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Pharmacokinetics and whole-body distribution of the new chemotherapeutic agent β-D-glucosylisophosphoramide mustard and its effects on the incorporation of [methyl-^{3}H]-thymidine in various tissues of the rat

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Abstract β-D-Glucosylisophosphoramide mustard (β-D-Glc-IPM) is a new, potential chemotherapeutic agent currently under investigation. Its pharmacokinetics in plasma and elimination of the parent drug and its metabolites via urine, bile, and exhaled air were studied in female Sprague-Dawley rats after bolus injection of 315 mg/kg. Typically, the drug's disposition from plasma follows a linear two-compartment model with half-lives ($t_{1/2}$) of 1.8 ($t_{1/2\alpha}$) and 32 min ($t_{1/2\beta}$). The rate of clearance is 0.0046 (range 0.0030–0.0071) l min^{-1} kg^{-1}, and the steady-state volume of distribution (V_{ss}) is 0.18 (0.08–0.042) l/kg (mean ± inter-individual standard deviation). In human plasma, 28.1 ± 2.6% (mean ± SD) of the drug (concentration range 0.5–5 mg/ml) is bound to plasma proteins (predominantly to albumin). Biliary excretion of the parent drug accounts for 2.9 ± 1.7% of the dose; its elimination in the form of $^{14}CO_2$ via exhaled air is less than 1%. Within 24 h, 63.5 ± 4.9% of the ^{14}C-labeled drug is excreted unchanged in the urine, whereas 17.5 ± 5.1% is excreted in the urine as metabolites. In addition, β-D-Glc-[^{14}C]-IPM was given as a bolus injection to female Sprague-Dawley rats at dose levels of 315 and 56.2 mg/kg. The distribution of radioactivity into tissue was examined qualitatively by whole-body autoradiography (WBA). Parallel experiments were carried out using the high dose of the L-derivative. After dosing with the D-compound, the highest levels of radioactivity were found in the liver, kidneys, thymus, thyroid gland, and central nervous system, including the brain. A similar distribution pattern was observed for the L-compound, except in the brain, which contained negligible levels of radioactivity. The distribution of the D-compound (high dose) was also investigated in male Copenhagen rats bearing a Dunning prostate tumor. The results were similar to those obtained in healthy Sprague-Dawley rats. Additionally, radioactivity was found in the tumor at 1 h after dosing with the drug and remained there even after 24 h. The effects of β-D-Glc-IPM on the incorporation of [methyl-^{3}H]-thymidine into the DNA of the liver, kidneys, thymus, spleen, esophagus, and bone marrow of the rat were examined following tissue excision and liquid scintillation counting at 2, 8, and 24 h after administration of the drug. β-D-Glc-IPM showed no effect on the incorporation of [methyl-^{3}H]-thymidine in the liver and an insignificant reduction in kidney DNA (maximal reduction: −27.3%). However, after 8 h there was a marked reduction in the incorporation rate in the thymus (−83.7%), spleen (−74.6%), and esophagus (−87.2%), with a tendency toward recovery within 24 h. In bone marrow cells a reduction of −75.5% (8 h) and −73.3% (24 h) was observed.

Key words β-D-Glucosylisophosphoramide mustard · Pharmacokinetics · Plasma protein binding · Whole-body autoradiography

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Introduction

Research and development of chemotherapeutic agents has generated effective drugs for the treatment of cancer. However, their use is frequently limited by severe side effects and by the low specificity of these substances. Therefore, new compounds with a better therapeutic index are urgently needed.

We are currently investigating the new chemotherapeutic compound β-D-glucosylisophosphoramide mustard (β-D-Glc-IPM, D19575). It belongs to the group of nitrogen mustard-derived alkylating agents. Cyclophosphamide and ifosfamide, the best known representatives of this class, play an important role in the chemotherapy of infant and adult malignancies [14]. They are prodrugs that require metabolic activation by cytochrome P-450 enzymes of the liver. Ifosfamide is enzymatically activated to form 4-hydroxyifosfamide, which builds up an equilibrium with its tautomer aldo-ifosfamide. The latter decomposes spontaneously into the active metabolite, isophosphoramide mustard, by β-elimination of acrolein. Free acrolein is responsible for the severe side effects in the urinary tract that are typical for this class of alkylating agents. Isophosphoramide mustard itself is too toxic and chemically unstable to be used directly. The conjugation of the mustard as an aglycon to the reducing end of glucose has resulted in a new compound with major advantages. The new drug is chemically stable, and it can be split by intracellular glycolytic enzymes. Furthermore, side effects in the urinary tract are avoided since no acrolein is formed. This substance shows remarkable antitumor activity and very low myelotoxicity. Its selectivity for tumor cells is probably due to an active transport by glucose carriers [17].

This paper deals with the pharmacokinetics and distribution (studied by whole-body autoradiography, WBA) of β-D-Glc-IPM in rats. A comparison between the distribution of β-D-Glc-IPM and that of β-L-Glc-IPM allows further conclusions to be drawn about a possible transport by glucose carriers, given that the L-compound (as L-glucose) is no substrate for glucose carriers. The effects of the drug on target tissues as elucidated by autoradiography were also studied.

Materials and methods

General information

β-D-Glc-IPM (Fig. 1) was synthesized in the Chemical Research Laboratories of Asta Medica AG (Frankfurt, Germany) according to the method described by Dickes [5]. β-L-Glc-IPM, β-D-[^{14}C]-Glc-IPM, and β-L-[^{14}C]-Glc-IPM were synthesized in the Division of Molecular Toxicology according to a method described by Wießler (unpublished data). The ^{14}C label was situated in the β-chloroethylamine side chain of the compounds; the specific radioactivity was 15 mCi/mmol. The α-/β-anomers were separated by recrystallization and subjected to column chromatography. The purity of the substances was >99%. The absence of isophosphoramide mustard was ascertained by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC).

Female Sprague-Dawley (SD) rats weighing 120 ± 10 (WBA) or 150 ± 10 g (kinetics experiments) were purchased from Charles River Wiga (Sulzfeld, Germany). Male Copenhagen rats weighing 180 ± 10 g were obtained from Harlan Sprague-Dawley (Indianapolis, Ind., USA). The animals were housed under specific pathogen-free, controlled environmental conditions.

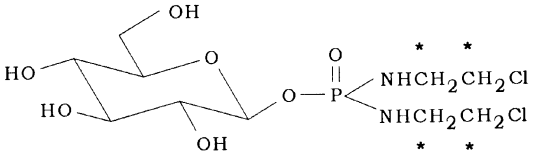

Fig. 1 Chemical structure of β-D-Glc-IPM, a prototype of a new class of sugar-linked chemotherapeutic agents. The position of ^{14}C label in the radioactive material is marked by *asterisks*

Fresh pieces (2 × 2 mm) of tumor tissue from the Dunning prostate tumor (subline R3327-AT1) were transplanted s.c. into the right thigh of Copenhagen rats. The fresh tissue was obtained from tumors grown on donor animals from frozen stock material maintained as a first passage of the original tumor tissue. The rats were kindly supplied by Dr. U. Haberkorn (German Cancer Research Center, Heidelberg). The tumors were allowed to grow for approximately 1 week. The animals were treated when the tumors were well palpable but not yet necrotic.

