Permeation Studies *In Vitro* and *In Vivo* of Potential Radiopharmaceuticals with Affinity to Neuro Receptors

Matthias Friebe,[1] Kayoshi Suda,[1] Hartmut Spies,[2] Rosmarie Syhre,[2] Ralf Berger,[2] Bernd Johannsen,[2] Efstratios Chiotellis,[3] Stefanie D. Kramer,[4] and Heidi Wunderli-Allenspach[1,5]

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Purpose. To check the influence of structural characteristics on their permeation through the blood–brain barrier (BBB), a set of radioactive [^{99m}Tc]chelates bearing amine groups was synthesized and tested *in vitro* as well as *in vivo*.

Methods. Compounds with different log P and pK_a values were obtained by complex forming reactions of [^{99m}Tc]pertechnetate with varying substituents. Transport was studied in rats and mice, as well as in an ECV304 cell culture model.

Results. *In vitro* higher permeation was found for compounds with electron attracting substituents in β-position to the amine group (pK_a values 7.4 to 8.3) than for those with more basic amine groups (pK_a values > 8.9) even for similar log $D_{pH\,7.4}$. *In vivo* brain uptake between 0.8 and 4.8% of the injected dose (ID) per organ was found for the former, whereas <0.4% ID were present for the latter.

Conclusions. Three structurally diverse classes of [^{99m}Tc]chelates showed distinct patterns with regard to brain uptake in vivo and BBB permeability *in vitro* which could not be predicted by their lipophilicity alone. The close correlation between the data from rats and mice and those obtained with cell cultures render the ECV304 cells an attractive model for the screening of new compounds.

KEY WORDS: radioactive tracer molecules; technetium; blood–brain barrier (BBB); permeation; log P; ECV304 cells; brain uptake.

INTRODUCTION

For application in nuclear medicine the γ-emitter ^{99m}Tc has become the nuclide of choice due to its ideal radiation properties and instant availability. Especially Tc-chelates bearing amine groups play an important role in the development of compounds which are able to image specific receptors and transporters. A first *in vivo* image of the dopamine transporter in the human brain was obtained with a ^{99m}Tc compound, TRODAT-1 (1) (Fig. 1). Such metal bearing compounds must be able to pass the intact blood-brain barrier (BBB) in order to reach their targets, however, the polar groups of the ligands, which are necessary for receptor binding, often render passage difficult (2,3). While brain uptake of TRODAT-1 is 0.4% of the injected dose (ID) 10 min post injection (p.i.), brain uptake of other compounds with similar structure is below 0.1% ID, i.e. they are practically excluded from brain entry (1,4). For a rational tracer design a detailed knowledge of the correlation between structure and permeation through the BBB is needed. It is known that the lipophilicity (partition coefficients P and $D_{pH7.4}$, respectively) and the charge of a compound (2,5) strongly influence permeation. Here we report on the synthesis and characterisation of a set of amine group bearing ^{99m}Tc chelates obtained by systematic variation of substituents, which were tested in rats and mice as well as in an ECV304 BBB cell culture model.

MATERIALS AND METHODS

Chemicals

The tertiary aminoethanethiol ligands **a** and **b** (hydrochlorides) as well as the alkyl thioles **o**, **p**, **q**, **s**, **t** (Fig. 2) were purchased from (FLUKA). All other ligands were prepared and characterised as described (6). All chemicals were of analytical grade (p.a.). The pH range of phosphate buffer (PB) 0.01 M containing Na_2HPO_4 and KH_2PO_4 was extended to pH 3 by addition of 0.1 M citric acid and to pH 11 with 0.1 N NaOH. Where indicated PBS was used, i.e. PB 0.01 M at pH 7.4 containing 0.13 M NaCl. [^{99m}Tc]pertechnetate was obtained by a generator "ULTRA-TECHNECOW" (MALLINCKRODT). [^{99g}Tc]pertechnetate was purchased from ISOCOMMERZ (GDR). The Ethylene Cysteinate Dimer (ECD) was obtained as an instant kit (Neurolite) from DUPONT.

