Europ. J. Cancer Vol. 17, No. 6, pp. 595–607, 1981
Printed in Great Britain

0014-2964/81/060595-13$02.000/0

Perspectives in Cancer Research

Studies on the Urotoxicity of Oxazaphosphorine Cytostatics and its Prevention—I

Experimental Studies on the Urotoxicity of Alkylating Compounds*

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Abstract—*The urotoxic potency of various (mainly alkylating) drugs was studied in an extensive series of experiments. It was found that damage to the kidneys and to the efferent urinary tract (haemorrhagic cystitis) is a specific side effect of compounds possessing the oxazaphosphorine ring. This effect is due to the renal excretion of toxic metabolites. The only carriers of urotoxicity are the renally eliminated fractions of the 4-hydroxy-oxazaphosphorines and acrolein which is formed spontaneously therefrom. Other oxazaphosphorine metabolites and breakdown products such as the directly alkylating phosphoric acid diamides, 4-keto-derivatives and carboxyderivatives, have at most only a very slight urotoxic potency. The relationships between the chemical structure and the urotoxicity have been clarified in the group of oxazaphosphorines. Using a standardised test model (rat) the urotoxic side effects of cytostatics were studied experimentally and were measured quantitatively. The urotoxic effects were found to be dose- and concentration-dependent and also showed a marked dependence on the pH. The manifestations of inflammation were more pronounced in an alkaline than in an acidic milieu.*

INTRODUCTION

DESPITE the advances achieved in recent decades in the domain of cancer chemotherapy, the selectivity and thus the therapeutic range of many cytostatics are still unsatisfactory. The achievable therapeutic results are limited by toxic side effects. At the same time, it is becoming increasingly difficult to synthesise novel chemical compounds with higher carcinotoxic efficacy. Great practical importance therefore attaches pharmacologically to detoxification, for reducing the general toxicity and for preventing undesirable organotropic side effects [1].

One of the researchers who has contributed considerably to the pharmacological study of detoxification [2] is Abraham Goldin. He celebrates his 70th birthday this year and our thoughts turn to him in friendship.

*This work was supported by the Bundesministerium für Forschung und Technologie, Bonn.

In principle, reduction in the toxicity of active agents can be achieved in various ways:

Chemically, by alteration of the molecular structure. This path has led from the toxic nitrogen mustard to the development of the much better tolerated cytostatics Endoxan®, Ixoten® and Holoxan® [3–5].

Pharmacologically, by administration of an "antidote". The pharmacologically detoxifying interaction may take place locally, regionally or systemically.

The occurrence of nephrotoxic and urotoxic side effects, i.e. of damage to the kidneys and to the efferent urinary system in the course of medicinal tumour therapy, has been described for many cytostatics (see survey [6]). In therapy with oxazaphosphorine cytostatics, e.g. with cyclophosphamide and ifosfamide, the urotoxicity—mainly in the form of sterile haemorrhagic cystitis—is quite often a therapy-limiting factor [6–9]. It is due to renal elimination of activated aggressive metabolites,

and its intensity depends on the concentration of those metabolites in the urine [10].

Various prophylactic measures have been recommended in the past [11], including raised fluid intake, administration of diuretics, alkalisation of the urine and, as an example of *local* detoxification, instillation of mercapto-compounds into the urinary bladder [12, 13]. The efficacy and practicability of these measures, however, were by no means satisfactory.

The *systemic* use of mercapto-compounds, for example of *N*-acetyl-*l*-cysteine, has also remained clinically unsuccessful so far because these compounds become distributed throughout the organism and reduce not only the urotoxicity but also the anti-tumoral efficacy of oxazaphosphorines [14, 15]. It was our aim to develop a systemically administrable drug which would not impair the chemotherapeutic efficacy of oxazaphosphorines on the tumour whilst definitively preventing the urotoxicity of these cytostatics regionally in the kidneys and the urinary tract ("regional detoxification").

The development of this "uroprotector" encompassed three phases. In the first phase we studied the mechanisms and parameters governing the urotoxicity of compounds with an alkylating action component, especially the relationships between the chemical constitution and the urotoxic potency of such compounds. The second phase was devoted to experimental testing of the uroprotective efficacy of various active agents, particularly of mercapto-compounds. The third phase was concerned with the pharmacotherapeutic characterisation of sodium 2-mercapto-ethane sulfonate which emerged as a suitable uroprotective compound.

The results of these three study phases are reported in the three consecutive parts of this communication, of which this is the first part.

In this first phase of these investigations we had to clarify whether the clinically known urotoxicity of oxazaphosphorine cytostatics is an obligate side effect of alkylating compounds as such or whether it is attributable to a specific mechanism of action based on the distinctive feature of the metabolism of oxazaphosphorines.