β-D-Glc-IPM (315 mg/kg) was given by i.v. bolus injection. Unless stated otherwise, the concentration of the drug in body fluids was determined directly using ^{31}P-nuclear magnetic resonance (^{31}P-NMR) spectroscopy. Spectra were obtained at 5.4 T (101.26 MHz) and 28°C using a Bruker AC-250 spectrometer. Known volumes of the biofluid (0.15–0.4 ml) were diluted as necessary with physiological saline to give a sample volume of 0.4 ml in a 5-mm NMR tube. Spectra were acquired under the following standardized conditions: spectral width 3548 Hz, 8 K data points, 1.5 μs rf pulse (flip angle 36°), and repetition time 2.0 s. In all, 1–30 blocks of 600 transients were acquired (0.33–10 h) as needed to obtain a sufficient signal-to-noise ratio according to the concentration of drug in each sample. A reference sample containing a known amount of drug was measured under the same conditions, and the integral of the drug's NMR peak was used to calibrate the integrals obtained from the spectra of the biofluids. The integrals were scaled according to the number of transients acquired and the known dilution factors, and absolute concentrations of drug were calculated. A typical spectrum is shown in Fig. 2. ^{31}P chemical shifts (δ_p) were referenced to phosphoric acid at 0 ppm.

Kinetics in plasma

The jugular veins of six rats were catheterized under chloral hydrate (400 mg/kg i.p.) anesthesia [1]. The catheter was sutured to the neck of the animals and the external part was protected by a metal coil. The end of the catheter was fixed to the cage lid, allowing the rats to move freely. At 1 day after implantation, the drug was given through the catheter as a bolus injection, and blood samples of 0.4–0.5 ml were drawn at 2, 5, 10, (20), 30, 60, 90, and (120) min after injection. Blood cells were immediately spun down, and plasma samples were stored at −80°C until measurement.

The plasma concentration data obtained for four animals were analyzed simultaneously using a mixed-effect model [18]; this technique allows estimation of the mean kinetic parameters ("fixed effects") of a group of subjects along with their interindividual variances ("random effects") without the need of estimating individual parameters. (Two animals were excluded because they showed no drug elimination at times later than 10 min after injection. This was probably due to injuries and inner bleeding caused by a misplaced catheter. The animals had to be killed in a moribund state at about 75 min after injection.) Inspection of the data suggested using a linear two-compartment model as the "structural" part of the mixed-effect model that was defined by the parameters V_1 (central distribution volume), CL_{10} (elimination clearance from the central compartment), CL_{121} (distribution clearance between the central and the peripheral compartment), and V_{ss} [the apparent total

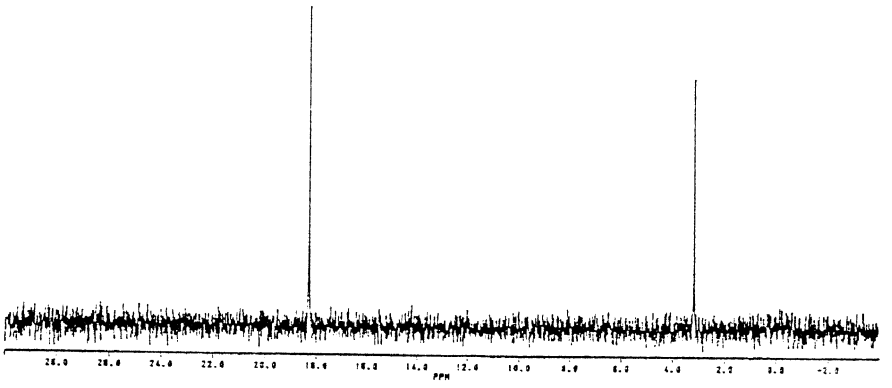

Fig. 2 ^{31}P-NMR spectrum (600 transients in 20 min) of a plasma sample taken 2 min after the administration of β-D-Glc-IPM. The signal from the drug appears at the *left* ($\delta_P = 18.3$ ppm); the signal from inorganic phosphate in plasma appears at the *right* ($\delta_P = 3.2$ ppm)

distribution volume (central plus peripheral compartment) under steady-state conditions, i.e., when the drug concentration is the same in both compartments]. All of these parameters are assumed to vary randomly between individuals:

$$V_{1,i} = V_1 \exp(\eta_1, i), \tag{1}$$

$$CL_{10,i} = CL_{10} \exp(\eta_{2,i}), \tag{2}$$

$$CL_{121,i} = CL_{121} \exp(\eta_3, i), \text{ and} \tag{3}$$

$$V_{ss,i} = V_{ss} \exp(\eta_4, i), \tag{4}$$

where the index i refers to the individual animals; V_1, CL_{10}, CL_{121}, and V_{ss} are the mean kinetic parameters; and η_1, η_2, η_3, and η_4 are normally distributed random variables with mean zero, variances ω_{11}, ω_{22}, ω_{33}, and ω_{44}, and covariances ω_{12}, ω_{13}, ω_{14}, ω_{23}, ω_{24}, and ω_{34}.

The residual variation of observed plasma drug concentrations (C_P) around predicted concentrations was modeled according to the equation

$$C_{Pi,j,\ \text{observed}} = C_{Pi,j,\ \text{predicted}} \exp(\varepsilon_{i,j}),$$

where $j = 1, \ldots,$ the number of measuring time points in animal i; $C_{Pi,j,\text{predicted}}$, is the C_P predicted for time j in animal i on the basis of the individual parameters $V_{1,i}$, $CL_{10,i}$, $CL_{121,i}$, and $V_{ss,i}$; and ε is normally distributed with mean zero and variance σ^2.

In model building the interindividual variance and covariance parameters were introduced one by one in the order ω_{11}, ω_{22}, ω_{12}, ω_{33}, ω_{13}, ω_{23}, and ω_{44} and were kept only when the addition improved the fit significantly as judged by the likelihood-ratio criterion [18] with $\alpha < 0.05$.

The estimated covariance between individual η on V_1 and CL_{10} resulted in a correlation coefficient of 1.00; therefore, individual η values on V_1 and CL_{10} were linked by a proportionality factor:

$$CL_{10,i} = CL_{10} \exp(\theta \eta_{1,i}), \tag{2a}$$

where $\eta_{1,i}$ is the individual η on V_1 and the proportionality factor θ is the same for all individuals (final estimate 0.521).

