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ORIGINAL ARTICLE

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D-19575—a sugar-linked isophosphoramide mustard derivative exploiting transmembrane glucose transport

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Abstract D-19575 is a glucose derivative of ifosfamide mustard with a broad spectrum of antitumor activity in animal models. In comparison with ifosfamide, D-19575 is less toxic and is better tolerated by tumor-bearing animals, achieving a better therapeutic efficacy. D-19575 is directly cytotoxic in vitro—in contrast to ifosfamide—and it is possible to modulate this cytotoxicity by inhibition of transmembrane glucose transporters. Correspondingly, renal reabsorption of filtered D-19575 could be blocked by pre- and cotreatment with phlorizin, resulting in a higher urinary excretion of the unchanged drug. The toxicity to white blood cells, colony-forming units (CFU-C), and spleen-cell colony-forming units (CFU-S) is considerably lower for D-19575 as compared with ifosfamide. In conclusion, D-19575 is a new alkylating cytotoxic agent with increased antitumor selectivity, probably caused by an active transmembrane transport mechanism.

Key words Sugar-isophosphoramide mustard · Active glucose transport · Antitumor activity

Introduction

Despite remarkable success in the development of chemotherapeutic agents against cancer, there is a further demand for more selective drugs. Most therapeutic drugs used today exhibit very little site specificity, and the present challenge in therapy research is the development of systems that can selectively deliver drugs to their sites of action within the organism. The concept of destroying tumor cells by reactive chemical compounds is conclusive and effective only if it is possible to target tumor cells selectively.

In recent years a lot of attempts have been started to reduce the incidence of side effects by developing conjugates of established drugs. The most promising techniques used were the conjugation of alkylating agents to monoclonal antibodies directed against tumor-associated antigens, the encapsulation of reactive compounds into liposomes, and the synthesis of hormone conjugates to address tumor cells rich in hormone receptors. Furthermore, the importance of oligosaccharides in recognition and cell interaction has been widely accepted, and research to provide a better understanding of these processes has evolved [20]. Endogenous lectins are involved in these processes [22], but knowledge about their structures, their distribution, and their significance in biological processes is limited [25]. Lectins have been described to be associated with different cell lines [9] and are also involved in the metastatic process [8, 19]. Detailed analysis of the interaction between lectins, their receptors, and branched synthetic oligosaccharides can be fruitful for the design and synthesis of oligosaccharides as carriers.

In recent years, monosaccharide conjugates of different types of compounds have been synthesized to improve the therapeutic efficiency of their aglycons; e.g., azidothymidine (AZT), used to treat patients infected with the human immunodeficiency virus (HIV), was conjugated with glucose to provide better transport to the brain [15]. Glycosylation of antileishmanial drugs led to better uptake by macrophages [16], and porphyrins, which are used in photodynamic therapy, were rendered more water-soluble by glycosylation [6]. Glycosylation of small peptides resulted in an attenuated in vivo clearance [7].

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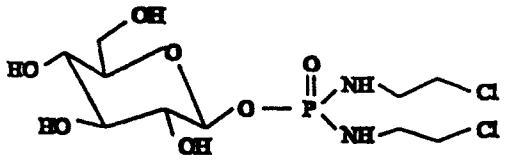

D-19575 M.w.: 383.2

Fig. 1 Chemical structure of D-19575 (*M.w.* Molecular weight in KDa)

Oxazaphosphorines are a class of alkylating agents with major experimental and clinical antitumor activity. Cyclophosphamide and its position isomer ifosfamide are examples of two clinically widely used agents of this class of drugs [3]. They are prodrugs and need metabolic activation by cytochrome P-450 enzymes of the liver to generate the cytotoxic species. The goal of analogue research has to date been to synthesize 'activated' oxazaphosphorines, which hydrolyze spontaneously to form primary 4-hydroxy-metabolites. However, one of these agents, mafosfamide, was difficult to develop clinically because of severe pain at the injection site, which probably resulted from acrolein generated during the spontaneous degradation of the molecule during infusion [12, 17].