Synthesis of Compounds

$^{99m/99g}$Tc compounds on the millimolar level were synthesised in yields between 96 and 99% according to the literature (7) ^{99m}Tc complexes were prepared from [^{99m}Tc]pertechnetate (1.35–2.70 mCi in 0.5–1.0 ml generator eluate) by sequential addition of propylene glycol (400 μl), the monodentate ligand (~0.5 mg in 100 μl of methanol), the tridentate ligand (~0.05 mg in 100 μl of methanol), 50 μl of sodium hydroxide (0.1 N) and 20 μl of a stannous chloride solution (1.0–2.0 mg in 0.1 N HCl) for reduction. Yields were 90 to 95% after heating for 20 min at 45°C (molar concentration 10^{-9}–10^{-12} M).

[^{99m}Tc] compounds were purified by HPLC (model 1020, VIS/UV detector LC290, Perkin Elmer) with a Hypersil-ODS column (250 × 8 mm, 10μm, Knauer). Runs were performed under isocratic conditions with a solvent mixture of methanol/PB (0.01 M, pH 7.4) 80:20 (v/v) and a flow rate of 2 ml/min. The effluent was monitored by UV absorption at 254 nm for Re complexes and γ-detection for [^{99m}Tc] complexes. The compounds are chemically stable for hours after purification in methanol, acetonitrile and dichloromethane as well as in aqueous solutions of pH 3 to 8 and in serum (pH 7.4).

Determination of Lipophilicity and pKa Values

Log $D_{pH\,7.4}$ and pK_a values were determined by HPLC on a Perkin-Elmer system 1020 coupled with a PRP-1 (250 ×

[1] Department of Pharmacy, Biopharmacy, Swiss Federal Institute of Technology, Zurich, Switzerland.
[2] Institute of Bioinorganic and Radiopharmaceutical Chemistry, Research-Center-Rossendorf, Germany.
[3] National Research Center, Athens, Greece.
[4] Centre for Neuroscience Research, King's College London, UK.
[5] To whom correspondence should be addressed at Department of BioSciences, Winterthurerstr. 190, CH-8057 Zurich, Switzerland. (e-mail: wunderli-allenspach@pharma.ethz.ch)

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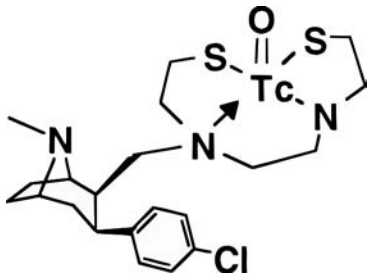


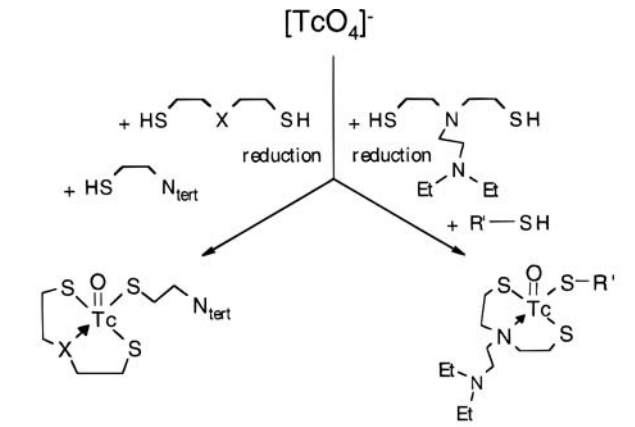
Fig. 1. First [^{99m}Tc]-based imaging agent for the dopamine transporter within the human brain.

4.1 mm; 10 μm; Hamilton) reversed phase column run under isocratic conditions with a flow rate of 1.5 ml/min at room temperature. Volume ratios of 3:1 of acetonitrile and 0.01 M PB pH between 3 and 11 were used as a mobile phase (3,8).

The pK_{HPLC} values were obtained as the fitted points of inflection from the sigmoidal D_{HPLC}/pH profiles. The aqueous ionisation constants pK_a were calculated from the pK_{HPLC} values by correction with a determined correction summand (3,8). Log P values of the neutral compound were estimated from the respective plateau of the sigmoidal log D/pH curve.

