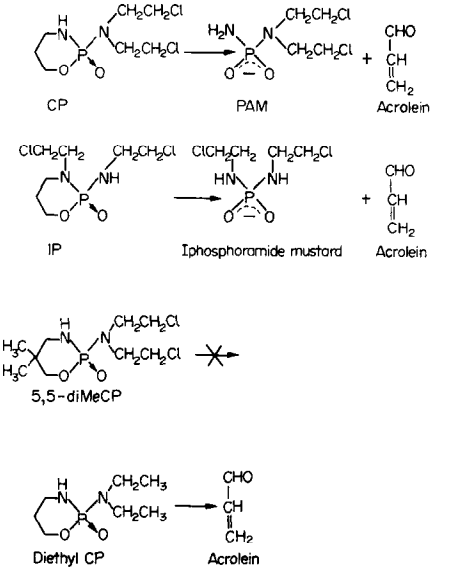

Fig. 1. The structures of CP and the analogues used, showing the principal cytotoxic metabolites that can be formed.

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† Abbreviations used: CP—cyclophosphamide {2-[bis(2-chloroethyl)aminotetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide}; IP—ifosfamide [3-(2-chloroethyl)-2-(2-chloroethylamino)-tetrahydro-1,3,2-oxazaphosphorine 2-oxide]; 5,5-diMeCP—5,5-dimethylcyclophosphamide; Diethyl CP—diethylcyclophosphamide [2-(diethylamino)-tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide]; PAM—phosphoramide mustard [*N*,*N*-bis(2-chloroethyl)phosphorodiamidic acid]; NAC—*N*-acetyl-L-cysteine.

metabolite of CP [12] and is a moderately good antitumour agent in its own right [13] in experimental systems. Acrolein is forty-fold less toxic than PAM when tested against tumour cells *in vitro* [14]; when released *in vivo* by metabolism of diethyl CP it is totally inactive as an antitumour drug [15]. However, although acrolein is inactive alone against tumour cells, it cannot be ruled out that, as PAM and acrolein are formed together by ring scission of the primary hydroxylated metabolite of CP [12, 16], acrolein may act synergistically with the alkylating agent.

Clearly, if the alkylating metabolite is responsible for cystitis, PAM should be toxic to the bladder. If toxicity is due to the primary metabolite before ring scission, then 5,5-diMeCP, which forms such a primary hydroxylated metabolite but no strongly alkylating metabolites [17], should cause cystitis, and if acrolein is the causative agent, then diethyl CP should be effective.

MATERIALS AND METHODS

Cyclophosphamide monohydrate was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England. The following were obtained as gifts: Ifosfamide (Dr. B Dean, W. B. Pharmaceuticals Ltd., Bracknell, Berks, England); 5,5-dimethylcyclophosphamide (Professor N. Brock, Asta-Werke, Bielefeld, West Germany); phosphoramide mustard cyclohexylammonium salt (Dr. H. B. Wood, N.C.I. Bethesda, Maryland 20014, U.S.A.). Bis(chloroethyl)amine hydrochloride, diethylcyclophosphamide [15] and 3-hydroxypropylmercapturic acid dicyclohexylammonium salt [18] were synthesised at this institute. Acrolein was purchased from Aldrich Chemical Co. Ltd., Gillingham, Dorset, *N*-acetyl-L-cysteine, gum arabic and gum tragacanth from Sigma London Chemical Co. Ltd., Poole, Dorset and diethylamine from Hopkin and Williams, Chadwell Heath, Essex.

Male and female Wistar rats, bred in this institute were used. Drugs were administered intraperitoneally (i.p.) in aqueous solution except for acrolein (subcutaneously in aqueous solution) and NAC [i.p. in 0.1 M phosphate buffer adjusted to pH ~ 5 or orally in phosphate buffer, aqueous gum tragacanth (3% w/v) or aqueous gum arabic (3% w/v)]. After 48 hr, the rats were killed by cervical dislocation, the bladders removed, expressed and weighed wet. After drying overnight at 110°, the tissue dry weight was obtained. Results are expressed as mg wet (or dry) weight/100 g body weight ± S.E. and as mg water/100 g body weight. Statistical analysis of the data was made using N − 1 weighting for the standard deviation, and N weighting for the variance. Values of P less than 0.02 obtained from Student's *t* test were considered significant.