All model fits were done using the program system NONMEM [3]; the "first order" (FO) method was used for initial model building, and the "first order conditional estimate" (FOCE) method was used for the final elimination of unnecessary interindividual variance or covariance parameters and for the estimation of the final results. The dose was entered into the calculations as the dose per kilogram of body weight such that all clearance and volume estimates are scaled by kilograms of body weight. In model building the ratio of the peripheral compartment volume to the central comparment volume, V_2/V_1, was used as the second volume parameter instead of V_{ss} because, in our experience, model fits appear to be less sensitive to initial parameter estimates with this parameterization. Confidence limits on parameter estimates were computed as $P \pm 1.96\sigma_P$, where P is the parameter estimate and σ_P is the standard deviation of the estimate.

The expected time course of C_P after a bolus injection can be expressed as

$$C_P(t) = \text{dose } [L_1 exp(-\lambda_1 t) + L_2 exp(-\lambda_2 t)], \tag{5}$$

where

$$\lambda_1 = 0.5\,[(k_{10} + k_{12} + k_{21} + ((k_{10} + k_{12} + k_{21})^2 - 4k_{10}k_{21}))^{1/2}], \tag{6}$$

$$\lambda_2 = 0.5\,[(k_{10} + k_{12} + k_{21} - ((k_{10} + k_{12} + k_{21})^2 - 4k_{10}k_{21}))^{1/2}], \tag{7}$$

$$L_1 = (\lambda_1 - k_{21})/V_1/(\lambda_1 - \lambda_2), \tag{8}$$

$$L_2 = (k_{21} - \lambda_2)/V_1/(\lambda_1 - \lambda_2), \text{ and} \tag{9}$$

$k_{10} = CL_{10}/V_1$; $k_{12} = CL_{121}/V_1$; $k_{21} = CL_{121}/V_2$ (23). This allows a convenient calculation of the total AUC (area under the plasma concentration-time curve) as $(L_1/\lambda_1 + L_2/\lambda_2)$, where L_2/λ_2 is the part due to the slow component of drug disposition. The half-lives of the fast and the slow components of disposition are obtained from $In(2)/\lambda_1$ and $In(2)/\lambda_2$, respectively.

Urinary excretion

For measurement of urinary excretion, rats were placed for 24 h in metabolic cages with separate collection of urine and feces. The urine-collecting vials were cooled in a mixture of ice and salt to prevent degradation of the drug. Urine was collected in periods of 0–8 and 8–24 h. During the collection time the animals were fasted but received tap water ad libitum. Unlabeled β-D-Glc-IPM was given to five animals (rats 1–5) so as to determine the amount of unchanged drug excreted in the urine. ^{14}C-labeled β-D-Glc-IPM (synthesized in the Division of Molecular Toxicology; ^{14}C-label in the β-chloroethylamine side chain; sp. act. 15 mCi/mmol) was given to a second group of four animals (rats 6–9) so as to verify the results obtained with the NMR method and to determine the amount of labeled metabolites excreted. The radioactivity in urine samples was measured with a liquid scintillation analyzer (Packard, Groningen, The Netherlands). In addition, 10-µl samples were analyzed by TLC using F254 silica plates (Merck, Darmstadt, Germany) in chloroform: methanol: water (70:40:10, by vol.). The radioactivity on thin-layer plates were determined with an automatic linear analyzer (Berthold LB 2842, Wildbad, Germany).

Binding of drug to plasma proteins

The amount of β-D-Glc-IPM bound to plasma proteins was determined by ultrafiltration of test solutions containing one of the

following: 2% or 4% human albumin; 0.2%, 0.066%, or 0.033% α_1-acidic glycoprotein; human serum; human plasma; or rat serum. All proteins were purchased from Sigma (Deisenhofen, Germany) and were dissolved in 10 m*M* phosphate-buffered saline (PBS, pH 7.4; Sigma, Deisenhofen, Germany). Pooled samples of human serum and plasma were obtained from four healthy volunteers. Protein electrophoresis of the individual samples before pooling guaranteed that all protein parameters were within the normal range. Concentrations of 5, 2.5, and 0.5 mg β-D-Glc-IPM/ml with a trace amount of ^{14}C-labeled substance were investigated. A test volume of 1 ml was pipetted into a Centrisart I SM 13229 ultrafiltration vial (Sartorius, Göttingen, Germany). A 20-µl aliquot was immediately taken out, and the radioactivity was measured in a liquid scintillation counter. The vials were incubated at 37°C for 15 min. After filtration by centrifugation at 1500 *g* for 10 min, a second 20-µl aliquot was measured. All measurements were done in triplicate. The percentage of bound drug was obtained by subtracting the filtered amount of drug and the amount of unspecifically bound drug (i.e., drug bound to the filter and filtration vial) from the original amount. The amount of unspecifically bound drug was determined in control experiments under the conditions described above using PBS (pH 7.4) without protein.

Creatinine clearance and partition coefficient

The creatinine clearance was determined in four healthy female SD rats. The analysis was carried out in 24-h urine samples and in plasma taken from the bulbus retroorbitalis using methofane anesthesia. The determination was performed with the kinetic Jaffé method described elsewhere [10].

The partition coefficient of β-D-Glc-IPM was determined according to the OECD guideline No 107 in 1-octanol/PBS pH 7.4 and n-heptane/PBS pH 7.4. PBS was dissolved in bidest water to give a 10 mM concentration. A stock solution of β-D-Glc-IPM in PBS was mixed with a small portion of ^{14}C-labelled β-D-Glc-IPM. Three concentrations of β-D-Glc-IPM were examined: 5 mg/ml; 0.5 mg/ml; 0.05 mg/ml. Three ml buffer were mixed with 100 µl stock solution and carefully overlayed with 3 ml 1-octanol or n-heptane. The probes were shaken in order to achieve an equilibrium concentration and were then centrifuged at 1500*g*. Aliquots of 0.5 ml were taken after 10', 20', 30', and 40' from the different layers and counted in a liquid scintillation counter. All experiments were carried out in triplicate at 21°C.

Autoradiography

Groups of five healthy SD rats received i.v. bolus injections of either β-D-Glc-[^{14}C]-IPM (315 and 56.2 mg/kg) or β-L-Glc-[^{14}C]-IPM (315 mg/kg). In addition, five tumor-bearing Copenhagen rats received the high dose of radiolabeled β-D-Glc-IPM. Each animal received on average 20 µCi of radioactivity. One animal per group was euthanized by CO_2 inhalation at 10 min, 1 h, 2 h, 8 h, and 24 h after drug administration. The rats were quickly shaved and immediately frozen, first in a mixture of methanol and dry ice and later in an aqueous gel of carboxymethyl cellulose, and were stored at −75°C. Then 20-µm-thick sagittal sections were prepared with a Cryo-Polycutt microtome (Cambridge Instruments, Nussloch, Germany) and collected on tape (tape D, Beiersdorf, Hamburg, Germany) for whole-body autoradiography as described by Ullberg [21, 22]. Series of adjacent freeze-dried sections derived from areas of interest were exposed to X-ray film (Scientific Imaging Film X-Omat, Eastman Kodak, New York) and stored for 5 weeks at a temperature of −75°C.