Biodistribution Studies

Experiments with animals adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). Swiss albino mice (**7o–7t**)(20 g average weight, 3 to 4 weeks of age) and male Wistar rats (**1a-e–6a-e**) (150–200 g weight, 5 to 6 weeks) were used. Due to the strict regulations in terms of animal use and a limited amenability of rats, uptake studies for complexes **7o–7t** were performed in mice. Complexes **3b**, **3c**, and **5b** were crosschecked in both species. No differences in brain uptake were found. The purified ^{99m}Tc labelled compounds (**7o–7t**) were dissolved in 300 μl of ethanol and these solutions diluted with saline to 3 ml (final activity 40 μCi/ml). Compounds **1a-e–6a-e** were dissolved in saline/HCl (pH 5 to 6) to 14–28 μCi/ml. Portions of 4 μCi (0.1 ml **7o–7u** or 0.2 ml **1a-e–6a-e**) were injected via the tail vein. At least three animals were sacrificed at 2, 5, 10, 30 and 60 min p. i. by heart puncture under ether anaesthesia and brains were isolated for weighing and counting. The total brain radioactivity was expressed as % ID with a standard deviation of ≤10% ($n \geq 3$) for all measured time points.



X / -N_{tert}	S (1)	O (2)	N-Me (3)	N-Et (4)	N-Pr (5)	N-Bu (6)
-NMe_2 (a)	1a	2a	3a	4a	5a	6a
-NEt_2 (b)	1b	2b	3b	4b	5b	6b
-NBu_2 (c)	1c	2c	3c	4c	5c	6c
-piperidine (d)	1d	2d	3d	4d	5d	6d
morpholine (e)	1e	2e	3e	4e	5e	6e

R'	No.
-Me (o)	7o
-Et (p)	7p
-Bu (q)	7q
-Pn (s)	7s
-*c*Hex (t)	7t

Fig. 2. Synthesis and classification of Tc complexes (Me -methyl, Et - ethyl, Pr -propyl, Bu -butyl, Pn -pentyl, *c*Hex -cyclohexyl).

Transport Studies with *In Vitro* Cell Cultures

ECV304 cells (ATCC CRL-1998, passage 149–152) were grown in M199 medium (Sigma) with 10% fetal calf serum (FCS), 10 mM HEPES, and 50 μg/ml penicillin/streptomycin. For transport studies ECV304 cells were cultivated with one part M199 and 1 part supernatant of C6 glioma cells (DKFZ, Heidelberg), grown in the same medium for two days. Cells were seeded on Falcon® inserts with PET membranes (0.4 μm, #3090; Becton Dickinson) in 6-well plates (Falcon® #3502). The transendothelial electrical resistance (TEER) was measured at 37°C with the Millicell-ERS system (MER 000 01, Millipore). Cell cultures were characterised regarding their cytoarchitecture (actin cytoskeleton, nuclei, tight junctions) with a confocal laser scanning microscope (CLSM, Zeiss LSM 410 inverted microscope) as previously described (9). Transport experiments were performed at 37°C in medium without C6 supernatant: 3.1 ml total volume in the donor (insert), and 3.5 ml in the receiver (well) chamber. Tightness of the cell layers was tested with [^{3}H]inulin (0.64 μCi in 10 μl). [^{99m}Tc]-compounds were prepared as described above, the organic solvents were evaporated in an argon stream and the complexes dissolved in M199 containing 10% FCS (final radioactive concentration 27 μCi/ml). 100 μl were added to 3.0 ml medium in the donor chamber. At the times indicated samples (100 μl) were collected from the donor as well as the receiver chamber. For each time point a separate sample well and a separate insert were used. After removal of the samples, inserts with cells were washed with PBS and the cells scraped. Cells and inserts were separately counted in a γ-counter to determine the recovery. After 60 min incubation less than 1% of each compound was detected in the membrane, whereas the cell-associated portion was 2.7% (**3b**, **5b**), 2.9% (**5e**) and 3.8% (**7q**, Tc-ECD), respectively. Permeation (% dose) at the respective incubation times was calculated as follows:

$$\%\ \text{dose in receiver chamber} = \frac{A_{receiver}}{A_{receiver} + A_{donor} + A_{insert}} \quad (1)$$

$A_{receiver}$ and A_{donor} correspond to the total radioactivities in the respective chambers as calculated from the cpm of 100 μl samples and the total volumes of medium in each chamber (A_{insert} see above). To test the stability of the ^{99m}Tc-compounds, complexes were dissolved in M199 containing 10% FCS to

give a final radioactivity of 130 μCi/ml. After incubation for 60 min at 37°C they were analysed by HPLC. Decomposition was <2% for all compounds except ^{99m}Tc -ECD for which about 4% were found.