Measurement of the inhibition of the Walker 256 carcinosarcoma tumour *in vivo* were essentially as described by Rosenoer *et al.* [19] except that ascites cells were used for the intramuscular implant.

RESULTS AND DISCUSSION

As reported by Levy and Harris [11], CP caused gross haemorrhagic cystitis when administered at 100 mg/kg (0.365 mmoles/kg) in female rats. In male rats, the effect was more pronounced (see Table 1). However, protection by orally administered NAC in gum arabic solution was not particularly effective, presumably for pharmacokinetic reasons (i.p. injection of cyclophosphamide has been used in this study as opposed to intravenous administration). Thus various timings and routes of administration of NAC were investigated (Table 1). Injection i.p. 30 min after CP appeared to give consistent protection against cystitis in both male and female rats and was thus chosen as standard. To demonstrate that no significant reaction occurred under these circumstances between CP and NAC in the peritoneal cavity, a comparative test against the Walker tumour was conducted (Fig. 2). No significant difference between CP and its combination with NAC was found (LD_{50}—both 480 mg/kg; ID_{90}—16.5 mg/kg alone, 20 mg/kg in combination). This is in agreement with the finding [11] that NAC did not interfere with the production of alkylating metabolites from CP *in vivo*.

Figure 3 shows the effects on wet and dry bladder weights of CP (100 mg/kg; 0.365 mmoles/kg), IP (100 mg/kg; 0.365 mmoles/kg), 5,5-diMeCP (105 mg/kg; 0.365 mmoles/kg), PAM (120 mg/kg; 0.365 mmoles/kg) and diethyl CP (100 mg/kg; 0.521

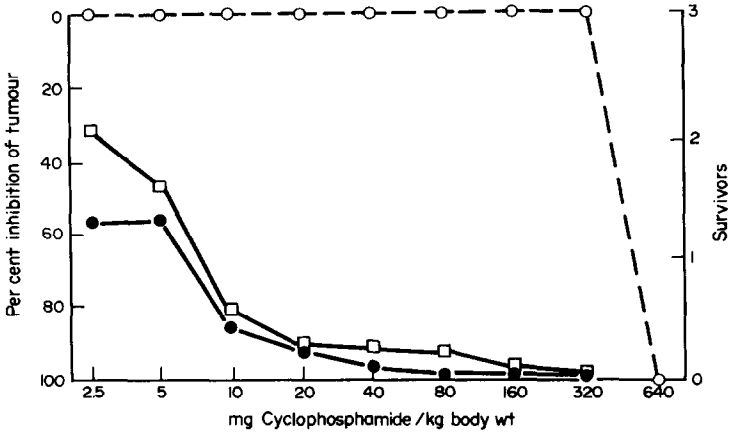

Fig. 2. Inhibition of growth of the intramuscular Walker 256 carcinosarcoma in rats by CP alone (—●—) and in combination with NAC (—□—). Survivors (- - ○ - -) with both treatments were identical.