Unlike the highly reactive compounds with direct alkylating action, the oxazaphosphorine cytostatics cyclophosphamide and ifosfamide represent primarily inactive transport forms. Their wide therapeutic range and high carcinotoxic selectivity are based on enzymatic activation (4-hydroxylation) in the liver. These activation products are the primary metabolites with a higher reactivity. These primary metabolites are themselves transport forms which later give rise to the actual oncocidal active alkylating forms (toxification).

By virtue of their high reactivity, directly alkylating compounds expend their reactivity rapidly in the organism and reach the kidneys only in small amounts if at all, whereas oxazaphosphorines and their metabolites are eliminated renally.

To obtain precise information on the urotoxicity of cytostatics it was necessary to develop an experimental model which would enable rapid and reliable evaluation of the pathological changes occurring after systemic and local administration of test substances and, later, of prophylaxis against these changes.

MATERIALS AND METHODS

Chemical compounds

The compounds listed below were synthetised for these investigations by the Chemical Research Laboratories of the Asta-Werke under the guidance of Dr. Niemeyer and Dr. Scheffler, to whom we wish to express our thanks:

Bis-(2-chloroethyl)-amine (NH-mustard), mechlorethamine, mechlorethaminoxide, busulfan, cyclophosphamide, ifosfamide, trofosfamide, sufosfamide, 4-hydroperoxy- and 4-hydroxy-derivatives of cyclophosphamide and ifosfamide, 4-keto-cyclophosphamide, carboxyphosphamide, *N*,*N*-bis-(2-chloroethyl)-phosphorodiamidic acid, *N*,*N*′-bis-(2-chloroethyl)-phosphorodiamidic acid, 2-diethylamino-tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide and its 4-hydroxy-derivative and 5,5-dimethylcyclophosphamide.

We also used compounds procured from other sources, namely, mannitol-busulfan, BCNU, CCNU and Me-CCNU, kindly provided by the National Cancer Institute, Bethesda, U.S.A., chlorambucil and melphalan (Burroughs-Wellcome, London), mannomustin (Medimpex, Budapest), procarbazine (Hoffman-La Roche, Basle), triaziquone (Bayer AG, Wuppertal) and acrolein (Merck, Darmstadt). The compounds procured from these sources were tested for identity and purity by the Chemical Research Laboratories of the Asta-Werke.

Test animals

Species: Rats.

Strain: Sprague–Dawley SPF and BD II SPF.

Breeder: Asta-Werke AG, Bielefeld.

Sex: Male and female. Weight: about 250 g.

Feed: Altromin® 1324, no feed withdrawal, water *ad libitum*.

Keeping: Standard conditions.

Groups: 5 or 10 rats per test group.

Procedure

It was confirmed in preliminary experiments that induction of haemorrhagic cystitis in the rat (and also in the dog) can be achieved particularly easily and reliably by treatment with oxazaphosphorines [10]. In the rat, a single intravenous or oral administration of cyclophosphamide or ifosfamide is sufficient to induce inflammation of the urinary bladder which is dose- and time-dependent. This made it possible to develop an appropriate test and to achieve qualitative and quantitative characterisation of the urotoxic potency of various cytostatics

Experimental induction of haemorrhagic cystitis in the rat with systemic administration. With systemic administration, the test substances were administered intravenously or intraperitoneally depending on their solubility in water. The highest dose used in each series of tests was the LD_{50} for the test substance concerned. The lower doses, used for determining the dose-dependence of the urotoxic effect, if one was found, were scaled down in a descending geometric progression with a decrement factor of 2.15 (in some cases 1.47).

In order to obtain information on a possible influence of the physiological milieu (especially on the pH-dependence) the rats in some of the experiment series were pre-treated with NH_4Cl (681 mg/kg p.o.) or with Na_2CO_3 (562 mg/kg p.o.) 3 hr before administration of the test substances. This pretreatment resulted in highly uniform pH values of the urine of about 5.0 (NH_4Cl) and 8.0 (Na_2CO_3) which were maintained for between 8 and 10 hr.

Assays of alkylating activity in the urine after systemic administration. In orienting experiments conducted with a few selected test substances, the alkylating activity occurring in the urine within the first hour after systemic administration was assayed by the NBP test (4,4'-nitrobenzyl-pyridine) according to Friedman and Boger [16]. The rats used in these tests were each given an oral load of 5 ml water before the test in order to ensure sufficient enuresis.

Experimental induction of haemorrhagic cystitis in the rat with intravesical administration. With intravesical administration the test substances were instilled in increasing concentrations (increment factor 3.16) into the urinary bladder of female rats under Nembutal anaesthesia, by means of a fine PVC catheter. The urethra was then clamped shut by means of a fine clamp, thus ensuring a uniform dwelling time of the test substances of 1 hr. After the lapse of this time the bladder was voided by gentle pressure on the abdomen. Flushing the bladder was impossible for technical reasons.