RESULTS

Preparation and Characterisation of Tc Compounds

The [^{99m}Tc]chelate probes (Fig. 2) were synthesized (pico molar level) and the identity of the purified ^{99m}Tc compounds was determined by comparison of HPLC and TLC chromatograms with the respective patterns of the well characterised Re analogous (Fig. 3) (6). The structures of **1a**, **2d** and **3e** were also confirmed by Extended X-ray Absorption Fine Structure (EXAFS) spectroscopy (data not shown) on $^{99m/99g}$Tc-compounds.

Partition Coefficients and Protonation Constants

The partition coefficient P, i.e. the absolute partition coefficient of the nonionised species, and $D_{pH\ 7.4}$, i.e. the apparent partition coefficient at pH 7.4, as well as the protonation constant pK_a were determined by RP-HPLC (Table I). Three classes can be defined. Complexes with aliphatic substituted amine groups at the monodentate ligand (**1a-d–6a-d**) have log $D_{pH\ 7.4}$ values between −0.30 and 1.56 with pK_a values >8.9 (**class I**). **Class II** complexes have an amine group bearing monodentate ligand with a morpholinyl group (**1e–6e**). They show log $D_{pH\ 7.4}$ values from 0.95 to 1.78 and a pK_a value around 7.4. **Class III** comprises compounds bearing the amine group in the tridentate ligand at the position indicated in Fig. 2 (**7o–7t**), which have log $D_{pH\ 7.4}$ values between 1.63 to 2.76 and a pK_a value around 8.3.

Biodistribution Studies

Brain uptake studies were performed in rats and mice for all complexes shown in Fig. 2. In all cases, highest brain uptake was found at 2 min p.i. At later times amounts decreased due to clearance from the system. Brain uptake data (~2 min p.i.) are presented in Fig. 4, which illustrates the clustering of the three classes regarding *in vivo* brain uptake and log $D_{pH\ 7.4.}$ Complexes of **class I** (**1a-d–6a-d**), which were tested in rats, showed a low initial brain uptake, between 0.1 and 0.5 % ID/

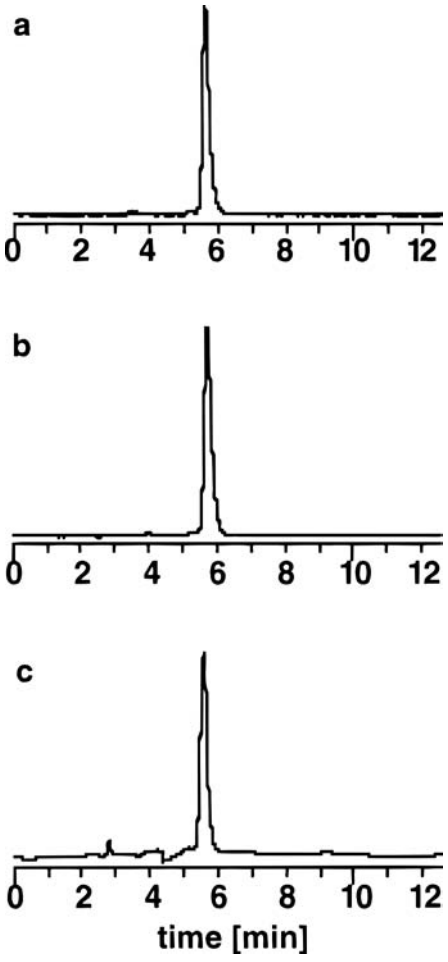


Fig. 3. Comparison by HPLC of compound **3e**: (a) γ detection of [^{99m}Tc]- **3e**; (b) γ detection of [$^{99m/99g}$Tc]- **3e**; (c) UV detection at 254 nm of [$^{185/187}$Re]-**3e** (for details see Methods).

