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Pharmacokinetics of Repinotan in Healthy and Brain Injured Animals

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ABSTRACT: Repinotan hydrochloride (repinotan) is a highly potent and selective 5-HT_{1A} full receptor agonist. The ability of repinotan to cross the blood–brain barrier (BBB) and penetrate into rat brain tissue was investigated, because rapid penetration into brain tissue is thought to be essential for neuroprotective efficacy. Intravenous (i.v.) repinotan was rapidly distributed into brain, and the distribution equilibrium between blood and brain was reached immediately after the start of infusion. Free concentrations of repinotan were identical in brain and plasma, indicating that repinotan crosses the BBB freely in both directions with diffusion as a driving force. The brain concentration of repinotan was determined by the free plasma concentration. Thus, the total plasma concentration of repinotan (sum of bound and unbound compound) is only indicative for the brain concentration as long as the unbound fraction remains constant. Metabolites of repinotan do not penetrate the BBB and are retained in the perfusing blood due to their increased polarity. The penetration of [^{14}C] repinotan into ischemic areas of the brain was dependent on time. In studies using injured animals (pMCAO), high levels of [^{14}C] repinotan could be detected in ischemic areas when the compound was administered up to 5 h post injury. [^{14}C] repinotan radioactivity could no longer be detected in ischemic areas when administered 18 h after pMCA-O. After the end of infusion, repinotan was rapidly and completely eliminated from rat brains. Elimination occurred in parallel from plasma and brain with half-lives of about 1 h. In conclusion, repinotan rapidly and to a considerable extent penetrates into brain tissue of healthy and injured animals. Copyright © 2005 John Wiley & Sons, Ltd.

Key words: repinotan; pharmacokinetics; brain penetration; rat

Introduction

Repinotan hydrochloride (repinotan) is a highly potent and selective 5-HT_{1A} full receptor agonist [1]. Repinotan induced a long lasting but reversible inhibition of neuronal firing *in vitro* [2] and *in vivo* [3], demonstrating the hyperpolarizing properties of repinotan. *In vivo*, repinotan displayed neuroprotective efficacy in animal models of stroke [4,5], transient ischemia [2] and traumatic brain injury (TBI) [6,7], and repinotan reduced ischemia-induced glutamate release *in vivo* [8]. Additional investigations demonstrated that repinotan was effective even when intravenous (i.v.) administration was delayed 5 h [9].

Drug delivery to the brain poses unique challenges. Specialized anatomical and physiological features of the brain vasculature and cerebral tissue fluids result in barriers, which

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restrict the delivery of a wide range of possible therapeutic agents. Following acute stroke and trauma, a disruption of the blood–brain barrier (BBB) occurs [10–12], and it is assumed that all compounds can penetrate the BBB into brain tissue. However, the situation is more complex because alterations in BBB permeability after acute injury can follow a heterogeneous spatial and temporal pattern. Biphasic openings of the BBB are common in cerebral ischemia, and the precise timing depends on the severity and duration of the ischemic insult [13]. At least one study suggests that the ability of drugs to penetrate the BBB in ischemic brain is not assured [14]. As the degree of ischemic BBB disruption is variable, complete penetration of a drug through the BBB is not guaranteed. The present study investigated the ability of repinotan to cross the BBB and penetrate into rat brain tissue in healthy and brain injured animal.

Methods

Chemicals and reagents

Repinotan (R-(-)-2-{4-[(chromane-2-ylmethyl)-amino]-butyl}-1,1-dioxo-benzo[d]-isothiazolone hydrochloride) and [^{14}C] repinotan were synthesized by Bayer Health Care AG, Wuppertal, Germany. Repinotan was labeled uniformly with ^{14}C in the chromane system [15]. The chosen labeling position was shown to be metabolically stable. The [^{14}C]-labeled compound had a specific activity of 3.71 MBq/mg and a radiochemical purity of >97%. All chemicals used for the preparation of biological samples and for analysis were of highest commercially available purity.

Experimental animals

Male Wistar rats with body weights of about 200 g were used for most of the studies. The animals were purchased from Harlan Winkelmann, Borchen, Germany. Nagase analbuminemic rats were used for pharmacokinetic studies to characterize the blood/brain penetration in dependence on the extent of the free fraction of repinotan in plasma. The animals were obtained from the same supplier as the Wistar rats. Male Long Evans rats were purchased from Mollegard, Denmark for whole-body autoradiography study of time-dependent distribution of radioactivity into ischemic brain areas. All experimental animals received solid feed (Altromin® 1324, Altrogge, Lage/Lippe, Germany) as restricted feeding (15 g per rat and day). Animals were kept in special restraining cages for the infusion period and housed in Macrolon® cages until killing.