Table 1. Protection of the rat bladder from cyclophosphamide induced cystitis by *N*-acetylcysteine

		Time of dosing of NAC relative to CP (min)	Number of animals	Male Wet weight (mg/100 g body wt)	Male Dry weight (mg/100 g body wt)	Male Water content (mg)	Female Wet weight (mg/100 g body wt)	Female Dry weight (mg/100 g body wt)	Female Water content (mg)
Control		—	20	19.3 ± 0.6	7.3 ± 0.3	12	23.0 ± 0.9	7.0 ± 0.2	16
CP		—	20	82.6 ± 4.6*	13.0 ± 0.5*	70	72.2 ± 6.6*	12.7 ± 0.8*	60
CP + NAC (in phosphate buffer)	NAC orally	−30	5	79.5 ± 6.3*	12.4 ± 0.7*	67	—	—	—
CP + NAC (in gum tragacanth)	NAC orally	−30	5	77.8 ± 12.9*	12.4 ± 1.1*	65	—	—	—
CP + NAC (in gum arabic)	NAC orally	−30	5	49.4 ± 3.9*†	11.0 ± 0.5*†	38	52.1 ± 7.2*	10.6 ± 0.8*	41
CP + NAC (in gum arabic)	NAC orally	+30	5	42.0 ± 7.9*†	10.3 ± 1.2*	32	—	—	—
CP + NAC	NAC i.p.	−30	5	21.0 ± 0.7†	7.8 ± 0.4†	13	31.8 ± 2.1*†	8.6 ± 0.3*†	23
CP + NAC	NAC i.p.	0	5	29.9 ± 3.3*†	8.0 ± 0.4†	22	23.7 ± 1.3†	9.1 ± 0.5*†	15
CP + NAC	NAC i.p.	+30	10	26.1 ± 3.5†	8.0 ± 0.4†	18	20.3 ± 0.7*†	7.9 ± 0.2*†	12

Cyclophosphamide (CP) was injected i.p. at 0.365 mmoles/kg. *N*-Acetylcysteine (NAC) was dissolved in 3% gum tragacanth, 3% gum arabic or 0.1 M phosphate buffer pH 7.4 (pH readjusted to approx. 5), and was dosed orally or i.p. at 2.45 mmoles/kg, either 30 min before, 30 min after or concurrently with the cyclophosphamide. Bladder weights are given as mg/100 g body weight ± S.E.

* $P < 0.02$ compared to control animals.

† $P < 0.02$ compared to cyclophosphamide-treated animals.

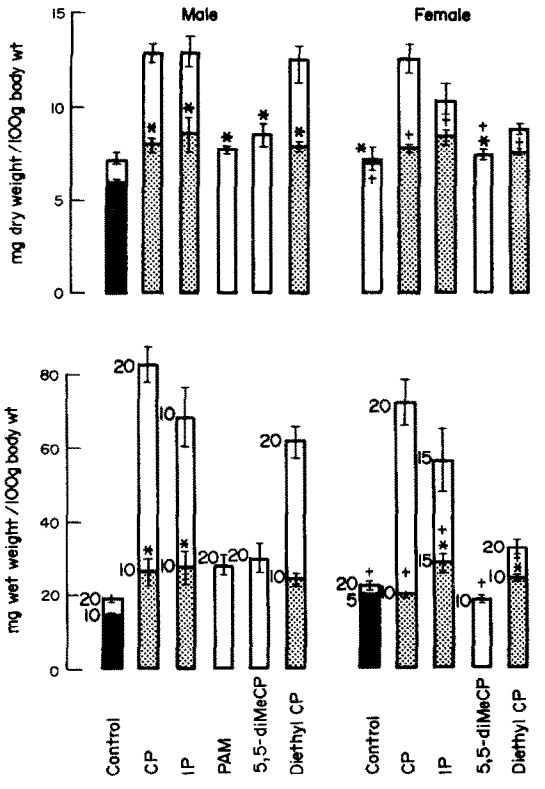

Fig. 3. Bladder weights, expressed as mg wet (or dry) wt/100 g body wt in male and female rats treated i.p. with CP and its analogues (0.365 mmoles/kg, except diethyl CP—0.521 mmoles/kg). Box-hatched bars indicate combination of the test compound with NAC (2.45 mmoles/kg i.p. 30 min after test drug). Gray bars indicate control animals injected with water. Error bars show the standard error. Figures show the number of animals in each group. All differences from controls and the combinations with NAC were statistically significant except those marked * (not significantly different from untreated controls) and † (not significantly different from water-injected controls) above the error bar.

mmoles/kg). The reason for a higher molar dose of diethyl CP than of the other compounds was that measurements of the production of acrolein *in vitro* by male rat liver microsomes and cofactors showed that diethyl CP released approx. 60–80 per cent of that formed from CP [20]. As insufficient material was available for a dose–response study, it was decided to increase the dose level to compensate for a comparable effect *in vivo*.