One of the attempts to eliminate acrolein formation during hydrolytic degradation was the substitution of the oxazaphosphorine ring by sugar moieties. D-19575 is an ifosfamide mustard in which glucose has an ester-like link to the phosphoric acid part of the molecule [5]. The resulting structure (Fig. 1) bears a certain similarity to glucose 1-phosphate, a major source of cellular energy. The present communication describes the experimental profile of D-19575 as a cytotoxic agent and attempts to elucidate some of the specific pharmacological characteristics of its extraordinarily high selectivity, which might be related to transmembrane glucose transport mechanisms [21].

Materials and methods

Compounds

D-19575 and ifosfamide mustard were synthesized in the Chemical Research Laboratories of ASTA Medica AG (Frankfurt/Main, Germany) ifosfamide and cyclophosphamide were used as bulk material of pharmaceutical grade. Phloretin and phlorizin, two specific inhibitors of transmembrane glucose transport [18], and all other chemicals used, were commercially obtained (Serva/Heidelberg and Sigma/Deisenhofen, Germany).

Cell cultures

Mouse bone marrow was harvested from the femora of female CD2FI mice weighing approximately 20 g. Mouse leukemia L1210 and human KB squamous epithelial carcinoma cell lines were obtained from the American Type Culture Collection (ATCC) and were propagated in suspension culture according to standard techniques using commercially available and individually supplemented tissue-culture media.

In vitro assays for inhibition of colony formation in soft agar (colony assay) were performed according to the method of Hamburger and Salmon [10, 11] with some modifications. In short, cells were incubated in medium solidified with 0.3% agar in the presence of different concentrations of test substance. Tumor cells were incubated for 6 (L1210) or 8 (KB) days in RPMI-1640 medium containing 20% fetal calf serum at 37°C in an atmosphere containing 95% relative humidity and 7.5% CO_2. Bone marrow cells were incubated for 7 days in Iscove's medium [13] containing 20% horse serum in the presence of 300 units GM-CSF at 37°C in an atmosphere containing 95% relative humidity and 10% CO_2. The experiments were performed in triplicate. At the end of the incubation period the number of colonies consisting of more than 50 cells were counted. The concentration of test substance resulting in an inhibition of colony formation by 90% (EC_{90}) was determined graphically.

Animals

All animals had unrestricted access to food and water (acidified to pH 3) and were fed a standard pellet diet (Altromin 1324). Female Sprague-Dawley rats weighing 180–220 g were purchased from Möllegard Breeding Center (Elby, Denmark). Female BD IX rats in the same weight range and female mice of the strains NMRI, DBA2, and CD2F1 weighing 18–22 g were obtained from the Zentralinstitut für Versuchstierkunde (Hannover, Germany). The animals were housed under specific pathogen-free (SPF) conditions at a constant temperature of 22°C, at 50% ± 5% relative humidity, and on a 12-h/12-h light/dark cycle.

Peripheral blood cell counts and assays of colony-forming units

NMRI female mice aged 10–12 weeks and weighing 20–25 g were used both for treatment with cyclophosphamide, ifosfamide, or D-19575 and as recipients for assays of spleen-cell colony-forming units (CFU-S). Drugs were appropriately dissolved in 0.5 ml 0.9% NaCl and then given ip. Control animals received the same volume of diluent. Drug effects on peripheral white blood cells (WBC) and hematopoletic progenitor cells (CFU-S and CFU-C) in the bone marrow were determined on the basis of injections of 0.5 mmol/kg of each drug on days 0, 3, and 7. Single-cell suspensions were derived from flushed femoral marrow of three mice per group per point, and blood samples for individual WBC counts were obtained by severing the axillary vessels. WBC were counted using an automatic blood cell counter (Coulter).