Dosage and administration of repinotan

Repinotan or [^{14}C] repinotan was dissolved in buffered physiological saline at specified concentrations and the pH was adjusted to 5. The test compound was stable in the dosage form for at least 4 h as tested by HPLC. Intravenous bolus doses (<30 s) were injected by puncture of a lateral tail vein. A cannula (Introcan®, 22G1, Braun, Melsungen, Germany) was placed in a lateral tail vein for i.v. infusion with a Harvard pump (Technical and Scientific Equipment, Bad Homburg, Germany).

Measurement of [^{14}C] radioactivity

Radioactivity was measured in the biological samples by liquid scintillation counting (LSC). This analytical technique does not distinguish between parent compound and radioactive metabolites. Plasma was pipetted directly into the scintillation cocktail. No further sample preparation was necessary for radioanalysis. Brain tissue was lyophilized and homogenized. Radioactivity was measured in the biological samples after combustion and collection of $^{14}CO_2$. The concentration of radioactivity was calculated as mg repinotan HCl equivalents/l.

Determination of repinotan

The determination of unchanged repinotan was carried out in the biological samples after addition of an internal standard and liquid/liquid extraction with diethyl ether. The ether extract was evaporated to dryness and the residue was taken up in a mixture of acetonitrile and ammonium acetate buffer pH 3. The analysis was performed by high pressure liquid chromatography (HPLC) coupled to an atmospheric pressure ionization/tandem mass spectrometer (LC-MS/MS) via the heated nebulizer interface

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(SCIEX). The limit of quantification was 0.1 ng/ml with a linear working range between 0.1 ng/ml and 80 ng/ml. The accuracy (deviation from nominal concentrations) ranged between −8.2% and +8.6%. The precision (relative standard deviation) was between 2.6% and 18.6%. Both, accuracy and precision were calculated from QC-samples employed in three validation runs on three different days.

Blood brain penetration of repinotan/[^{14}C]repinotan radioactivity

Male Wistar rats received a 2 h i.v. infusion of either repinotan or [^{14}C] repinotan at a rate of 5 mg/kg · h. At fixed time points during and after the infusion (0.25 0.5, 1, 2, 2.25, 3, 4, 6, 7, 9 and 26 h after start of infusion), groups of animals (n=4) were killed by heart puncture under ether anesthesia. Blood was collected and plasma was prepared by centrifugation. During subsequent preparation, the brain (cerebrum and cerebellum) was removed, and the sample material was used for analysis of unchanged repinotan by LC–MS/MS or for measurement of radioactivity by LSC. The concentrations of unchanged repinotan or radioactivity in plasma and brain were determined in separate studies. Thus, the concentration vs time data for radioactivity and unchanged substance represent an interindividual and not an intraindividual comparison.

Free blood and brain concentrations

Implantation of the dialysis probe into the jugular vein. In preparation for the study, dialysis probes were implanted into the jugular vein and into the brain of Wistar rats 1 day before the administration of repinotan. Rats were anesthetized with chloral hydrate (intraperitoneal administration of 80 mg/kg; 5 ml/kg, Fluka Chemie GmbH, Deisenhofen, Germany). A small incision through the skin was made at the back of the neck and on the right shoulder. The jugular vein was exposed and a small nick made in the vein. The microdialysis probe (CMA 20, CMA Microdialysis AB, Solna, Sweden) was inserted through this nick, threaded through the vein to a region near the heart (~2.5 cm) and fixed with a silk suture. The jugular vein was then ligated, the inlet and outlet tubing of the dialysis probe were threaded under the skin and out the incision on the back of the neck, and the rat was attached to the cage.

Implantation of the dialysis probe into the brain. After implantation of CMA 20 probes into the vein, the animals were placed in a stereotaxic frame. After exposing the skull, holes were drilled (interaural line coordinates: frontal +9.2, lateral −2.8, horizontal +6.5, according to Paxinos and Watson [16], allowing implantation of the guide cannula (CMA Microdialysis AB, Solna, Sweden) into the striatum. The cannula was secured with dental cement (Palavitt 55, Kulzer, Wehreim, Germany) and two anchoring screws on the top of the skull. After surgery, rats were housed individually in cages and given free access to food and water.

Performance of microdialysis perfusion experiments All microdialysis perfusion experiments commenced 20–24 h after surgery with awake rats. A microdialysis probe (CMA 10, CMA Microdialysis AB, Solna, Sweden) was inserted into the brain through the implanted guide cannula. The microdialysis probes placed in the jugular vein and brain were connected to a CMA/100 microinjection pump (CMA Microdialysis AB, Solna, Sweden) by means of polyethylene tubing and to a CMA/140 microfraction collector (CMA Microdialysis AB, Solna, Sweden). Flow was set to 2 µl/min, both in brain and blood. The dialysis fibers were perfused with Krebs-Ringer solution consisting of 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 10 mM glucose, 25 mM $NaHCO_3$, pH 7.4, 0.5% BSA. Repinotan was infused at a rate of 5 mg/kg·h i.v. for 3.5 h. Microdialysis samples were collected at 15 min intervals from the microdialysis probes in the jugular vein and in the brain during and after the end of i.v. infusion. Free concentrations of repinotan were determined in the samples by LC/MSMS.