Table I

No.	mw	p*K*$_a$	log $D_{(pH\ 7.4)}$	log P	Class
1a	372	8.87	0.18	1.40	**I**
1b	400	9.51	0.50	2.12	**I**
1c	456	9.91	1.40	3.88	**I**
1d	412	8.99	0.84	2.50	**I**
1e	414	7.20	1.15	1.36	**II**
2a	356	9.05	−0.15	1.32	**I**
2b	384	9.59	0.23	2.08	**I**
2c	440	9.68	1.48	3.94	**I**
2d	396	9.24	0.78	2.58	**I**
2e	398	7.11	1.28	1.46	**II**
3a	369	9.15	−0.30	1.04	**I**
3b	397	9.71	0.00	1.67	**I**
3c	453	9.93	1.00	3.51	**I**
3d	409	9.50	0.65	2.19	**I**
3e	411	7.17	0.95	1.15	**II**
4a	383	8.98	0.15	1.51	**I**
4b	411	9.51	0.40	2.14	**I**
4c	467	—	—	—	—
4d	423	9.42	0.95	2.49	**I**
4e	425	7.17	1.20	1.41	**II**
5a	397	—	—	—	—
5b	425	9.33	0.60	2.46	**I**
5c	481	—	—	—	—
5d	437	9.49	1	2.87	**I**
5e	439	7.57	1.28	1.72	**II**
6a	411	9.15	0.70	2.09	**I**
6b	439	9.76	1.18	2.79	**I**
6c	495	—	—	—	—
6d	451	9.56	1.54	3.20	**I**
6e	453	7.37	1.78	2.05	**II**
7o	397	8.25	1.63	2.72	**III**
7p	411	8.15	1.78	2.96	**III**
7q	439	8.32	2.32	3.64	**III**
7s	453	8.12	2.76	4.01	**III**
7t	465	8.31	2.65	4.08	**III**

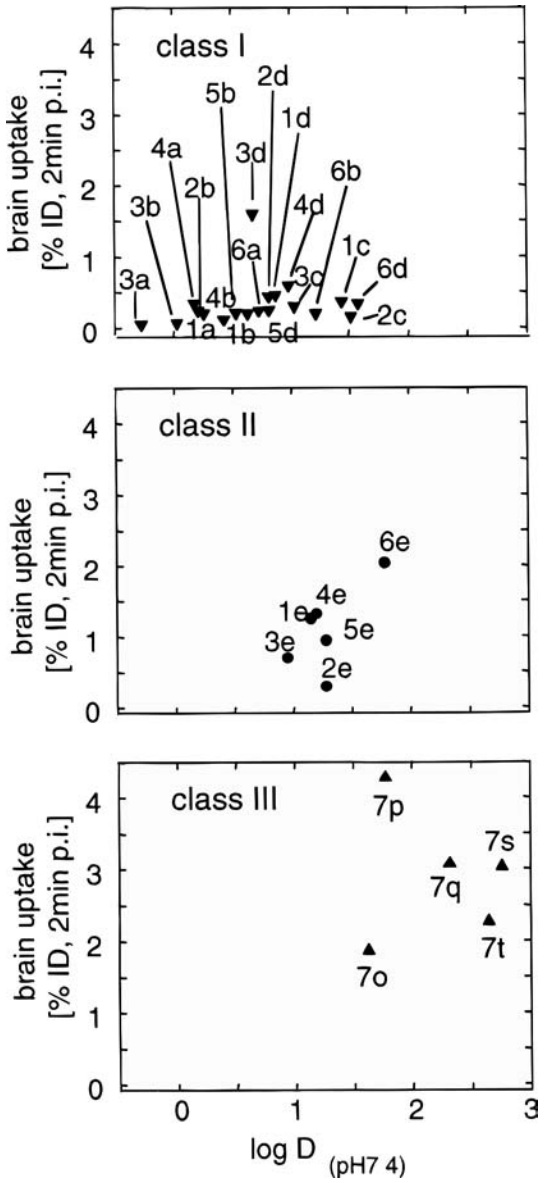


Fig. 4. Influence of structure and lipophilicity $D_{pH\ 7.4}$ and pK_a values on initial brain uptake in mice (**7o**–**7s**) and rats (**1a**–**6e**) (for details see Methods). Standard deviations were ≤10% (n ≥ 3) for all uptake measurements.