The intravesical milieu was adjusted to the desired pH values (pH 5, 7.4 and 8) by buffering the test substance solutions in appropriate isotonic phosphate buffers.

Evaluation

The results were evaluated 24 hr after systemic administration or intravesical instillation of each test substance. The rats were killed with CO_2 and laparotomised, and the bladder was excised.

To evaluate the increase in capillary permeability in the inflammation area, each rat was given an intravenous injection of an aqueous solution of trypan blue (21.5 mg/kg in 3.16 ml/kg) 30 min before being killed.

The evaluation criteria were development of oedema, increase in capillary permeability and the occurrence of bleeding. The severity of the oedema was measured quantitatively by weighing the bladder (wet weight). The increase in capillary permeability was measured quantitatively by extraction and photometric assay of trypan blue in the extravasal space.

These two criteria were also used as the basis of a point-scoring system for semi-quantitative macroscopic evaluation. The point scores ranged from 0 to 3. The severity of the bladder damage was plotted against the dose (for systemic administration) or against the concentration (for intravesical instillation) and the effective dose $ED_{1.5}$ or the effective concentration $EC_{1.5}$ of each test substance, inducing bladder damage scoring 1.5 points, were determined graphically. The comparative urotoxicity of each test substance was assessed on the basis of these $ED_{1.5}$ and $EC_{1.5}$ data.

RESULTS

Pathogenesis and evaluation of the urotoxicity, taking ifosfamide-induced cystitis as an example

Within a few hours after administration of

ifosfamide there develops an oedema of the bladder wall and, owing to increased capillary permeability, escape of trypan blue (bound to plasma albumins) into the exudate. The development of the oedema is protracted and, accordingly, the wet weight of the bladder and the amount of extravasal trypan blue increase steadily (see Table 2). The inflammatory reaction reaches its peak about 24 hr after the administration of ifosfamide and then recedes relatively rapidly within 6–9 days [17–19]. In other words, the full development of cystitis requires considerably more time than the metabolism of ifosfamide and the renal elimination of the metabolites which takes its course within a few hours [20–22]. This is confirmed by the investigations with intravesical administration in which the dwelling time of the noxious agents in the bladder was only 1 hr but the peak of damage was only reached after about 24 hr, as with systemic administration.

After high systemic doses of ifosfamide (>50 mg/kg) the damage to the urinary bladder exceeds the stage of odema. The observed changes include necroses of the mucosa, ulcerations and petechial or confluent haemorrhages

Bladder weight as criterion of urotoxic damage. Various authors have used the wet or the dry weight of the urinary bladder as a quantitative parameter for assessing urotoxic potency, in some cases the absolute weight and in some cases the relative weight referrred to the body weight [10, 23]. We present in Table 1 the results of a representative experiment with systemic administration of ifosfamide.

Ifosfamide doses of over 50 mg/kg induce increased incidence and severity of bleeding and large-area detachments of the urinary epithelium. The exudate can no longer be retained in the tissue, and the mean bladder weight drops accordingly. The weight of the bladder can therefore be used as a valid criterion of the urotoxic potency of ifosfamide only within a limited dosage range, between about 10 and 50 mg/kg.

Table 1. Dependence of the bladder wet weight on the ifosfamide dose

Ifosfamide i.v. (mg/kg)	Bladder wet weight (mg)
—	86±13
31.6	136±27
68.1	171±23
147	174±25
316	136±24

Ten rats per dosage group, mean±S.D.

To illustrate the time-dependence of the development of oedema, Table 2 presents the results of experiments in which the rats were killed at various times after the administration of ifosfamide. After administration of ifosfamide the weight of the bladder increases nearly proportionally to the duration of the experiment. At high ifosfamide doses, the occurrence of bleeding and thus of losses of oedema in the tissue is reflected in lesser increases in the bladder weight with time.

The problems inherent in the use of the bladder weight as an assessment criterion prompted us to carry out statistical analyses of the relationships between the bladder weight, the body weight and the dosage of the urotoxic agent. These studies will be reported separately [23].

Extravasation of trypan blue as criterion of urotoxic damage. The trypan blue administered intravenously to the rats shortly before they are killed becomes bound to plasma albumins intravasally. With the development of oedema some of the dyestuff escapes into the tissue together with the plasma proteins. This extravasated amount of dyestuff can be extracted and assayed photometrically [24]. It correlates closely with the increase in the bladder wet weight. At high doses of ifosfamide

Table 2. Dependence of the bladder wet weight on the ifosfamide dose and on the duration of the experiment

Ifosfamide i.v. (mg/kg)	Bladder wet weight (mg) for various durations of the experiment (hr) 6	12	24
0	85.7±10.4	87.6±15.8	84.6±8.1
68.1	113.0±12.3	155.0±15.0	184.2±33.5
147	119.0±21.8	144.6±16.3	165.0±12.8

Ten rats per dosage and time group, mean±S.D.