Determination of relative recovery response for microdialysis probes. The relative recovery response, defined as the ratio between the concentration of drug in the dialysate to that in the environment outside the dialysis membrane (i.e. the extracellular space), was individually characterized *in vitro* for each microdialysis

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probe used in the animal experiments. At least six samples were collected over 15 min intervals from reservoir standards containing 1 or 2 μg/ml repinotan prepared in either rat blood (CMA 20) or in normal saline (CMA 10). The ratio of the concentration determined in the dialysate to that remaining in the reservoir sample was used as a measure of relative recovery. The relative recoveries were used to calculate free concentrations of repinotan in blood and brain using the measured dialysate concentrations from *in vivo* sampling.

Influence of the free fraction on blood–brain barrier penetration

The influence of the plasma repinotan free fraction (= fraction unbound to plasma proteins) on blood brain penetration was assessed in a study with Nagase analbuminemic rats (NA-rats). Nagase analbuminemic rats are a mutant strain of Sprague-Dawley rats, which are deficient in serum albumin, but exhibit normal concentrations of alpha-1-acid glycoprotein. The extent of protein binding of unchanged repinotan was determined in plasma of NA-rats and Wistar rats *in vitro* by ultrafiltration method prior to the infusion experiments. In the main experiment repinotan was infused at a rate of 5 mg/kg · h i.v. for 2 h to Wistar and NA-rats. At the end of infusion groups of animals ($n = 4$) were killed by heart puncture under ether anesthesia. Plasma and brain tissue samples were collected. After appropriate preparation, the sample material was used for analysis of unchanged repinotan by LC–MS/MS.

Distribution of [^{14}C]repinotan radioactivity into ischemic rat brain

Permanent middle cerebral artery occlusion (pMCA-O). Under general anesthesia (Forene®, Abbott GmbH, Wiesbaden, Germany or Isoflurane-Baxter, Baxter Deutschland GmbH, Unterschleißheim, Germany mixed with ≅28% O_2 in N_2O to 5%–1% v/v concentration) the MCA was occluded unilaterally based on the surgical procedure described by [17]. The left temporal-parietal region of the head was shaved, and the skin was disinfected and opened between the orbit and the external ear canal. Following a midline incision, the temporal muscle was divided and pulled aside with surgical hooks to make the lateral aspect of the skull free. The facial nerve, major facial arteries and veins, the lateral eye muscles, the intra- and extraorbital lacrimal glands and the zygomatic bone were left intact. Under an operating microscope a small burr hole was drilled directly under the zygomatic arc, 1–2 mm rostrally to its caudal origin from the aquamosal bone. After careful opening of the dura, the exposed MCA and its branches were permanently occluded between the olfactory tract and the inferior cerebral vein proximal to the lenticulostriate branch by microbipolar electrocoagulation (Bipolator 50, Fischer MET GmbH, or Bipol 50, Stockert GmbH, Freiburg, Germany). To avoid recanalization, the occluded vessels were cut and removed. Muscle and skin wounds were closed with surgical suture or with tissue glue (Histoacryl, B. Braun Surgical GmbH, Melsungen, Germany). During surgery and subsequent drug infusion the body temperature was monitored using a rectal temperature probe and maintained between 36.5°C and 37.5°C with a heating pad.

Performance of the animal experiment. At different time points (control without MCA-O, 13 min, 5 h, 18 h) after unilateral occlusion of the MCA and its branches according to [17] [^{14}C] repinotan was administered to male Long Evans rats as a single i.v. bolus dose of 1.0 mg/kg body weight. Thirty minutes after administration of the radiolabeled test compound the animals were killed. The distribution of [^{14}C] repinotan radioactivity in the brain was evaluated with whole-body autoradiography.

Detection of radioactivity by whole-body autoradiography. Whole-body autoradiography was carried out according to the procedure originally described [18,19]. The detection method was radio-luminography [20,21]. With this technique it is not possible to differentiate between parent compound and radioactive metabolites. The animals were killed by CO_2-inhalation, deep-frozen (−70°C), and embedded in carboxymethyl cellulose. Sagittal whole-body sections of 50 μm thickness were cut at −25°C using a cryomicrotome (PMV 450) and freeze-dried for at least 24 h. Dry whole-body sections as a whole or as separated head areas were exposed to Imaging Plates (BAS III®, Fuji Photo Film Ltd., Tokyo) for

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4 h in a shielding box to detect the distribution of radioactivity. The Imaging Plates were scanned with the Laser Scanner BAS 2000 (Fuji Photo Film Ltd., Tokyo). Evaluation of the distribution pattern was performed qualitatively, i.e. visual ranking of the radioactive concentrations by using colored gradation bars. Radio-luminographic intensities can be compared between different animals or sections, since radio-doses, detection processes and display presettings of images were kept constant.