It should be seen that only three of the five compounds produce significant increases in bladder weight and that the three compounds are those which release acrolein. Whilst in male rats the extent of bladder damage was comparable for these compounds, in female rats the response was graded—CP produced damage comparable to that in male rats, whereas diethyl CP caused less than 20 per cent of the effect found in males. This sex difference is almost certainly due to pharmacokinetic factors. The 6-fold difference in the rate of the microsomal metabolism of cyclophosphamide between uninduced male and female rats [21] serves to illustrate the potential for wide variation between the sexes. PAM, the active antitumour principle of CP, caused only a mild oedema of the bladder in male rats, as did 5,5-diMeCP. No haemorrhage or thickening of the bladder wall was observed. Acrolein itself could not be compared as i.p. injection caused gross peritonitis and subcutaneous injection proved extremely irritant to the rats. Direct instillation of acrolein into the bladder was not attempted because this would be a poor model both quantitatively and kinetically.

The three compounds causing haemorrhagic cystitis were also tested in combination with NAC (400 mg/kg, 2.45 mmoles/kg). As can be seen from Fig. 3, protection was almost complete and, in many cases, bladder wet and dry weights did not differ significantly from control values. In all cases, the protection by NAC against haemorrhagic cystitis was highly significant.

Other potentially irritative compounds which may be formed during metabolism are bis(chloroethyl)amine (from CP) and diethylamine (from diethyl CP). However, neither compound, administered i.p. at 0.365 mmoles/kg caused oedema or any increase in the bladder tissue dry weight (Table 2). An important urinary excretion product formed from acrolein is 3-hydroxypropylmercapturic acid [22, 23]. This compound was identified in the urine of rats treated with CP, diethyl CP and IP, using a paper chromatographic technique [18]. It also failed to elicit any bladder toxicity when given i.p. (Table 2).

The data show that acrolein is the causative agent in CP cystitis. It is likely that the acrolein formation occurs within the bladder by breakdown of aldophosphamide, CP's primary metabolite, for two reasons. Firstly, acrolein is very reactive and will thus have a

Table 2. Effects on the bladders of male rats of three cyclophosphamide related metabolites

	Number of animals	Wet weight	Dry weight	Water content
Control	20	19.3 ± 0.6	7.3 ± 0.3	12
Bis(chloroethyl)amine hydrochloride	10	20.8 ± 1.1	7.5 ± 0.3	13
Diethylamine	10	16.3 ± 0.9	6.8 ± 0.3	10
3-Hydroxypropyl-mercapturic acid, dicyclohexyl-ammonium salt	9	20.7 ± 0.9	6.6 ± 0.8	14

Compounds were injected i.p. at 0.365 mmoles/kg. Bladder weights are expressed as mg/100 g body weight ± S.E. Water content is expressed as mg/100 g body weight.

short biological half-life and, secondly, acrolein administered by injection or released by metabolism of CP, IP and related compounds is largely excreted into the urine as 3-hydroxypropylmercapturic acid. The data also support the observations of Levy and Harris [11] that CP cystitis may be prevented successfully with NAC. Recent studies by Brock *et al.* using sodium 2-mercaptoethane sulphonate as a protective agent [24] show considerable advantages of this compound compared to NAC for clinical use.

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Note inserted in proof: Some of the observations and conclusions of this paper have been duplicated recently by N. Brock, J. Stekar, J. Pohl, U. Niemeyer and G. Scheffler, whose data are to be published in *Arzneimittel-Forschung*.