CFU-S were assayed by the method of Till and McCulloch [23]. Recipient mice (at least ten mice per point per group) were exposed to 7.5 Gy total-body irradiation (X-ray machine; rate, 1.43 Gy/min; focal distance, 40 cm) before the injection of 4×10^5 nucleated cells from the marrow. Macroscopic surface colonies in the spleens were counted 9 days later. CFU-C were assayed according to the method of Bradley and Metcalf [1] as modified by Iscove [13]. Marrow nucleated cells (10^5) were cultured in 1 ml alpha-medium containing 0.8% methylcellulose, 20% horse serum, and 20% mouse-heart conditioned medium [4]. The results were converted to percentages of the control values.

Tumors

L5222 monocytic leukemia is transplantable in syngenic BD IX rats and kills the hosts within 8–10 days of the i.p. transplantation of 10^6 cells freshly harvested from a donor animal. AH13r hepatoma cells

(2×10^5) were transplanted s.c. into the right flank of Sprague-Dowley rats. At the transplantation site a palpable solid tumor grew within 16 days, and it killed the animals by day 20 (median). Mouse P388 leukemia (10^5 cells/animal) was transplanted i.p. into DBA2 mice, which died within 10–12 days of systemic leukemia. Ehrlich ascites carcinoma (10^6 cells/animal) was transplanted into the peritoneal cavity of female NMRI mice (age) 10–12 weeks; weight, 20–25 g), killing the animals by progressive ascites tumor growth within 16–19 days (median, 18 days). Drug effects on survival were determined on the basis of i.v. injections of 0.5 mmol/kg of each drug on days 7, 10, and 13 after tumor inoculation.

Acute toxicity

The approximative dose lethal to 50% of the animals tested (LD_{50}) was determined by single logarithmically spaced doses of ifosfamide or D-19575, respectively. Treatment was given by either the i.v. or the oral route.

Venous pain

Induction of venous pain was investigated according to a previously published method [12]. Briefly, conscious but rather sleepy rats had continuous infusions of physiological saline into the tail vein. After switching the infusion to a solution of the test substance, we observed the animals for behavioral alterations and vocalization.

Urinary excretion

In the context of broader pharmacokinetics studies (part of the Ph.D. thesis of J. Stüben) with unlabeled and ^{14}C-labeled D-19575, the urinary excretion of D-19575 was investigated by means of ^{31}P-nuclear magnetic resonance (NMR) spectrometry. Female SD rats received 315 mg/kg D-19575 i.v. ($n = 5$) and were placed for 24 h in metabolic cages for separate sampling of urine and feces. Urine-collecting vials were cooled in a mixture of ice and salt throughout the sampling period to prevent degradation. Three more animals were treated s.c. with phlorizin at 400 mg/kg per day divided into three fractions over the day prior to and the day of D-19575 i.v. treatment. All animals were fasted but had access to tap water ad libitum. Urine was sampled over the 0- to 8-h and the 8- to 24-h periods.

Table 1 Cytotoxic activity in vitro: molar concentrations that produced a 90% inhibition of colony formation in different cell lines

	IPM	D-19575
L1210	1.4×10^{-6}	6.3×10^{-6}
KB	8.1×10^{-6}	5.2×10^{-6}
Bone marrow Stem cells (mice)	4.5×10^{-6}	3.9×10^{-6}

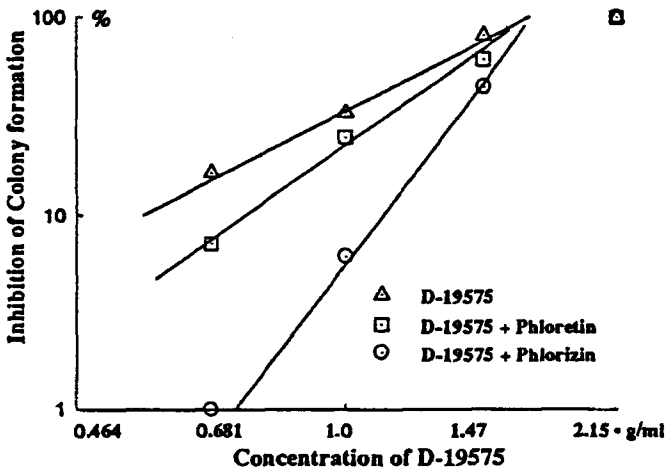

Fig. 2 Cytotoxic activity of D-19575 in vitro on L1210 colony formation and its inhibition by 10^{-7} M phloretin or phlorizin