Pharmacokinetic evaluation

The pharmacokinetic parameters for unchanged substance and for radioactivity (unchanged substance and radioactive metabolites) for the plasma and brain were calculated with non-compartmental analysis using KINCALC Vers. 2.33 software, 1994 [22].

Pharmacokinetic parameters were derived from the area under the plasma concentration vs time curve (*AUC*) and the area under the first statistical moment curve (*AUMC*) [22,23]. Both *AUC* and *AUMC* were calculated as the sum of partial areas. The partial areas were calculated with the linear (if $C_{i+1}>C_i$) and the logarithmic trapezoidal rule (if $C_{i+1}\leqslant C_i$). The *AUMC* was calculated according to the formulae published by Charter [24]. The extrapolated portion of the *AUC* from the last measured data point $C(t_n)$ to infinity was calculated using the concentration $C'(t_n)$ which was calculated from the log-linear regression line through the last points of measurement instead of $C(t_n)$.

The symbols used for the pharmacokinetics parameters are in accordance with ACCP Nomenclature Recommendations [25]. Pharmacokinetic parameters and concentration values are given as geometric means ($\bar{x}_g$) and geometric standard deviations (s_g). The 1 s interval ranges from $\bar{x}_g/s_g$ to $\bar{x}_g \cdot s_g$).

Results

Penetration into brain and elimination from brain

Repinotan and radioactivity (parent compound and radioactive metabolites) were determined in plasma and brain tissue of Wistar rats at different time points during and after a 2 h constant rate i.v. infusion of [^{14}C] repinotan or repinotan at 5 mg/kg·h. Repinotan was distributed rapidly and to a considerable extent from blood into brain. The ratio of brain:plasma concentrations remained constant from the start of the infusion until the end of the infusion. This indicates that the distribution equilibrium was reached immediately after the start of the infusion. The total concentration of unchanged repinotan (sum of repinotan bound and unbound to proteins) was higher in brain tissue than in plasma with the exception of the last sample collection time (Figure 1). The *AUC* of parent compound was 1.57 times higher in brain than in plasma (Table 1).

The *AUC* calculated for unchanged repinotan (6.58 mg·h/l) and for radioactivity (5.36 mg-eq.h/l) were similar in the brain. The pharmacokinetic parameters were derived from two different experiments and thus, represent an inter-individual comparison. This might explain slight differences in the *AUC* values from the two studies. In plasma, however, the equivalent concentrations of radioactivity and of repinotan began to diverge increasingly after the start of the

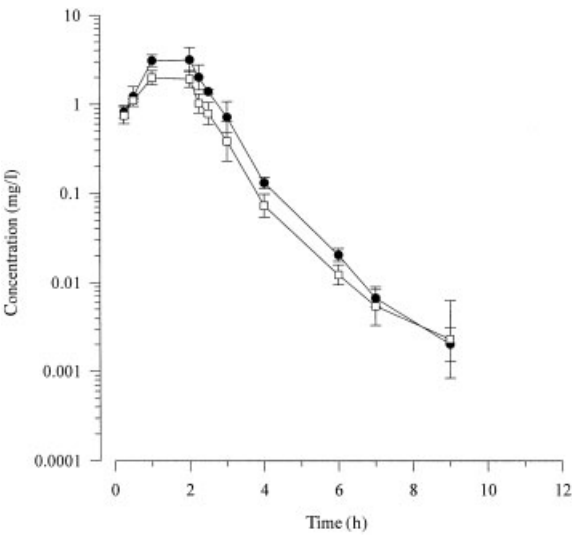

Figure 1. Concentration vs time curves of unchanged repinotan in brain (●) and plasma (□) of Wistar rats after i.v. infusion of repinotan at 5 mg/kg·h for 2 h. Geometric mean of 4 animals per time point

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Table 1. Pharmacokinetic parameters derived from concentration vs time data of radioactivity and of unchanged repinotan in plasma and brain of Wistar rats after intravenous infusion of [^{14}C]repinotan /repinotan at 5 mg/kg.h for 2 h (total dose: 10 mg/kg). Values are the geometric mean of 4 animals per time point

		Plasma		Brain	
		Radioactivity	Repinotan Unchanged	Radioactivity	Repinotan Unchanged
AUC	[mg · h/l][a]	7.85	4.20	5.36	6.58
C_{max}	[mg/l][a]	2.18	1.98	2.41	3.14
t_{max}	[h]	2.00	1.00	2.00	2.00
$t_{1/2}$	[h]	n.c.	n.c.	0.58	0.55
Points[b]				5	6
Interval[b]	[h]			2–4	2–6
$t_{1/2}$	[h]	39.7	1.28	5.14	1.16
Points[b]	Term.	2	3	2	2
Interval[b]	[h]	26–50	6–9	6–9	7–9

n.c., not calculated.
[a] mg-eq in the case of [^{14}C]-studies.
[b] Used for regression to determine $t_{1/2}$.

i.v. infusion. This reflects progressive biotransformation of repinotan with the formation of radioactive metabolites. The radioactivity concentration in plasma reflects repinotan but also various metabolites. The corresponding radioactivity *AUC* (7.85 mg-eq·h/l) was significantly higher in comparison with the *AUC* of repinotan (4.20 mg·h/l) (Table 1).