(>50 mg/kg) this measured parameter also loses its information value.

Macroscopic assessment. For these experiments, involving large numbers of rats, we devised a semi-quantitative macroscopic assessment method independent of the quantitative determinations of the bladder weight and of the extravasal amount of trypan blue. With this method the extent of the swelling and the extent of extravasation of the dyestuff were assessed visually and were recorded on a 4-point scoring scale ranging from 0 to 3. A score of 0 was used to describe no swelling and coloration. A score of 1 was used for a slight swelling and for a pale blue colour, corresponding to an increase in the bladder weights of <30%. A score of 2 was used for moderate swelling and for a frankly blue colour, corresponding to an increase in the bladder weight of between 30 and 60%. A score of 3 was used for severe swelling and for a dark blue colour, corresponding to an increase in the bladder weight of >60%. The scores of the individual rats of each test group (of 5 rats) were averaged, yielding group-mean scores of bladder inflammation ranging between 0 and 3 (Table 3).

Besides these inflammation signs we also used as an independent criterion the percentage incidence of bleeding in each test group.

Urotoxic potency with systemic administration

The oxazaphosphorine cytostatics cyclophosphamide, trofosfamide, ifosfamide and sufosfamide are characterised by a high urotoxic potency (Table 4). Under the experimental conditions used, bladder damage was demonstrable at doses as low as 10 mg/kg, i.e., in the chemotherapeutic dosage range. At doses corresponding to a mean inflammation score of 1.5 ($ED_{1.5}$ > 50 mg/kg) all the test rats showed swelling of the urinary bladder associated with a strong blue coloration. Haemorrhagic foci were also observed in some of these bladders.

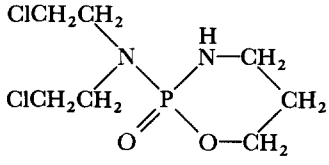
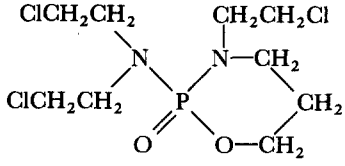
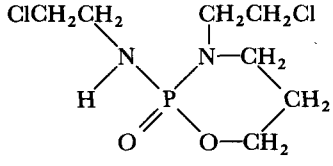
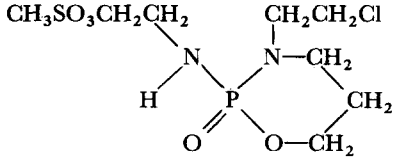
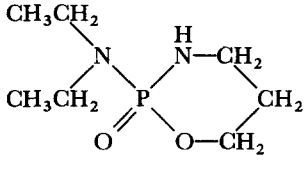
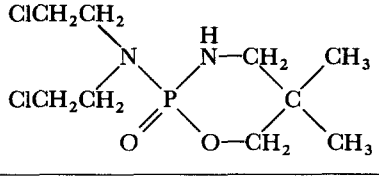
The common structural feature of the urotoxically active compounds shown in Table 4 is the oxazaphosphorine ring. The non-chlorinated and chemotherapeutically inactive cyclophosphamide analogue ASTA 7019 also possesses this ring. Despite the absence of alkylating chloroethyl groups, ASTA 7019 does possess a urotoxic activity.

The primary 4-hydroxy-metabolites of cyclophosphamide, ifosfamide and ASTA 7019 (ASTA 6635, 6569 and 7169) and the corresponding 4-hydroperoxy-compounds (ASTA 6496, 6760 and 7037), administered intravenously, also induce dose-dependent bladder damage. On the other hand, the compound ASTA Bx 707 which is dimethylated in position 5 of the oxazaphosphorine ring is inactive both chemotherapeutically and urotoxically. ASTA Bx 707 does become hydroxylated in position C-4 of the oxazaphosphorine ring, but the subsequent cleavage of the ring with release of acrolein and phosphoric acid diamide cannot take place [25, 26]. In the case of the primary metabolites, after the ring has been opened and acrolein split off by β-elimination, the directly alkylating phosphoric acid diamides ASTA 5317 and ASTA 5333 are formed. These compounds, when adminis-

Table 3. Comparison of the weight and of the macroscopic assessment of the bladder after a single i.v. injection of ifosfamide (10 rats per dosage group)