Table 2 Acute toxicity: approximate LD_{50} values obtained after single i.v. or oral administration to mice and rats

		Rat mg/kg (mmol/kg)	Mouse mg/kg (mmol/kg)
Ifosfamide	i.v.	350(1.34)	700(2.68)
	p.o.	370(1.42)	1000(3.83)
D-19575	i.v.	1575(4.11)	1575(4.11)
	p.o.	1470(3.84)	1470(3.84)

Results

The cytotoxicity in vitro of D-19575 against L1210 leukemia, KB squamous-cell carcinoma, and bone marrow stem cells is shown in Table 1. As can be seen, D-19575 showed activity comparable with that of ifosfamide mustard, the final active metabolite of ifosfamide. As expected, ifosfamide itself was completely inactive in these assays (data not shown).

Figure 2 demonstrates the in vitro dose-response relationship of D-19575 in L1210 leukemia cells in the presence of 0.1 mmol phloretin and 0.1 mmol phlorizin dissolved in phosphate buffer. Neither compound had an intrinsic effect on the number of colonies formed at concentrations of 10^{-4}–10^{-7} M (data not shown). The colonies were, however, considerably smaller than those observed in the controls. It is evident that phlorizin reduced the cytotoxicity of D-19575, whereas phloretin had only minor effects on the cytotoxic action on the compound. The inhibition was observed only in the lower concentration range of the cytostatic agent. Higher concentrations overcame the inhibitory potency of the two glucose channel inhibitors, probably by diffusion.

The approximate LD_{50} values obtained for ifosfamide and D-19575 in rats and mice are summarized in Table 2. On a weight basis, D-19575 appeared to be less toxic by a factor of 2–4. On a molar basis, this factor ranged between 2 and 3. The comparison of the oral and i.v. LD_{50} values suggested a bioavailability for D-19575 in the range of 100% in both species. This was similar for ifosfamide in rats but not in mice.

The effects of various i.p. doses of ifosfamide and D-19575 given as five daily treatments on the survival

Table 3 Antitumor activity in murine P388 cells. Treatment was given i.p. on 5 consecutive days beginning on day 1 after i.p. tumor implantation (LTS Long-term survivors)

	Single dose mg/kg (mmol/kg)	Median survival time (day 60)	T/C %	L.T.S. > day 60
Control	–	9.1	–	0/22
Ifosfamide	75 (0.29)	26.8	294	0/6
	150 (0.57)	> 60.0	> 660	6/6
	300 (1.15)	9.8	107	1/6
D-19575	125 (0.33)	29.0	318	4/6
	250 (0.65)	> 60.0	> 660	6/6
	500 (1.31)	> 60	> 600	5/6

Table 4 D-19575 given i.v.—therapeutic efficacy in rat tumors

Tumor model	Dose (mmol/kg)	Growth delay (days)	LTS > day 90
L5222	0.08	–	6/6
	0.26	–	6/6
AH13r	0.8	5	0/6
(solid)	1.78	12 (2/6)	4/6

of mice transplanted with P388 leukemia are shown in Table 3. Obviously, 250 mg/kg D-19575 and 150 mg/kg ifosfamide were equipotent curative doses in this tumor system. Doubling of the doses to 500 mg/kg D-19575 and 300 mg/kg ifosfamide indicated that ifosfamide had reached the toxic range, whereas D-19575 produced five survivors among six animals. A similar phenomenon was seen after dose reduction: 125 mg/kg D-19575 produced four survivors among six animals, whereas no animal treated with 75 mg/kg ifosfamide was cured (this experiment was performed by Dr. E. Tueni, Laboratory for Experimental Chemotherapy and Screening, Institute Jules Bordet, Brussels).