[methyl-^{3}H]-Thymidine incorporation study

For this study, 18 female SD rats divided into 3 groups were used; each group was divided into 2 subgroups of 3 animals each, representing a control group and a group receiving treatment. Treated rats were given β-D-Glc-IPM (315 mg/kg) as an i.v. bolus injection in saline (0.35 ml), and control animals received physiological saline alone. [methyl-^{3}-H]-Thymidine (New England Nuclear, Boston; 5.0 mCi/0.061 mg) was injected i.p. in saline at 0.5 µCi/g body weight according to Hellman and Ullberg [11] at 2, 8, and 24 h after administration of the cytostatic compound. The animals were euthanized 2 h later by CO_2 inhalation. Target organs, including the liver, kidneys, thymus, spleen, esophagus, and bone marrow, were rapidly dissected. The organs were freed of fat and connective tissue. The marrow from both femora was pooled and treated as one sample. The organs were homogenized in 5 ml physiological saline, and the same volume of ice-cold 10% trichloroacetic acid (TCA; Roth, Karlsruhe, Germany) in physiological saline was added. The samples were shaken for 30 min at 4°C and then centrifuged for 5 min at 400 *g*. The resulting pellet was treated twice with 5% TCA as described above to remove non-incorporated [methyl-^{3}H]-thymidine and its acid-soluble metabolites. It was shown that the amount of radioactivity detected in the TCA precipitate was almost equal to that found in isolated and purified DNA [11]. The last pellet was dissolved in 10% sodium dodecyl sulfate (SDS; Serva, Heidelberg, Germany) in physiological saline at 60°C. Then, 500-µl aliquots were used to determine the TCA-insoluble radioactivity using a liquid scintillation analyzer (Packard, Groningen, The Netherlands).

The results were expressed as counts per minute per milligram of fresh tissue. In the case of bone marrow and esophagus the more exact unit of measure, counts per milligram of protein, was used. Protein determination was carried out according to Bradford [4]. All measurements were done in triplicate. For statistical analysis the *t*-test was used, assuming a normal distribution of the data. The homogeneity of the distribution of the data was tested with the *F*-test. In case of heterogeneous distribution, the Welch approximation was used.

Results

Kinetics in plasma

Plasma concentration data showing the disposition of β-D-Glc-IPM were obtained for four rats using ^{31}P-NMR spectroscopy. Drug disposition from plasma was biphasically linear for rats 1–3, whereas in rat 4 there was no evidence of an initial distribution phase. Observed C_P values differed between animals by a factor of up to 12 for a given time point. There was no increase in the terminal half-life with increasing concentration (Fig. 3, rat 4 versus rat 3).

The final interindividual variance model includes the variance of V_1 and CL_{121} (ω_{11}, ω_{33}), a fixed proportionality between the η on V_1 and CL_{10} such that $\eta_{2,i} = 0.52\ \eta_{1,i}$ (see Materials and methods), and equal η values on V_1 and V_{ss} ($\eta_{4,i} = \eta_{1,i}$). The mean kinetic parameters estimated with this model, along with confidence limits and estimated interindividual standard deviations, are given in Table 1.

C_P predictions, which are based on the mean kinetic parameters of the population and on individual body weights, are shown in Fig. 3 as solid lines, whereas additional empirical Bayesian estimates of the individual random deviations of V_1, CL_{10}, CL_{121}, and V_{ss} from the corresponding population means produce the predictions shown as dotted lines.

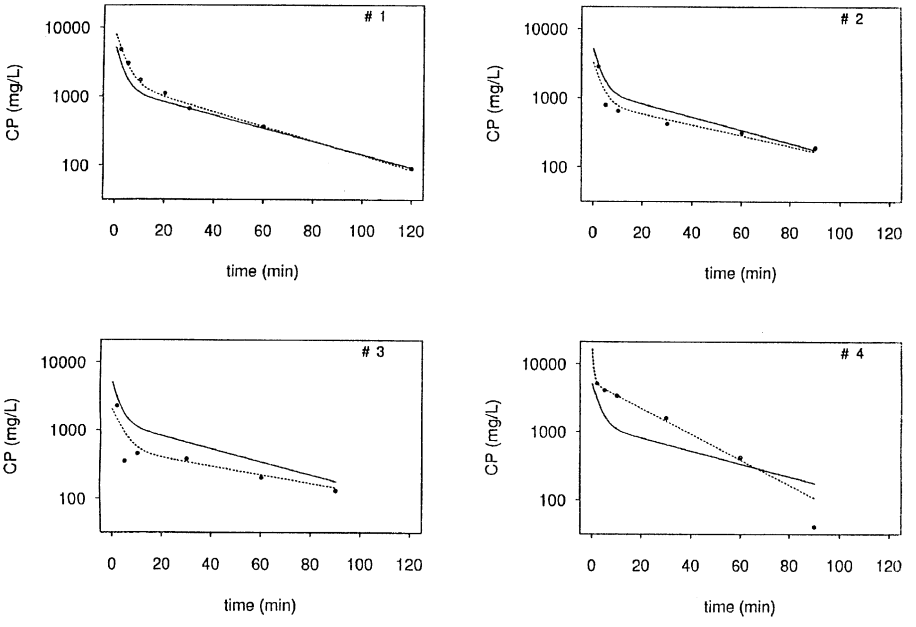

Fig. 3 Observed (*dots*) and predicted (*lines*) plasma concentrations of β-D-Glc-IPM versus time. *Solid lines* represent predictions based on mean population parameters; *dotted lines* represent predictions taking into account individual estimates of V_1/kg, CL_{10}/kg, and CL_{121}/kg

Table 1 Estimated population parameters of β-D-Glc-IPM pharmacokinetics in rat plasma. SD rats received 315 mg/kg β-D-Glc-IPM i.v.; plasma samples were taken and analyzed by ^{31}P-NMR. Parameters were estimated using a linear two-compartment population model

Parameter	Population mean	95% Confidence interval for the population mean	Factor for typical individual random deviation[a]
V_1 (l/kg)	0.061	± 38%	2.3
CL_{10} (l min^{-1} kg^{-1})	0.0046	± 41%	1.6
CL_{121} (l min^{-1} kg^{-1})	0.013	± 79%	2.0
V_{ss} (l/kg)	0.18	± 67%	2.3

[a] The factors listed in this column are computed as $\exp(\omega^{1/2})$, where ω is the interindividual variance of the corresponding kinetic parameter (see Materials and methods). The typical random deviation of the individual kinetic parameter is obtained by multiplying or dividing the population mean by this factor

The estimated mean population parameters were converted into the more commonly used parameters L_1, L_2, λ_1, and λ_2 to give the following expression for the expected time course of plasma drug concentration after bolus injection:

$$C_P(t) = \text{dose}\,[12.3\exp(-0.377\,t) + 4.03\exp(-0.0220\,t), \quad (10)$$

where C_P is expressed in milligrams per liter; dose, in milligrams per kilogram; and t, in minutes. The half-lives of the fast (λ_1) and slow (λ_2) components of disposition are 1.8 and 32 min, respectively, and the second component accounts for 85% of the total AUC.