Ifosfamide (mg/kg)	Bladder weight (mg) Mean ± S.D.	% increase	Individual assessment Colour	Swelling	Mean scores
—	73.0 ± 8.2	100	0,0,0,0,0 0,0,0,0,0	0,0,0,0,0 0,0,0,0,0	0.0
10.0	72.4 ± 9.1	99	0,0,1,1,1 1,0,1,0,1	1,1,0,1,1 0,0,1,0,1	0.6
14.7	76.8 ± 13.4	105	0,1,1,1,2 1,1,1,1,0	1,1,1,1,1 0,1,1,0,0	0.8
21.5	82.2 ± 17.1	126	1,1,1,2,0 1,2,1,1,1	1,1,0,1,1 0,2,1,1,0	1.0
31.6	110.2 ± 21.2	151	1,1,3,0,1 1,2,1,1,1	1,1,2,0,1 1,1,2,1,1	1.2
46.4	120.8 ± 18.7	165	1,2,2,1,1 1,1,2,3,3	2,2,2,1,1 2,2,2,1,2	1.7
68.1	135.0 ± 24.6	185	3,2,3,2,2 2,3,3,2,2	2,2,2,2,2 2,3,3,2,3	2.4
100.0	130.3 ± 28.3	178	3,3,3,2,3 3,3,3,3,3	2,3,3,2,3 2,3,2,3,3	2.8

Table 4. (a). *Urotoxicity of oxazaphosphorines in rats. Assessment of urinary bladder damage* 24 hr *after i.v. administration*

Compound	Structural formula	Dose (mg/kg)	Assessment of urinary bladder: Inflammation score	Bleeding (%)	DE 1.5 (mg/kg)
Cyclophosphamide		14.7 31.6 68.1 100	1.1 1.4 2.6 2.5	0 20 45 52	28.5
Trofosfamide		23.7 51.1 68.1 110	0 1.8 1.5 1.5	0 60 30 75	57
Ifosfamide		14.7 31.6 68.1 147 316	0.8 1.1 2.5 2.8 3	10 0 27 66 100	33.5
Sufosfamide		38.3 46.4 82.5 100	0 1.2 1.9 2.2	0 20 40 55	66
ASTA 7019		56.2 215 316 464	0 2 3 3	0 0 80 80	165
ASTA BX 707		100	0	0	—

tered systemically, show only a slight urotoxic potency without clear dose–response relationships.

The acrolein, which is released concurrently in these metabolic reactions, is not urotoxic when administered systemically. Also non-urotoxic are the metabolic end products 4-keto-cyclophosphamide (ASTA 5160) and carboxyphosphamide (ASTA 5754).

The urotoxic potency of the aggressive oxazaphosphorine metabolites accumulating in the bladder is markedly pH-dependent. Bladder damage is significantly less pronounced in a weakly acidic milieu (pH 5) than in a weakly alkaline milieu (pH 8) (Table 5). After 68.1 mg/kg or higher doses of ifosfamide i.v., haemorrhages and necroses occurred regularly at urinary pH 8 but never at pH 5.

Table 4. (b) *Urotoxicity of oxazaphosphorine metabolites in rats. Assessment of urinary bladder damage* 24 hr *after i.v. administration*

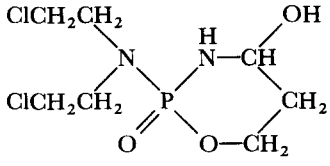
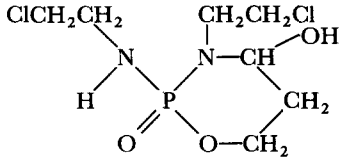
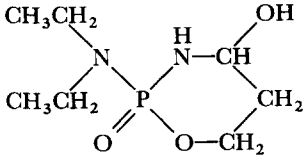
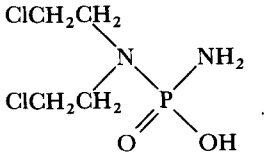
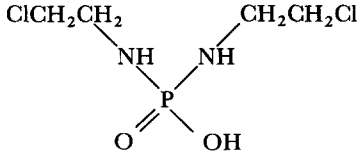
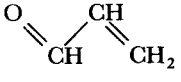
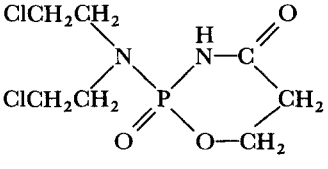
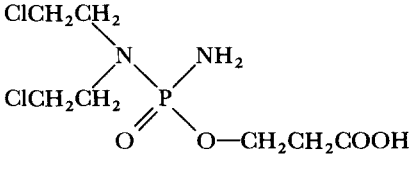
Compound	Structural formula	Dose (mg/kg)	Assessment of urinary bladder Inflammation score	Bleeding (%)	DE 1.5 (mg/kg)
ASTA 6635		14.7 31.6 68.1	0.6 1.9 1.8	0 40 60	36.2
ASTA 6569		14.7 31.6 68.1	0.0 0.4 1.0	0 0 0	~125
ASTA 7169		21.5 46.4 100	0.4 0.7 1.2	0 0 0	~118
ASTA 5317		26.1 56.2 121	1.2 1.0 1.2	10 3 0	—
ASTA 5333		38.3 82.5 178	0.1 0.2 0.1	0 0 0	—
Acrolein		7.32	0	0	—
ASTA 5160		800	0	0	—
ASTA 5754		600	0	0	—