D-19575 was highly active in the rat leukemia L5222 model. A single i.v. dose of 0.08 mmol/kg (21.5 mg/kg) produced 100% long-term survivors (> 90 days; Table 4). The compound was also curvative in four of six animals transplanted with the relatively chemoresistant AH13r hepatoma and treated with a single i.v. dose of 1.78 mmol/kg (464 mg/kg; Table 4). Similar activities have been reported for ifosfamide [2].

Figure 3 shows the antitumor activity of equimolar amounts (0.5 mmol/kg) of D-19575, ifosfamide, and cyclophosphamide given on days 7, 10, and 13 after i.p. transplantion of Ehrlich ascites carcinoma. The surviving fraction of each group is shown in relation to time. Cyclophosphamide was the least effective substance, followed by ifosfamide and D-19575. In this experiment the median survival time of untreated control animals was 18 (range, 16–19) days, for animals treated with cyclophosphamide it was 17 (range, 15–25) days, for those receiving ifosfamide it was 18 (range, 16–48) days, and for those given D-19575 it was 26 (range, 20–41) days, corresponding to a median increase in life span of 44%.

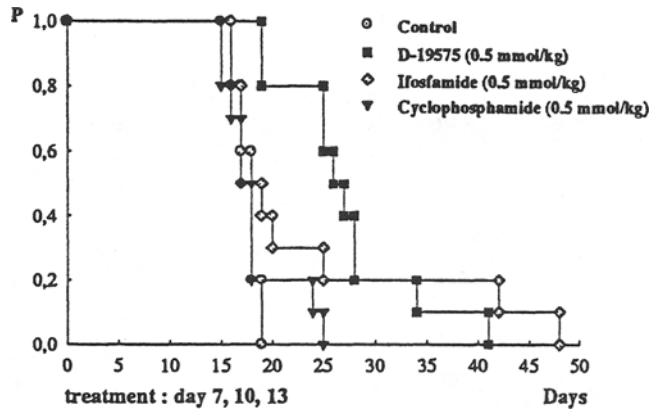

Fig. 3 Antitumor efficacy of three equimolar i.p. doses (0.5 mmol/kg; days 7, 10, and 13) of D-19575, ifosfamide, and cyclophosphamide against murine Ehrlich ascites tumors

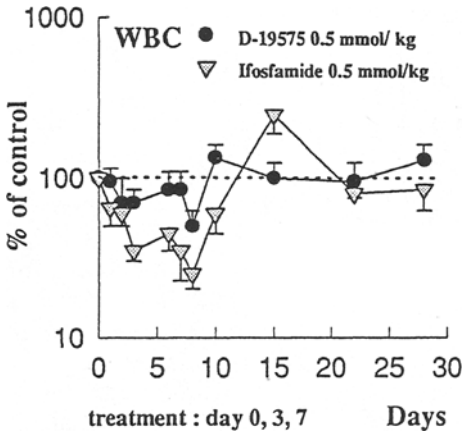

Fig. 4 White blood cell (*WBC*) counts obtained in mice after three equimolar (0.5 mmol/kg; days 0, 3, and 7) i.p. doses of D-19575 or ifosfamide

The same equimolar doses of D-19575 and ifosfamide as were given in the above experiment were used to monitor the WBC count in normal mice following treatment on days 0, 3, and 7. The initial WBC suppression observed was considerably lower for D-19575 as compared with equimolar ifosfamide (Fig. 4). Red blood cells (RBC) remained virtually unaffected (data not shown).