organ, with the exception of 3**d**. Although this complex belongs to **class I** it shows a brain uptake of 1.7% ID/organ. For comparison certain compounds of **class I** (complexes **3b, 3c, 5c**) were also studied in mice. Brain uptake in all cases was around 0.2% or even lower, i.e. no significant difference was found between the two species in accordance with the findings for similar Tc-compounds (10). *Class II* compounds (morpholinyl complexes *1e–6e*), which were also tested in rats, showed an initial brain uptake of 0.8–2.0% ID/organ. **Class III** complexes (amine functionality at the tridentate ligand **7o–7t**), which were tested in mice, had very high initial brain uptake values, up to 4.3% ID/organ. In general **class III** compounds with relatively high lipophilicities showed highest brain uptake, followed by **class II** with medium lipophilicity and finally **class I** with lowest lipophilicity showing lowest brain uptake. However, within the single classes no clear relationship between log $D_{pH\ 7.4}$ and *in vivo* brain uptake is visible. Within the investigated structures (**1–6**) of **class I** the lipophilicity follows -NBu_2 > -piperidine > -NEt_2 > -NMe_2. This trend is not fully reflected in brain uptake data where piperidine seems to be the most favourable ligand for structures **1–6**. The lipophilicity of the morpholino compounds (**e**-series, **class II**) increases with increasing length of the C chains at the tertiary amine (**3e–6e**). Again, this is not fully reflected in brain uptake data. Similar rules applied for the compounds of **class III** where the lipophilicity follows -Pn > -cHex > -Bu > -Et > -Me but with a different pattern in brain uptake.

Transport Studies with the ECV304 Cell Culture Model

ECV304 cells grown in the presence of C6 glioma supernatant were used for transport studies on day 14. At this stage TEER is stable around 300 Ωcm^2 and CLSM reveals flat monolayers of cells with a fairly complete tight junction network (Fig. 5). With [^{3}H]inulin the cell layer was shown to be tight, i.e. less than 1% dose permeated up to 60 min. The following selected compounds were tested (Fig. 6): **3b** and **5b** (**class I**), **5e** (**class II**), and 7q (**class III**). For comparison a non-protonable metal bearing compound agent [^{99m}Tc]-Ethylen Cysteinat Dimer (^{99m}Tc-ECD), was used (11). Drug increase in the receiver chamber is approximately linear between 2 and 60 min. Significant differences were found between the tested compounds. Highest permeation, i.e. about 7% dose in 60 min was observed for the ^{99m}Tc -ECD standard. For compound **7q** (**class III**) transport was slightly lower, i.e., 5.9% dose within 60 min, whereas the morpholinyl substituted compound **5e** (**class II**) showed a net transport of about 4.6% dose. Much lower permeation values were found for compounds **3b** and **5b** (**class I**), below 3.8% dose up to 60 min. At 30 min the pattern was the same as at 60 min.

DISCUSSION

Passive diffusion of molecules through the BBB largely depends on their physicochemical characteristics, i.e., the lipophilicity, the polarity, the molecular volume, the H-bonding capacity to name the most important ones (12–14). This is the first time that a set of ^{99m}Tc complexes with a wide range of log P as well as log $D_{pH\ 7.4}$ and pK_a values is available for systematic *in vivo* and *in vitro* studies. "3 + 1" mixed-ligand Tc compounds were synthesised with various ligands that bear amine groups with a low or high basicity, respectively, connected to the metal core, or with ligands containing different donor atoms and alkyl chains to modify the lipophilicity of the resulting compound. According to the substituents no big differences exist between the molecular volumes of the solutes (mw between 356 and 495). The lipophilicities, however, cover a range of log P_{HPLC} values between 1.04 and 4.08, with 12 out of 32 compounds between 1.5 and 2.5, the range reported for optimal brain uptake (17). The corresponding log $D_{pH\ 7.4}$ values are in the range of −0.30 to 2.76. In comparison the log $D_{pH\ 7.4}$ value (octanol/water) for the brain perfusion radiotracer ^{99m}Tc -ECD (11) is 1.11, whereas the amine group bearing tracer TRODAT-1 has a log $D_{pH\ 7.4}$ value (octanol/water) of 2.36 (1,15). Depending on the substituents three categories with typical pK_a ranges were defined: at pH 7.4 **class I** molecules are >99% protonated, **class II** compounds are protonated to about 50% and **class III** to >90 %.