Table 5. Severity of bladder damage induced by ifosfamide i.v. Bladder weight (mg) depending on the pH of the urine after oral administration of 681 mg/kg NH_4Cl (pH 5) *or of* 562 mg/kg Na_2CO_3 (pH 8) 3 hr *before the injection of ifosfamide (mean ± S.E.M.)*

Pretreatment	Ifosfamide dose (mg/kg) 0	31.6	68.1	147
NH_4Cl (pH 5)	78 ± 3.8	71 ± 2.1	135 ± 10.4	140 ± 7.5
Na_2CO_3 (pH 8)	75 ± 2.1	138 ± 9.1	163 ± 6.8	145 ± 4.6
P	NS	< 0.001	< 0.05	NS

The majority of the very many directly alkylating compounds which we tested showed no urotoxic action with i.v. administration, even at their mean lethal doses (LD_{50}). This applies in particular to the following compounds (the data in parentheses are the highest doses tested, in mg/kg):

NH-mustard (100), mechlorethamine (1.6), melphalan (18), chlorambucil (8.58), busulfan (31.6), mannitol-busulfan (1050), mannomustin (56), triaziquone (0.215), CCNU (40) and Me-CCNU (40). After high doses of mechlorethamine oxide (50 mg/kg) and of procarbazine (450 mg/kg) we did observe slight oedema of the bladder but never any bleeding. The only somewhat more urotoxic compound, without a clear dose dependence, was the nitroso-urea derivative BCNU administered intravenously. The mean scores were < 1, which shows that the inflammation manifestations were limited and were not observed in all the rats of a test group (see Table 3).

Assays of the alkylating activity in the urine after systemic administration

After intravenous administration of some directly alkylating compounds (at doses $\hat{=} LD_{50}$), only low alkylating activity levels in the urine were detected by the NBP test [16]. For example, after i.v. administration of 100 mg/kg NH-mustard ($\hat{=} LD_{50}$) only 188 nmol (about 0.09% of the dose) was eliminated renally within the first hour, and the concentration in the urine was 40 nmol/ml. After i.v. administration of 1.6 mg/kg mechlorethamine ($\hat{=} LD_{50}$) only 7 nmol (about 0.3% of the dose) was eliminated renally within the first hour, and the concentration in the urine was 2 nmol/ml. The alkylating activity in the urine was markedly higher after i.v. administration of 46.4 mg/kg mechlorethamine oxide ($\hat{=} LD_{50}$). Renal excretion within the first hour after administration amounted to 10 μmol ($\hat{=}$ 4.5% of the dose) and the urinary concentration was 16.3 μmol/ml.

Urotoxic potency with intravesical administration

For further clarification of the aetiology of the haemorrhagic oxazaphosphorine-induced cystitis, the compounds found to be urotoxic with systemic administration, and the metabolites and breakdown products of these compounds, were administered by direct instillation into the urinary bladder of female rats. The compounds concerned were administered in ascending concentration series (increment factor 2.15 or 3.16).

Control experiments showed that instillation of isotonic saline or of an isotonic phosphate buffer in the physiological range (pH 7.4) or in a weekly acidic range (pH 5) induced neither oedema nor a blue coloration of the urinary bladder. Instillation of alkaline buffer solutions (pH 8) occasionally induced a slight increase in the urinary bladder wet weight accompanied by slight extravasation of trypan blue.

The observations with intravesical instillation of urotoxic agents were similar to those with systemic administration. Urotoxicity manifested itself by oedema and extravasation of trypan blue into the tissue. The severity of the inflammation manifestations was concentration-dependent. As with systemic administration, the maximum increase in the wet weight of the bladder with intravesical instillation was about two-fold in comparison with the untreated control rats. At higher concentrations of the urotoxic agents the incidence and severity of haemorrhages and detachments of the urinary epithelium increased. At such doses the oedema fluid was no longer retained in the tissue and the wet weight of the bladder decreased accordingly again (see also Table 1).