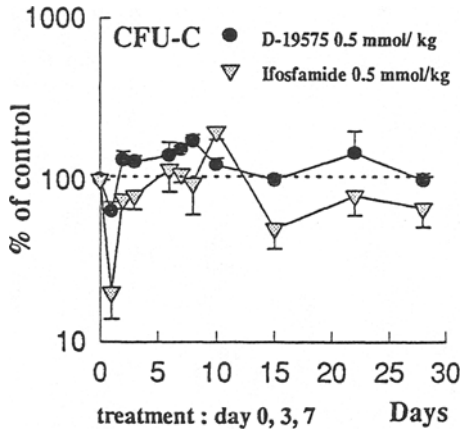

Fig. 5 Assay of colony-forming cells (*CFU-C*) from murine bone marrow after three equimolar (0.5 mmol/kg; days 0, 3, and 7) i.p. doses of D-19575 or ifosfamide

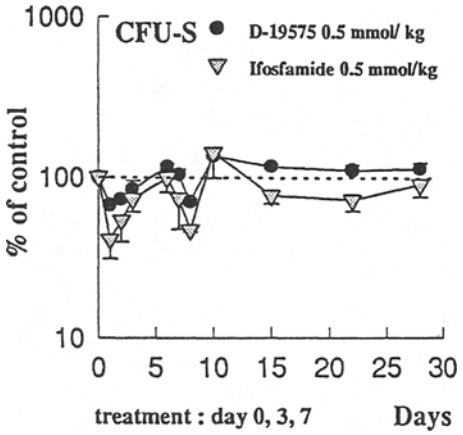

Fig. 6 Assay of spleen colony-forming cells (*CFU-S*) from murine bone marrow after three equimolar (0.5 mmol/kg; days 0, 3, and 7) i.p. doses of D-19575 or ifosfamide

When CFU-C were measured following the same treatment with D-19575 or ifosfamide, it was obvious that only ifosfamide produced a transient reduction in bone marrow stem cells. D-19575 had no in vivo effect on hemopoietic progenitor cells at the dose given to normal mice (Fig. 5). Similarly, D-19575 had only slight effects on spleen-cell colony formation (CFU-S), whereas equimolar ifosfamide produced a pronounced suppression (Fig. 6).

Single D-19575 doses of up to 1000 mg/kg given i.p. to rats resulted in no sign of myelotoxicity. Signs of venous pain were not observed with D-19575 of concentrations up to 25 mg/ml. Within the first 8 h of administration, 53.1% ± 18.5% (mean ± SD) of the delivered i.v. dose of D-19575 appeared as unchanged drug in the urine. Only 1.8% ± 1.4% of the dose was excreted during the second sampling period (8–24 h, Table 5). Pre- and cotreatment with phlorizin was performed to inhibit active renal reabsorption of glucose

Table 5 Urinary excretion of unchanged D-19575 by rats following D-19575 administration alone and in combination with phlorizin as determined by ^{31}P-NMR spectrometry. Data represent mean values ± SD, expressed as a percentage of the delivered dose

Sampling period	D-19575 ($n - 5$)	D-19575 + phlorizin ($n - 3$)
0–8 h	53.1 ± 18.5	79.4 ± 2.2
8–24 h	1.8 ± 1.4	2.0 ± 0.7

and was also assumed to block reabsorption of D-19575. The observed significant ($P < 0.05$) increase in the urinary excretion of unchanged D-19575, i.e., 79.4% ± 2.2% of the i.v. dose, during the first 8 h is in good accordance with this assumption.

Discussion

The development of oxazaphosphorines has resulted in a marked and important increase in selectivity as compared with that of the initial nitrogen mustard-like drugs, which has never been reached by analogue development in other classes of anticancer drugs. The complex metabolic activation and toxification of these compounds have been quite well explained, but a considerable interindividual variation among patients makes the clinical outcome unpredictable for the individual patient.

First attempts to circumvent the metabolic activation were aimed at stabilization of the activated primary metabolites, which are the carrier molecules of cytotoxic activity across cell membranes. This development eventually resulted in the promising drug mafosfamide. During clinical trials, however, the unexpected occurrence of venous pain limited further development of this drug. The venous pain was most likely produced by minute amounts of acrolein generated spontaneously after aqueous dissolution of mafosfamide.