The known main metabolites of [^{14}C] repinotan have increased polarity in comparison with the parent compound. Therefore, they are unable to diffuse or diffuse only to a small extent through the BBB [26]. This indicates that almost exclusively unchanged repinotan penetrated the BBB, whereas the metabolites of repinotan were retained in the perfusing blood due to their increased polarity and molecular weight [26].

After completion of the repinotan/[^{14}C] repinotan infusion, repinotan and radioactivity were very rapidly eliminated from brain and plasma. The elimination half-life of radioactivity in the brain ($t_{1/2}$: 0.58 h in the interval 2–4 h after the start of the infusion) was the same as for repinotan ($t_{1/2}$: 0.55 h in the interval 4–6 h after the start of the infusion) in comparable time intervals. The terminal elimination half-lives in the brain were 1.2 h for unchanged repinotan and 5.1 h for radioactivity. However, the terminal half-life of radioactivity was no longer exclusively determined by the diffusion of repinotan and radioactive metabolites from brain tissue back into the circulating blood. Rather the terminal half-life mainly describes the elimination of radioactivity from the residual blood contained in the cerebral vessels.

Free blood and brain concentrations

The mean steady-state concentration of unbound repinotan ($C_{max,u}$) was 0.030 mg/l in blood and 0.0239 mg/l in brain after a constant rate i.v. infusion of repinotan at 5 mg/kg·h for 3.5 h. The corresponding unbound *AUC* (AUC_u) was 0.129 mg · h/l and 0.124 mg · h/l, respectively, for blood and brain. Elimination half-lives of 1.22 h and 1.32 h were calculated for blood and brain in the time interval from 3.5 h to 7 h after the start of infusion (Table 2, Figure 2). The similar concentration vs time profiles, i.e. the equal $C_{ss,u}$, AUC_u and half-lives of unbound repinotan in blood and brain, indicate that there is no active transport of repinotan into or out of the brain. Repinotan can freely diffuse across the BBB in both directions.

Influence of the free fraction on blood-brain barrier penetration

The free fraction of repinotan (=fraction unbound, f_u) was significantly higher in the plasma

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Table 2. Pharmacokinetic parameters derived from the unbound concentration vs time data of unchanged repinotan in plasma and brain of Wistar rats after i.v. infusion of repinotan at 5 mg/kg · h for 3.5 h (total dose: 17.5 mg/kg). Values are the geometric mean of 4 animals per time point

		Blood	Brain
AUC_u	[mg · h/l]	0.129	0.124
$C_{max,u}$	[mg/l]	0.0300	0.0239
$t_{1/2}$	[h]	1.22	1.32
Points[a]	Term.	15	15
Interval[a]	[h]	3.5–7	3.5–7

[a] Used for regression to determine $t_{1/2}$.

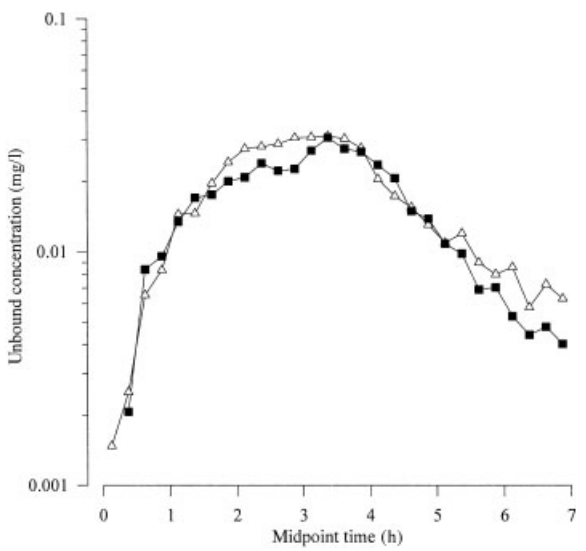

Figure 2. Free concentration vs time curves of unchanged repinotan in brain (■) and blood (△) of Wistar rats after i.v. infusion of repinotan at 5 mg/kg·h for 3.5 h. Values are the geometric mean of 4 animals per time point

of Nagase analbuminemic rats (NA-rats) than in Wistar rats. *In vitro*, the f_u of parent compound was more than 2 times higher in NA-rats in comparison with Wistar rats (Table 3). At a plasma concentration of 1 mg/l repinotan, the f_u was 6.1% in NA-rats compared with 3.15% in Wistar rats. In NA-rats the f_u was 17.0% and in Wistar rats 7.68% at plasma concentrations of 10 mg/l. The increase in the f_u at the higher concentration level was obviously the result of saturation of the binding sites at alpha-1-acid glycoprotein.