The results of the experiments with intravesical instillation are summarized in Table 6. Cyclophosphamide and ifosfamide, administered intravesically in the form of 2.6% solution in isotonic saline, induced no inflammation. The highest agent concentration used in these tests was 100 μmol/ml. The non-

Table 6. Urotoxicity of oxazaphosphorines and metabolites in rats (dissolved in normal saline). Assessment of urinary bladder damage 24 hr after intravesical instillation for 1 hr (0.5 ml of solution)

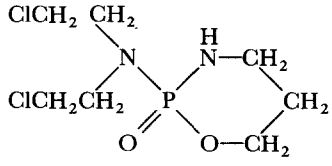
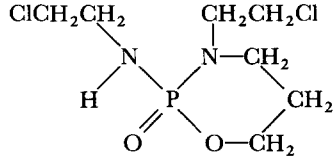
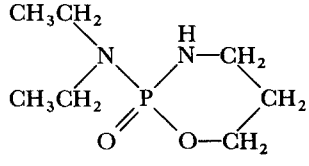
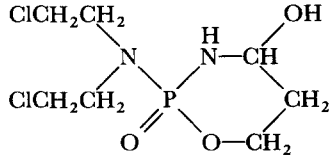
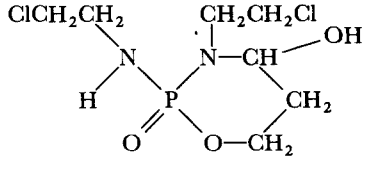
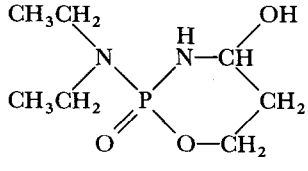
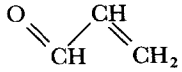
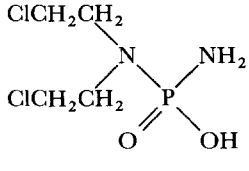
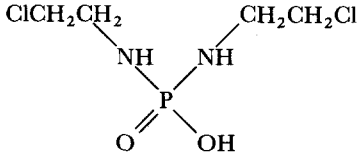
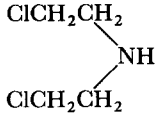
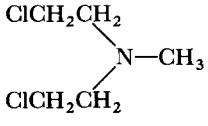
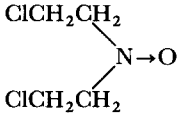
Compound	Structural formula	Concentration (μmol/ml)	Assessment of urinary bladder: Inflammation score	Assessment of urinary bladder: Bleeding (%)	CE 1.5 (μmol/ml)
Cyclo-phosphamide		100	0	0	—
Ifosfamide		100	0	0	—
ASTA 7019		100	0	0	—
ASTA 6635		1.0 1.47 3.16 6.81	0.0 0.6 1.6 2.0	0 0 20 50	3.2
ASTA 6569		1.47 3.16 6.81	1.3 2.1 2.3	0 22 42	1.9
ASTA 7169		1.0 1.47 3.16 6.81	0.2 0.4 1.3 1.6	0 0 20 45	4.4
Acrolein		0.100 0.316 0.681 1.000 1.470 3.160	0.5 0.7 1.2 2.3 2.6 2.8	0 0 0 20 60 100	0.65
ASTA 5317		31.6	0.9	0	—

Table 6 (*continued*)

Compound	Structural formula	Concentration (μmol/ml)	Assessment of urinary bladder Inflammation score	Bleeding (%)	CE 1.5 (μmol/ml)
ASTA 5333		31.6	0.9	0	—
NH-Mustard (pH 8)		10.0 31.6 100.0	0.4 0.7 1.7	0 0 20	~75.0
Mechlor-ethamine (pH 8)		0.316 1.000 3.160	0.4 0.7 2.5	0 0 100	~1.5
Mechlor-ethaminoxide (pH 8)		3.16 10.0 31.6	0.8 0.7 0.8	0 0 0	—

chlorinated analogue of cyclophosphamide (ASTA 7019), instilled intravesically in concentrations of up to 100 μmol/ml, also showed no urotoxic action.

By contrast, intravesical administration of the 4-hydroxy-metabolites induced inflammation of the urinary bladder, the severity of which was concentration-dependent. The urotoxic potency of the 4-hydroxy-metabolite of the non-chlorinated cyclophosphamide analogue (ASTA 7169) was about the same as that of 4-hydroxy-cyclophosphamide. Inflammation scoring 1.5 points was induced by concentration of about 5 μmol/ml. The urotoxic effects of the alkylating phosphoric acid diamides ASTA 5317 and 5333, on the other hand, manifest themselves only at concentrations some 5–10 times higher.

The urotoxically most potent compound when instilled intravesically was found to be acrolein, which is not urotoxic when administered systemically. The urotoxic concentrations of directly instilled acrolein were found to be lower by a factor of about 5–10 than the comparable urotoxic concentrations of the 4-hydroxy-compounds.