Starting from the observation that chlorozotocin showed reduced bone marrow toxicity in comparison with carmustine (BCNU) [18], N-mustard derivatives have been investigated in this respect [14] since their synthesis beginning in 1955 [24]. The participation of glucose carriers in the uptake of these compounds could not be shown [21]. To participate in these transport systems, the OH groups in positions 3, 4, and 6 should be accessible for interactions with the carrier system. Most of the compounds synthesized to date have not fulfilled these prerequisites. We directed our efforts to the synthesis of monosaccharide conjugates of reactive intermediates, where the 1-0-glycosidic bond can be cleaved either by an enzymatic attack or by hydrolysis to release the reactive intermediate. In our opinion, isophosphoramide mustard is a suitable reactive intermediate with known therapeutic efficacy,

because it is the alkylating intracellular metabolic end product of ifosfamide. A further benefit of this approach is the avoidance of acrolein production during ifosfamide metabolism. The phosphoramide mustard (PAM) from cyclophosphamide and the isophosphamide mustard (IPM) from ifosfamide have per se a poor ability to penetrate cell membranes, and they are consequently less cytotoxic in vitro than the primary 4-hydroxymetabolites.

The theoretical concept of conjugation of these mustard-like compounds with saccharides was to bind the resulting prodrugs preferentially to components of the cell membrane, perhaps lectins, and, after internalization, to cleave the aglycon from the reducing end of the saccharide by abundant intracellular glycosidases. The prototype compound D-19575 was part of a program to conjugate IPM with mono- and disaccharides. Glucose, galactose, and mannose were used as monosaccharides, and lactose, maltose, cellobiose, and mellibiose served as di-saccharides. Similar conjugates with PAM were also synthesized. As judged from the technical aspects and from the first biological observations, D-19575 was the most promising agent. The more detailed biological evaluation revealed some peculiar observations, which make D-19575 an outstanding new type of alkylating agent.

The direct cytotoxic activity determined in vitro for D-19575 was in the range of that of IPM and did not allow speculation about the mode of action, i.e., whether it is taken up by cells after membrane binding, internalization, and intracellular enzymatic cleavage or by extracellular enzymatic or spontaneous hydrolysis and uptake of free IPM, or by uptake caused by other transport mechanisms with or without intracellular cleavage. The in vivo observations, however, were rather surprising. From data generated by acute toxicity studies it was obvious that D-19575 was less toxic than the parent compound ifosfamide and much less toxic than IPM itself. Additionally, the lack of myelotoxicity observed even just below the approximative LD_{50} value was suggestive of a very special metabolic or distributional behavior for D-19575. In contrast to this reduced systemic toxicity, the antitumor activity seen in several rodent tumor models was not reduced but at least equal to that of ifosfamide [2]. It was therefore hypothesized that the overall similarity of D-19575 with glucose or, more specifically, with glucose-1 phosphate could contribute to these unexpected in vivo findings.

To study this possibility, specific inhibitors of glucose transporters in the cell membrane were added to in vitro cultures of murine leukemic L1210 cells. In fact, the cytotoxic activity of D-19575 could be at least partially inhibited. Phlorizin, which specifically blocks the sodium-dependent glucose cotransporter, was much more inhibitory than phloretin, which blocks sodium-independent transporters. This hypothesis was further confirmed by preliminary ^{31}P-NMR observations on the urinary excretion of unchanged D-19575. During the first 8-h sampling period after i.v. administration of D-19575 the urinary recovery of D-19575 was significantly increased if the renal reabsorption of glucose had been inhibited by pre- and cotreatment with phlorizin.

These observations suggestive of an active uptake of D-19575 by the sodium-dependent glucose cotransporter could also explain the extraordinarily high bioavailability of oral D-19575, which resulted in nearly identical approximative LD_{50} values after single i.v. or oral administration to mice and rats, because transporters of this kind are present in the gut.

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