Table 3. Protein binding of unchanged repinotan in plasma of NA-rats and Wistar rats determined *in vitro* by ultrafiltration

NA-rat		Wistar rat	
C [mg/l]	f_u [%]	C [mg/l]	f_u [%]
1.01	6.1	1.03	3.15
11.2	17.0	10.3	7.68

Table 4. Unchanged repinotan concentrations [mg/l] in plasma and brain of NA-rats and Wistar rats after i.v. infusion of repinotan at 5 mg/kg for 2 h (total dose: 10 mg/kg). Time point: 2 h after start of infusion (C_{max}). Values are the geometric mean of 4 animals per time point

Organ/Tissue	NA-rat	Wistar rat	Ratio NA/Wistar
Plasma	0.839	1.92	0.44
Plasma, unbound conc.[a]	0.0512	0.0605	0.85
Brain	3.24	3.14	1.03
Ratio, brain/total plasma	3.86	1.63	
Ratio, brain/unbound plasma	63.3	51.9	

[a] Plasma concentration was multiplied with f_u determined *in vitro* at 1 mg/l.

Brain:plasma ratios for the total concentration (sum of bound and unbound compound) of repinotan (Table 4) were 3.86 in NA-rats vs 1.63 in Wistar rats. Thus, the higher the f_u in plasma the higher the ratio brain:plasma concentration. However, the corresponding ratios were similar in NA- and Wistar rats if only the free concentrations of repinotan were considered in plasma and related to total brain tissue concentrations (Table 4). The ratios of brain:unbound plasma were 63.3 in NA-rats and 51.9 in Wistar rats. This shows that the brain concentration is determined by the free plasma concentration of repinotan.

Penetration across the injured BBB

The control animal (without MCA-O) showed high affinity and a homogeneous distribution pattern of radioactivity throughout the brain. The autoradiograms of the rat brain did not distinguish from the control animals when [^{14}C] repinotan was administered either 13 min or 5 h after MCA-O (Figure 3). However, if the radiolabeled test compound was administered as late as 18 h after MCA-O, in the ischemic areas of the

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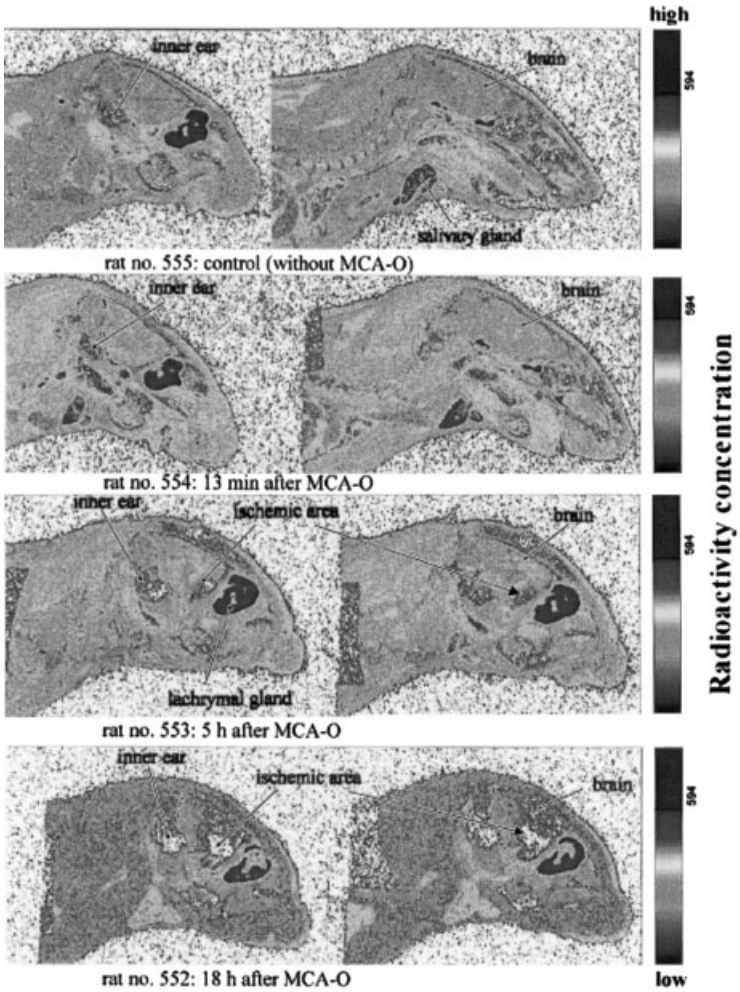

Figure 3. Distribution of [^{14}C] repinotan in rat brain with impaired BBB. [^{14}C] repinotan was administered at different times after permanent occlusion of the MCA (13 min, 5 h and 18 h) and the distribution pattern in brain was compared with control animals with intact BBB. Distribution of radioactivity in the rat brain was determined 30 min after i.v. bolus administration 1 mg/kg [^{14}C]repinotan

rat brains virtually no significant radioactivity penetration had occurred (Figure 3). This indicates that at 18 h after MCA-O, [^{14}C] repinotan radioactivity was no longer able to penetrate into the ischemic areas of the rat brain to any extent.