Urinary excretion

Quantitation by ^{31}P-NMR indicates that the average excretion of intact compound is 53.1 ± 18.5% within the first 8 h and 1.79 ± 1.4% over the period of 8–24 h. Spectra reveal three phosphorus-containing metabolites. One is known to be isophosphoramide mustard (δ_P = 13.5 ppm); the other two remain unknown (δ_P = 11.4 and 9.3 ppm). Experiments carried out with ^{14}C-labeled β-D-Glc-IPM verified these findings. Within 0–8 h, 59.7 ± 5.9% of the total drug dose was excreted unchanged, and 13.5 ± 4.0% was found as metabolites in the urine. In 8- to 24-h urine samples, 3.7 ± 2.4% of the drug was found unchanged and 3.7 ± 1.3% occurred as metabolites. Individual data obtained in these two experiments are shown in Tables 2 and 3.

Thus, over 24 h, an average of 63.5 ± 4.9% of the β-D-Glc-IPM dose is excreted intact and 17.5 ± 5.1% is excreted in metabolized form via the urine. This accounts for a total of 81% of a given dose. Other routes of excretion play only a minor role. Biliary excretion accounts for 2.9 ± 1.7%; excretion in the form of $^{14}CO_2$ via exhaled air is less than 1% (data not shown).

Plasma protein binding and creatinine clearance

In a preliminary test the amount of β-D-Glc-IPM bound unspecifically to filters and filtration vials was determined to be 2.88 ± 0.22%. This amount was judged to be sufficiently small to make ultrafiltration the method of choice. The data shown in Table 4 reveal that β-D-Glc-IPM binds predominantly to albumin

Table 2 Excretion of β-D-Glc-IPM in the urine of rats at 0–8 h after i.v. application. The drug was given at a dose of 315 mg/kg. The urine was collected and analyzed by ^{31}P-NMR (^{31}P) for rats 1–5 or by TLC with the aid of a linear analyzer (^{14}C) for rats 6–9

Rat	% Dose excreted unchanged (^{31}P)	Rat	% Dose excreted unchanged (^{14}C)	% Dose excreted metabolized (^{14}C)
1	66.7	6	59.5	12.7
2	76.4	7	67.0	8.4
3	45.3	8	59.8	17.7
4	29.7	9	52.5	15.3
5	47.4	–	–	–
Mean	53.1 ± 18.5	Mean	59.7 ± 5.9	13.5 ± 4.0

Table 3 Excretion of β-D-Glc-IPM in the urine of rats at 8–24 h after i.v. application. The drug was given at a dose of 315 mg/kg. The urine was collected and analyzed by ^{31}P-NMR (^{31}P) for rats 1–5 or by TLC with the aid of a linear analyzer (^{14}C) for rats 6–9

Rat	% Dose excreted unchanged (^{31}P)	Rat	% Dose excreted unchanged (^{14}C)	% Dose excreted metabolized (^{14}C)
1	0.35	6	–	–
2	2.70	7	1.5	3.3
3	1.25	8	3.5	2.7
4	3.74	9	6.2	5.2
5	0.91	–	–	–
Mean	1.79 ± 1.4	Mean	3.7 ± 2.4	3.7 ± 1.3

Table 4 Binding of β-D-Glc-IPM to plasma proteins, rat serum, human serum, and human plasma. The percentage of drug bound is shown, with the standard deviation being given in parentheses. The experiments were carried out using ^{14}C-labeled substance and the ultrafiltration technique (α_1-*AGP* α_1 Acid glycoprotein)

Test solution	5 mg/ml β-D-Glc-IPM	2.5 mg/ml β-D-Glc-IPM	0.5 mg/ml β-D-Glc-IPM
2% human albumin	17.2 (0.93)	19.1 (1.16)	18.1 (0.64)
4% human albumin	30.1 (2.45)	23.8 (3.97)	24.9 (1.75)
α_1-AGP 0.2%	5.9 (1.3)	4.5 (4.1)	0.1 (2.0)
α_1-AGP 0.066%	2.9 (3.1)	3.3 (2.27)	1.1 (2.29)
α_1-AGP 0.033%	0	0.6 (3.74)	1.4 (1.81)
Rat serum	25.8 (1.12)	27.0 (0.91)	23.4 (1.51)
Human serum	29.3 (4.79)	29.7 (1.73)	32.4 (1.7)
Human plasma	30.4 (2.77)	28.2 (0.82)	25.6 (1.27)

and only to a small extent to the α_1-acidic glycoprotein. The overall binding to plasma proteins is about 30%. There is no major difference between rat serum and human serum or between human serum and human plasma. The creatinine clearance determined in four animals was $0.0053\,l\,min^{-1}\,kg^{-1}$ (range 0.0049–$0.0056\,l\,min^{-1}\,kg^{-1}$).

Whole-body autoradiography of β-D-Glc-[^{14}C]-IPM and partition coefficient

The distribution of β-D-Glc-[^{14}C]-IPM is demonstrated in selected sections shown in Figs. 4 and 5. Within 10 min of an i.v. dose, radioactivity was extensively distributed throughout the body. Negligible levels were found in the central nervous system, the lumina of the gastrointestinal (GI) tract, and the bones. The highest concentrations of radioactivity were detected in the liver, kidneys, skin, and cartilage. Within 2 h, marked concentrations of radioactivity were observed in the kidneys (inner cortex), liver, thymus, and thyroid and in the small/large intestines, indicating rapid biliary excretion. Low levels of radioactivity appeared in the central nervous system. Radioactivity levels measured in cartilage at 2 h declined and were barely detectable. After 8 h, most of the radioactive dose had been cleared from the peripheral tissues, with the highest levels being associated with the organs of excretion (kidney cortex and intestines). Low levels were also found in the thymus, thyroid, and central nervous system. There was no marked change after 24 h. No differences in the pattern of distribution of radioactivity between the low dose and the high dose was observed.