In order to obtain information on the influence of the pH of the instilled solutions on the severity of the urotoxic effects of the test compounds, we carried out comparative experiments in the acidic and in the alkaline pH range. The use of an isotonic phosphate buffer pH 8 increased the concentration-dependent urotoxic potency of 4-hydroxy-cyclophosphamide and of 4-hydroxy-ifosfamide by a factor of about 2 in comparison with the same solutions in an isotonic phosphate buffer pH 5 (Table 7). By contrast, the urotoxic potency of the breakdown products—phosphoric acid diamide and acrolein—was not affected by changing the pH.

For comparison purposes, the directly alkylating compounds NH-mustard, mechlorethamine and mechlorethamine oxide, which are not urotoxic with systemic administration and the alkylating activity of which in the urine had been determined earlier, were instilled into the bladder in ascending concentration series. The data presented in Table 6 show that the concentrations required to induce urotoxic damage are higher by some orders of magnitude than those found in the urine after systemic administration.

Table 7. pH-dependence of the urotoxic potency of 4-hydroxy-oxazaphosphorines with intravesical administration in phosphate buffers

Compound	Concentration (μmol/ml)	Score pH 5	Score pH 8
ASTA 6569	1.47	0.1	1.6
	3.16	1.6	2.3
	6.81	1.8	2.5
ASTA 6635	1.47	0.7	0.8
	3.16	1.1	1.8
	6.81	1.7	2.6
ASTA 7169	1.47	0.1	0.8
	3.16	0.8	1.8
	6.81	1.0	2.3

DISCUSSION

The development of uroprotective drugs requires information on whether the urotoxic action of oxazaphosphorine cytostatics is due to the alkylating properties of their renally excreted metabolites or whether it is due to other breakdown products. The experimental results on rats presented in this communication show clearly that systemically administered directly alkylating cytostatics induce no renal or vesical damage or induce such damage only in the systemically toxic dosage range. The chemical activity of these cytostatics is so high that they enter into alkylating reactions with blood and tissue proteins and are detectable in the urine (NBP test) only in very low concentrations, if at all. By contrast, a specific urotoxicity was found with systemic administration of oxazaphosphorines, but only of those capable of undergoing ring cleavage with formation of acrolein. This includes the "transport forms" cyclophosphamide, ifosfamide, trofosfamide and sufosfamide as well as their enzymatically activated primary 4-hydroxy-derivatives. The non-chlorinated cyclophosphamide analogue ASTA 5017, which has no alkylating action component, also possesses urotoxic potency when administered systemically. None of the other intermediate metabolites and end products, such as the phosphoric acid diamides, the corresponding 4-keto-derivatives and the carboxy-derivatives, showed any urotoxic potency.

The question of which compounds are directly responsible for the renal and vesical damage was clarified in experiments with direct instillation of test compounds into the bladder. As expected in view of their nature, the inactive "transport forms" such as cyclophosphamide and ifosfamide were fully tolerated when administered intravesically. The directly alkylating phosphoric acid diamides, formed by ring cleavage of cyclophosphamide and ifosfamide, were found to possess only a low urotoxic potency. The specific urotoxicity carriers are only the primary 4-hydroxy-metabolites and especially acrolein which is formed by their further degradation. Acrolein is the compound with the highest urotoxic potency. The reason why acrolein is not urotoxic when administered systemically is its high reactivity. As a result, systemically administered acrolein is rapidly detoxified in the blood and tissues and does not appear in the urine in a reactive form.

Of particular importance for the clarification of structure/action relationships are the findings with the compound ASTA Bx 707 which is dimethylated in position 5 of the oxazaphosphorine ring. After systemic administration, this compound does undergo enzymatic hydroxylation in position 4 in the liver of warm-blooded animals, but β-elimination with subsequent formation of acrolein cannot occur for structural reasons [26].

The findings of the experiments presented in the communication support the following interpretation of the urotoxicity of oxazaphosphorine cytostatics:

With systemic administration of the transport forms cyclophosphamide, ifosfamide, trofosfamide and sufosfamide, the decisively important event for the formation of urotoxic metabolites is the first metabolic step, i.e., enzymatic hydroxylation in position 4 of the oxazaphosphorine ring. Non-metabolised fractions of the transport forms, as well as the 4-keto-, carboxy- and phosphoric acid diamide compounds, are also eliminated renally but possess no urotoxic potency. As show by the experiments with direct intravesical instillation, urotoxic potency is possessed only by the renally eliminated fractions of the 4-hydroxy metabolites and by acrolein which is formed from these metabolites spontaneously in the kidneys and in the efferent urinary tract.

It was found both in the experiments with systemic administration and in those with direct intravesical instillation that the potency of the urotoxic action is pH-dependent. It is substantially stronger in the alkaline range than in the acidic range.

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