Discussion

Rapid penetration into brain is thought to be essential for the therapeutic efficacy of a neuroprotectant. Therefore, the ability of repinotan to cross the BBB and to penetrate into brain tissue was investigated in healthy and injured animals using different rat strains and experimental techniques. In healthy animals, repinotan penetrated the intact BBB rapidly and extensively. Several studies with i.v. infusion of either radiolabeled [^{14}C] repinotan or non-labeled repinotan demonstrated that the distribution equilibrium between the plasma and brain was reached almost immediately after the start of infusion. The total concentration of unchanged repinotan (sum of repinotan bound and unbound to proteins) was even higher in brain tissue than

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in plasma. The *AUC* of parent compound in brain was 1.57 times greater than in plasma. The concentration vs time profiles and the *AUC* calculated for unchanged repinotan and for radioactivity were in the same order of magnitude in the brain. This means that the radioactivity in brain consists almost exclusively of unchanged repinotan. In plasma, however, the equivalent concentration of radioactivity and the corresponding radioactivity *AUC* were significantly higher in comparison with the parent compound. The concentration vs time curves of the parent compound and radioactivity diverged progressively after the start of the i.v. infusion of [^{14}C] repinotan or repinotan. This was due to biotransformation of the parent compound with formation of radioactive metabolites. The plasma radioactivity represented not only unchanged repinotan but also numerous radioactive metabolites. This indicates that almost exclusively unchanged repinotan penetrated the BBB, whereas the known metabolites of repinotan were retained in the perfusing blood due to their increased polarity [26].

In most pharmacokinetic studies total drug concentrations (bound and unbound to proteins) are determined in the plasma and the target organ. However, it is believed that only the free fraction (=fraction unbound, f_u) of the drug produces the pharmacological effect in the target organ [27]. Therefore, the pharmacokinetics of the free fraction of repinotan were investigated in blood and brain in more detail. The free concentrations of repinotan were measured in rat blood and brain by the microdialysis method during and after constant rate i.v. infusion of repinotan. The concentration vs time profiles of unbound repinotan were almost identical in blood and brain. This indicates that there is no active transport of repinotan across the BBB. Unbound repinotan penetrates the BBB freely with diffusion as the driving force.

The influence of the free fraction of repinotan in plasma on the blood–brain penetration was addressed in a study with Nagase analbuminemic rats (NA-rats). NA-rats are deficient in albumin, the most important binding site of repinotan. *In vitro*, the free fraction of repinotan was 2 times higher in plasma of NA-rats than in Wistar rats. The brain:plasma ratios were also quite different in NA- and Wistar rats for the total concentration of repinotan. The higher the f_u in plasma the higher the ratio of brain:plasma levels. In contrast, the corresponding ratios were very similar in NA- and Wistar rats if only the free concentration of repinotan in plasma was related to total brain tissue concentration. This shows that the brain concentration is determined by the free plasma concentration of repinotan.

The total plasma concentration of repinotan is only representative of the brain concentration as long as the f_u remains constant. Any increase in the plasma f_u of repinotan either due to changes in the plasma protein composition or due to saturation of plasma protein binding sites is coincident with a change in the brain:plasma concentration ratio. Under these circumstances the brain concentration of repinotan increases more than proportionally in comparison with the plasma concentration. However, the unbound concentration of repinotan in plasma is predictive of the unbound and the total brain concentration of the compound, since the ratio unbound plasma:brain concentration was shown to be a constant.

Repinotan was rapidly eliminated from the rat brain and plasma. The elimination half-lives were similar for plasma and brain with 1.3 and 1.2 h after the end of the i.v. infusion.

In studies using injured animals (pMCAO), high levels of [^{14}C] repinotan radioactivity could be detected in the ischemic area when repinotan was administered immediately or up to at least 5 h post injury. This shows that repinotan penetrated well into the ischemic areas of the rat brain. However, penetration into ischemic areas was dependent on time. [^{14}C] repinotan radioactivity could not longer be detected in ischemic areas when administered 18 h after pMCA-O.

In conclusion, these results show that repinotan rapidly and to a considerable extent penetrates into brain tissue in healthy and injured animals.