In 1-octanol/PBS pH 7.4 the equilibrium was reached at 20' and therefore the median was calculated for this and the following time points to give $P_{o/w} = 2.54 \times 10^{-2}$ with a $\log P_{o/w} = -1.6$. The equilibrium in n-heptanol PBS was reached after 30 min; $P_{h/w} = 9.68 \times 10^{-2}$, $\log P_{h/w} = 3.02$.

Whole-body autoradiography of β-L-Glc-[^{14}C]-IPM

The tissue distribution pattern of radioactivity observed after administration of β-L-Glc-[^{14}C]-IPM was very similar to that obtained after administration of β-D-Glc-[^{14}C]-IPM, with the striking difference being that at no time point did radioactivity appear in the central nervous system. After 10 min, radioactivity was also seen in the small intestine, whereas for the D-compound this could not be shown before 1 h. For lack of substance, the time point "2 h after administration" had to be omitted.

β-D-Glc-[^{14}C]-IPM in tumor-bearing animals

The results were the same as those obtained in healthy female SD rats. Additionally, it could be demonstrated that β-D-Glc-[^{14}C]-IPM was present in the tumor tissue and in the testes.

[methyl-^{3}H]-Thymidine incorporation study

In this study the effects of β-D-Glc-IPM on the incorporation of [methyl-^{3}H]-thymidine were investigated. The amount of DNA-incorporated radioactivity

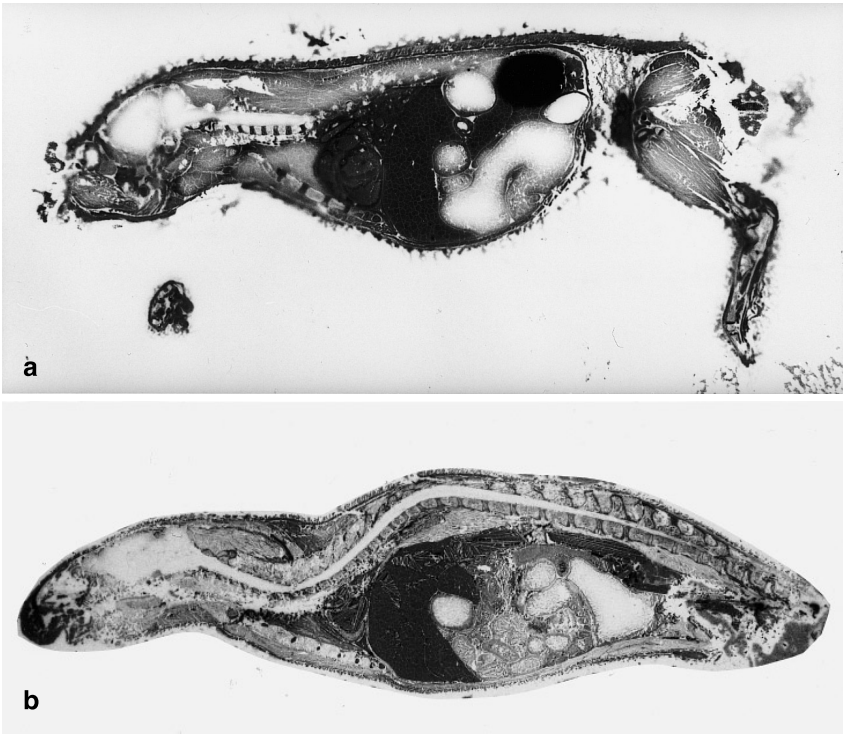

Fig. 4 a, b Autoradiographs of rats taken 10 min after the i.v. administration of **a** β-D-Glc-IPM and **b** β-L-Glc-IPM

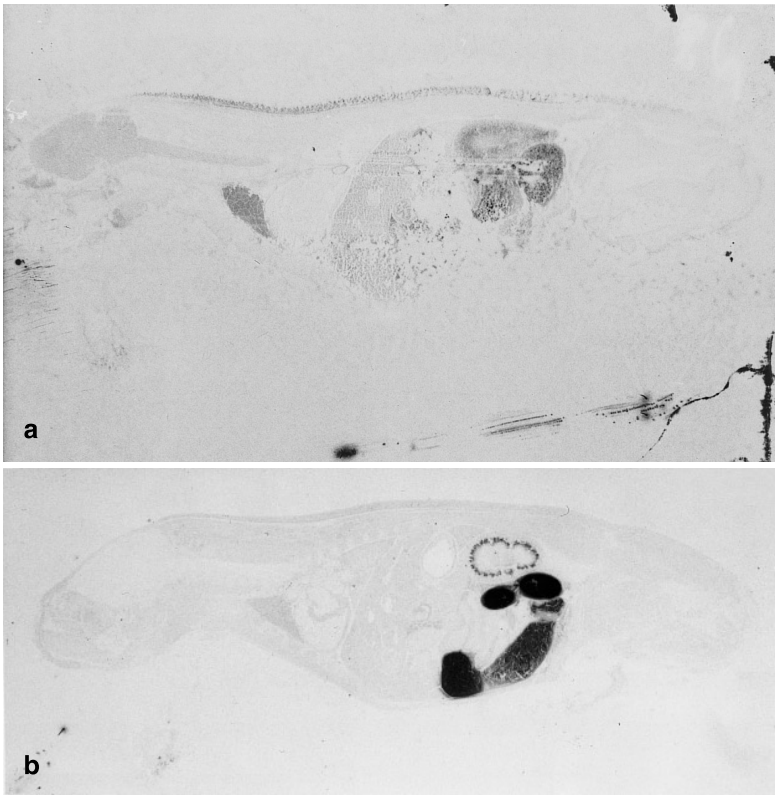

Fig. 5 a, b Autoradiographs of rats taken 8 h after the i.v. administration of **a** [^{14}C]-β-D-Glc-IPM and **b** [^{14}C]-β-L-Glc-IPM

(which equals the radioactivity remaining after TCA precipitation) was compared with that in a control receiving physiological saline. Figure 6 summarizes the findings. β-D-Glc-IPM influenced the uptake of [methyl-^{3}H]-thymidine in various organs of the rat to varying degrees. The liver and kidneys showed the smallest effects. Indeed, the incorporation of radioactivity into the liver was at no time point statistically