References

1. De Vry J, Schoe-Loop R, Heine H-G *et al*. Characterization of the aminomethylchroman derivate BAY × 3702 as a

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highly potent 5-hydroxytryptamine$_{1A}$ receptor agonist. *J Pharm Exp Ther* 1998; **284**: 1082–1094.
2. De Vry J, Dietrich H, Glaser T *et al*. BAY × 3702: *Drugs of the Future* 1997; **22**: 341–349.
3. Casanovas JM, Berton O, Celada P, Artigas F. *In vivo* actions of the selective 5-HT$_{1A}$ receptor agonist BAY × 3702 on serotonergic cell firing and release. *Naunyn Schmiedebergs Arch Pharmacol* 2000; **362**: 248–254.
4. Horváth E, Augstein K-H, Wittka R. Neuroprotective effect of the novel 5-HT$_{1A}$ receptor agonist BAY x 3702 in a rat model of permanent focal cerebral ischemia and traumatic brain injury. *Soc Neurosci Abstr* 1997; **23**: 1923.
5. Semkova P, Wolz J, Krieglstein J. Neuroprotective effect of 5-HT$_{1A}$ receptor agonist, BAY × 3702, demonstrated *in vitro* and *in vivo*. *Eur J Pharmacol* 1998; **359**: 251–260.
6. Alessandri B, Tsuchida E, Bullock RM. The neuroprotective effect of a new serotonin receptor agonist, BAY × 3702, upon focal ischemic brain damage caused by acute subdural hematoma in the rat. *Brain Res* 1999; **845**: 232–235.
7. Horváth E, Augstein K-H. Neuroprotection by the noval 5-HT$_{1A}$ receptor agonist BAY × 3702 in the rat model of acute subdural hematoma. *J Neurotrauma* 1997; **14**: 800.
8. Mauler F, Fahrig T, Horváth E, Jork R. Inhibition of evoked glutamate release by the neuroprotective 5-HT$_{1A}$ receptor agonist BAY × 3702 *in vitro* and *in vivo*. *Brain Res* 2001; **888**: 150–157.
9. Mauler F, Horváth E. Neuroprotective efficacy of repinotan HCl, a 5-HT$_{1A}$ receptor agonist, in animal models of stroke and traumatic brain injury. *J Cereb Blood Flow Metab* 2005; **25**(4): 451–459.
10. Cervos-Navarro J, Lafuente JV. Traumatic brain injuries: structural changes. *J Neurol Sci* 1991; **103**Suppl: S3–S14.
11. Hossmann KA. Pathophysiology of experimental brain edema. *Neurosurg Rev* 1989; **12**: 263–280.
12. Rosenberg GA. Ischemic brain edema. *Prog Cardiovasc Dis* 1999; **42**: 209–216.
13. Kuroiwa T, Ting P, Martinez H, Klatzo I. The biphasic opening of the blood–brain barrier to proteins following temporary middle cerebral artery occlusion. *Acta Neuropathol* 1985; **68**: 122–129.
14. Lo EH, Pan Y, Matsumoto K, Kowall NW. Blood–brain barrier disruption in experimental focal ischemia: comparison between *in vivo* MRI and immunocytochemistry. *Magn Res Imaging* 1994; **12**: 403–411.
15. Seidel D, Conrad M, Schoof Y, Schohe-Loop R. Synthesis of [^{14}C]-labelled repinotan hydrochloride and ist major metabolite M-6. *J Label Compd Radiopharm* 2002; **45**: 1115–1132.
16. Paxinos G, Watson C. *The Rat Brain in Stereotaxtic Coordinates*. Academic Press: Sydney, New York, London, San Diego, San Francisco, Sao Paolo, Tokyo, Toronto, 3rd edn, 1996.
17. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: Evaluation of the model and development of a neurologic examination. *Stroke* 1986; **17**: 472–476.
18. Ullberg S. Studies on the distribution and fate of ^{35}S-labelled benzyl-penicillin in the body. *Acta Radiol Suppl* 1954; **118**: 22–30.
19. Curtis CG, Cross SAM, McCulloch RJ, Powel GM. *Whole-body Autoradiography*. Academic Press: London, New York, 1981.
20. Hamaoka T. Autoradiography on new era replacing x-ray film. Bio-Imaging Analyser BAS 2000. *Coll Technol* 1990; **9**: 456–462.
21. Ahr HJ, Steinke W. Imaging plate: Validation and first experiences with quantitative studies in whole-body autoradiography during drug development. *Xenobiotic Metab Dispos* 1994; **9**: 371–378.
22. Yamaoka K, Nakagawa T, Uno T. Statistical moments in pharmacokinetics. *J Pharmacokinet Biopharm* 1978; **6**: 547–558.
23. Benet LZ, Galeazzi RL. Non-compartmental determination of the steady-state volume of distribution. *J Pharm Sci* 1979; **68**: 1071–1074.
24. Charter MK. The estimation of moments: A technical note. *J Pharmacokin Biopharm* 1989; **17**: 203–208.
25. Allen L, Kimura K, McKichan J, Ritschel WA. Manual of symbols, equations and definitions in pharmacokinetics. *J Clin Pharmacol* 1982; **22**: 1S–23S.
26. Pardridge WM. Transport of small molecules through the blood–brain barrier: biology and methodology. *Adv Drug Deliv Rev* 1995; **15**: 5–36.
27. Rowland M, Tozer TN. *Clinical Pharmacokinetics – Concepts and Applications*. Williams & Wilkins: Baltimore, Philadelphia, Hong Kong, London, Munich, Sydney, Tokyo, 3rd edn, 1995.

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