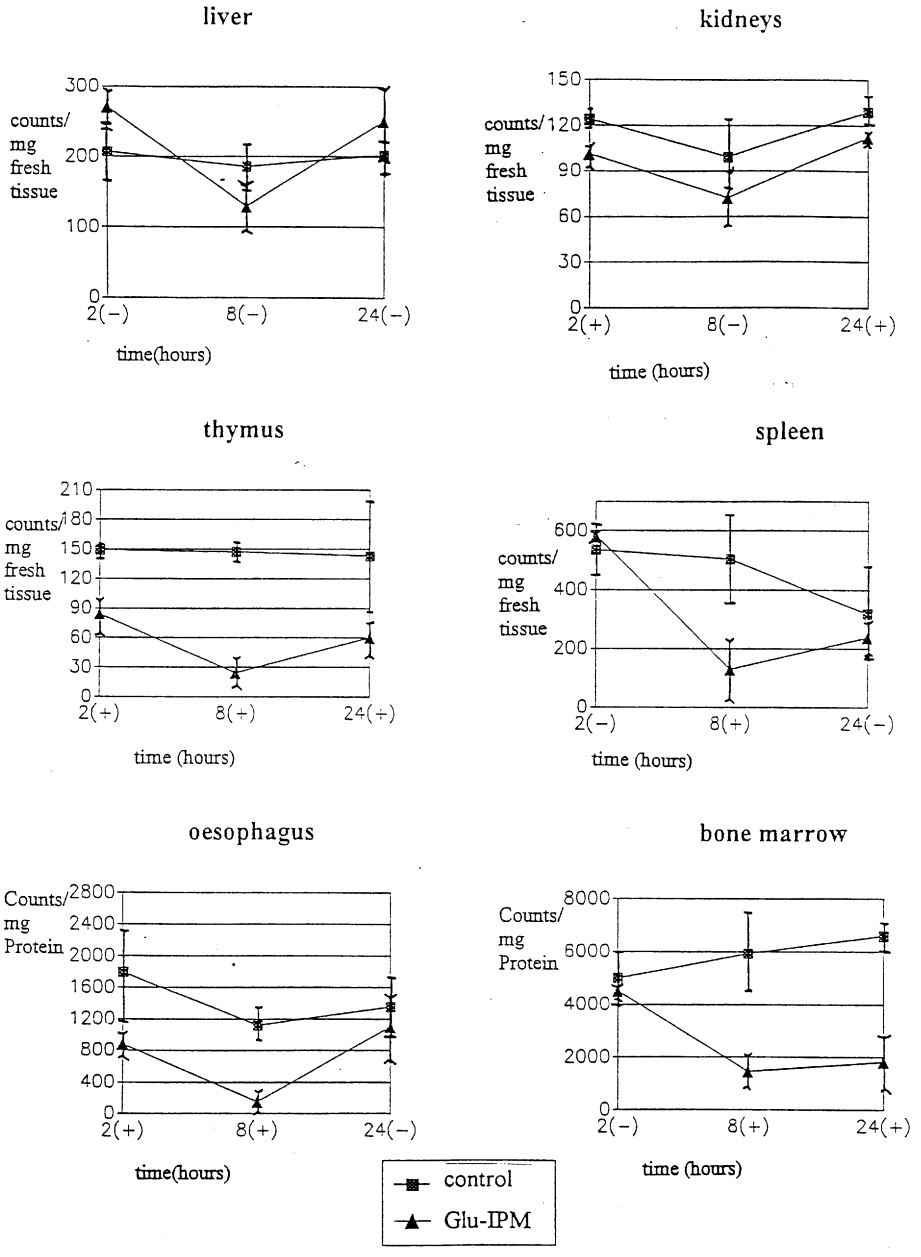

Fig. 6 Effects of β-D-Glc-IPM on the incorporation of [methyl-^{3}H]-thymidine in various tissues of the rat as based on the determination of acid-insoluble radioactivity, expressed as mean values ± SD for triplicate determinations (± Difference statistically significant, − difference not statistically significant; *t*-test)

different from that in the control ($0.1007 > P > 0.05$). In the kidneys, values of −18.5% (2 h), −27.3% (8 h), and −13.2% (24 h) were determined. Marked effects were found in the thymus (−43.6%, −83.7%, −58.0%), spleen (+9%, −74.6%, −25.2%), and esophagus (−48.9%, −87.2%, −18.7%). In all these organs, maximal inhibition was reached after 8 h, with a marked recovery occurring after 24 h. At 24 h the values observed in the spleen and esophagus were no longer significant as determined by decreased versus control values. The bone marrow (−9.4%, −75.5%, −72.3%) also showed a maximum after 8 h, but no recovery was found within the time range of the experiment (24 h).

Discussion

Pharmacokinetics

β-D-Glc-IPM is a very hydrophilic substance that proved to have a linear biphasic disposition from plasma and a short terminal half-life of about 30 min. Its estimated V_{ss} is 0.18 ± 0.12 l/kg (mean ± 95% confidence interval), thus being about 4 times lower than that of total body water and indicating little overall binding to tissues. In analysis of the β-D-Glc-IPM plasma concentration data, mixed-effect modeling has been preferred over more traditional approaches such as "naive aver-

aging of data" (NAD) or the "standard two-stage" (STS) method (estimating individual parameters first, and subsequently computing the mean and the variance of the individual parameter estimates) for the following reasons:

1. In this experimental situation, interest is focused on the mean kinetic parameters of the animals investigated and their interindividual variances; the individual kinetic parameters are of minor importance.
2. In general, mixed-effects modeling has been shown to yield less biased estimates of the mean kinetic parameters of a group of subjects than does NAD or STS and to avoid the overestimation of interindividual random variation, which is typical for STS [19].

Since β-D-Glc-IPM is thought to be transported by glucose carriers [17], its renal clearance (CL_R) may be compared with its glomerular filtration rate (GFR) to get information about a possible tubular reabsorption of this drug. CL_R can be estimated by multiplying the total clearance (4.6 ml min^{-1} kg) by the fraction of dose excreted unchanged in urine (55%), giving $CL_R = 2.5$ ml min^{-1} kg). The GFR of this low-molecular weight drug may be estimated by multiplying the endogenous creatinine clearance (5.25 ± 0.3 ml min^{-1} kg^{-1}) in this strain of rats as based on our own unpublished results) by the fraction of drug unbound in plasma (70%), which gives $GFR_{drug} = 3.7$ ml min^{-1} kg^{-1}). The difference is suggestive to tubular reabsorption; however, it is not statistically significant because of the large interindividual variation in total clearance (Table 1) and the fraction of dose extracted unchanged in the urine (Tables 2, 3).

The excretion data reveal that β-D-Glc-IPM is predominantly eliminated via the urine, as would be expected for a molecule of its size and hydrophilicity. The overall 24-h excretion via all routes (including metabolites) accounts for 85% of a given dose, with 15% remaining in the body. This is not surprising since we are dealing with an alkylating agent. The urine data presented herein with limited time resolution show that most of the drug is excreted within 8 h. Whole-body autoradiography carried out with ^{14}C-labeled β-D-Glc-IPM indicates that most of the drug is actually excreted within the first 1 or 2 h.

The two analytical methods used with two different groups of animals for determining the amount of drug in urine show similar results for the mean population parameters. The advantage of the ^{31}P-NMR method, however, is that it requires no radioactive label and can be used to analyze phosphorus-containing drugs and their metabolites in biofluids from patients without the need for derivatization or extraction procedures.

The partition coefficients of β-D-Glc-IPM as measured in 1-octanol or n-heptane were very low in both media and not concentration-dependent identifying the compound as strongly hydrophilic. Thus, a passive transport of the substance via the blood-brain barrier or the intestinal mucosa is not favored. Since strong hints point to a transport through both barriers it may be concluded that this transport is mediated by glucose carriers.

Whole-body autoradiography and partition coefficient

The results reveal an early distribution of radioactivity into the liver, kidneys, cartilage, and skin within 10 min of treatment with radiolabeled β-D-Glc-IPM. The excretion study mentioned above showed that most of the delivered dose of β-D-Glc-IPM (81%) was rapidly excreted in the urine at 24 h. This is consistent with the high level of radioactivity observed in the kidneys in this study. In addition, most of the dose appears to be excreted via the urine within the first 2 h. Significant levels of radioactivity were also detected in the liver, which is the main organ of metabolism of β-D-Glc-IPM.

Rather unexpected is the rapid distribution of radioactivity in the cartilage and skin and, during the 1st h, in the thymus and thyroid which cannot yet be explained. Radioactivity might be transported to the skin, since the blood supply to this area is high, which is also the case for the heart, lung, and striated muscle. The early appearance of radioactivity in the intestines is in good accordance with an observed biliary excretion of the drug.

The time pattern of the appearance of radioactivity in the brain is somewhat peculiar. Neither the parent compound nor any known metabolite should be capable of crossing the blood-brain barrier: β-D-Glc-IPM is a very hydrophilic agent; the primary metabolite isophosphoramide mustard is not known to cross the blood-brain barrier; and β-chloroethyl amine cannot cross this barrier either, being a primary amine. Metabolically generated $^{14}CO_2$, which could theoretically contaminate the brain, would have vanished during the freeze-drying process in the preparation of the autoradiographs.

A possible explanation for the appearance of radioactivity in the brain could be a transport of the whole molecule by glucose carriers. In the brain, three glucose transporters are known. The isoform GLUT 1 is present in plasma membranes of endothelial cells of the blood vessels that form the blood-brain barrier [6, 9, 12, 16, 20]. GLUT 3, the second isoform, is also present in these membranes as well as in the plasma membranes of glia cells and neurons [7]. The third isoform, GLUT 5, is expressed in microglia [15]. Transport by glucose carriers is thought to be a very rapid process. Therefore, one should expect an early uptake of the drug into the brain. Since this is not the case according to our autoradiography study, the affinity for the carrier seems to be low as compared with that for glucose. Both normal blood glucose and β-D-Glc-IPM might compete for the transporters, thus hampering a quick transport of the drug.

The experiment carried out with the analogous L-compound is a strong indication for transport of β-D-Glc-IPM by glucose transporters because β-L-glucose is not a substrate for the glucose carriers [2]. Accordingly, β-L-Glc-IPM should not be transported to the brain, which is actually demonstrated by our results. Moreover, after splitting of the linkage between the sugar and the mustard moiety, all possible further metabolites are chemically the same,regardless of their origin from the D- or L-moiety. However, this also rules out the possibility that a labeled metabolite could be responsible for the radioactivity in the brain. The distribution pattern of β-D-Glc-IPM and β-L-Glc-IPM is strikingly different only in the central nervous system, whereas in other organs the differences are slight. Therefore, other transport mechanisms (e.g., passive diffusion), which are most probably dependent on the concentration of the drugs, might be possible in these organs. Since the blood-brain barrier represents a tight control and protection system, these other transport mechanisms cannot be used; hence, carrier-mediated transport remains the only logical explanation for our findings. The observation of drug retention in the testes of tumor-bearing animals is another indication of carrier-mediated transport, since hydrophilic agents are normally incapable of crossing the blood-testes barrier.

Since β-D-Glc-IPM accumulates in the brain, it might be a potential chemotherapeutic agent for the treatment of brain tumors. In animal experiments, no sign of neurotoxicity has been observed thus far (Pohl, unpublished results). Most cytostatic agents only poorly cross the blood-brain barrier, if at all [13, 24]. Brain tumor tissue is known in many cases to express more glucose carriers than does normal brain tissue [8], thus further enrichening the compound. Our experiments carried out in tumor-bearing animals reveal that the drug accumulates in the tumor, underlining its specificity to tumor cells.

[methyl-^{3}H]-Thymidine incorporation study

[methyl-^{3}H]-Thymidine is rapidly taken up into proliferating cells and is selectively incorporated into DNA. It is therefore a very suitable agent for the study of the effects of cytotoxic/cytostatic substances and radiation under in vivo conditions. The amount that is not incorporated into DNA will be metabolized via derivatives of isobutyric acid to carbon dioxide, ammonia, and tritiated water, the main metabolite. To prevent disturbance by these agents, we investigated only the washed TCA precipitates of the organs. The amount of radioactivity that is incorporated into macromolecules other than DNA was shown to be very low, and there is good evidence that the amount of radioactivity detected after TCA precipitation is almost equivalent to the that associated with purified DNA [11]. In this experiment, target organs for chemotherapeutic treatment and proliferating organs were examined. The liver and kidneys showed no effect on the incorporation of [methyl-^{3}H]-thymidine into DNA. The incorporation rate is low since the turnover rate of the cells in these organs is also low. All other organs investigated showed a marked effect after 8 h, but all except for bone marrow recovered within 24 h. Other experiments carried out to evaluate the bone marrow toxicity of β-D-Glc-IPM have revealed a very low degree of myelotoxicity [17]. Therefore, it can be expected that the observed effects on bone marrow cells are temporary and cause a certain arrest in cell proliferation rather than a massive depletion of cells.

In conclusion, the substance β-D-Glc-IPM has been shown to possess interesting chemotherapeutic properties in several tumor models [17]. The experiments reported herein support the assumption that this new compound may represent a valuable new agent in the treatment of cancer. Investigations on its pharmacokinetic behavior as measured by ^{31}P-NMR showed fast elimination of the parent compound and metabolites, especially via the urine. Proliferation in target organs for chemotherapeutic treatment was strongly inhibited by β-D-Glc-IPM as found in experiments on the incorporation of [methyl-^{3}H]-thymidine. Whole-body autoradiography experiments with β-D-Glc-IPM that had been ^{14}C-labeled in the ifosfamide part showed that the substance crossed the blood-brain and the blood-testes barrier. Since the identical compound coupled to L-glucose was not capable of crossing the blood-brain barrier, we assume that the transport of β-D-Glc-IPM proceeds via glucose or glucose-related transporters.

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