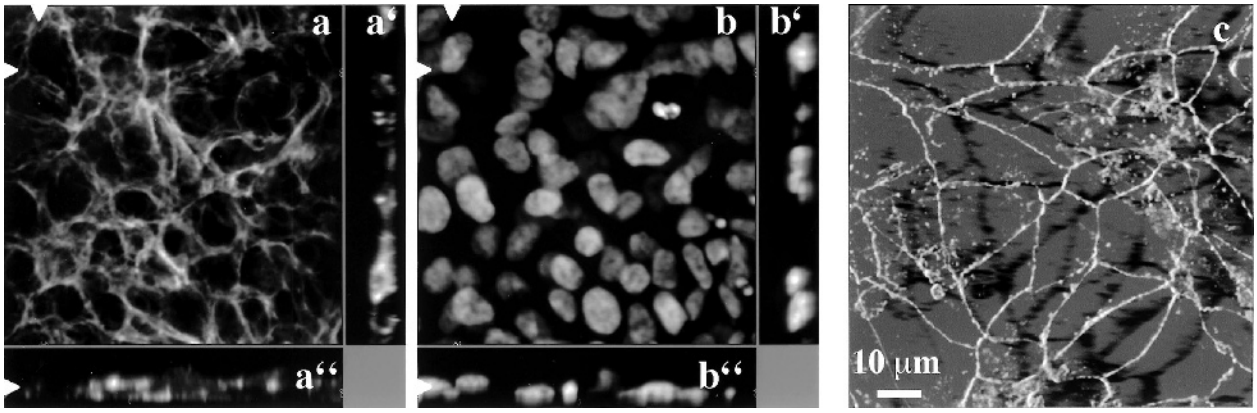


Fig. 5. Characterisation of the ECV304 *in vitro* model by CLSM. ECV304 were grown for 14 days on PET filter membranes with C6 supernatant, fixed and stained as described (see Methods): (a) F-actin; (b) nuclei (same area as (a)); (c) ZO-1 tight junction protein (3D reconstruction). (a, b) x,y-plane; (a', b') yz-projections; (a", b") xz-projections.

The *in vivo* studies in rats and mice revealed distinct patterns of brain uptake (% ID/organ) at 2 min p.i.. **Class I** molecules all have less than 0.5%. From the other molecules, only **2e** is in the same category. The others of the **e-series** (**class II**), are in the range of 0.7 (**3e**) to 2.0 (**6e**). Thus, **6e** is in the same range as **7o** and **7t** (**class III**). The other **class III** molecules, **7q**, **7s** and **7p** show the highest uptake of the whole set, namely >3%.

Class I compounds are defined by a set of simple alkyl substituents at their tertiary amine group of the monodentate ligand, whereas compounds of **class II** bear a morpholine amine group with a decreased basicity. Both classes are characterised by different neutral donor atoms at the tridentate ligand, i.e. sulphur, oxygen and nitrogen as well as alkyl chains of different length **3**(C1) < **4**(C2) < **5**(C3) < **6**(C4). In contrast to **class I** and **II**, compounds of **class III** all carry the same diethyl substituted amine group at the tridentate chelating ligand and only differ by the monodentate alkyl thiol substituents. The pKa value increases with longer alkyl substituents in **class I** (-NMe2 < NEt2 < piperidinyl < NBu2) from 8.9 to 9.9 as expected. The lower amine basicity caused by a morpholine amine group for **class II** decreases the pK_a to 7.1–7.6. The pK_a of a tri-ethyl amine group is expected to be >9, however, the coupling of the diethyl amine group (**class III**) to the co-ordinate nitrogen of the tridentate chelating ligand via an ethylene spacer produces pK_a values of 8.1 to 8.3. For compounds with the same monodentate ligand as in **1b–6b** or **1e–6e** the introduction of tris-chelating co-ligands with alkyl chains of various length on the coordinate nitrogen (**3–6**) leads to increasing log $D_{pH\ 7.4}$ and log P values with increasing number of carbon atoms, i.e. **3b,e**(C1) < **4b,e**(C2) < **5b,e**(C3) < **6b,e**(C4), in accordance with our knowledge about lipophilicity (e.g., 12). All **class III** compounds have an identical tridentate ligand, thus their log $D_{pH\ 7.4}$ and log P values are directly correlated with the alkyl substituent at the monodentate ligand (**7o** < **7p** < **7q** < **7t** < **7s** and **7o** < **7p** < **7q** < **7t = 7s,** respectively).

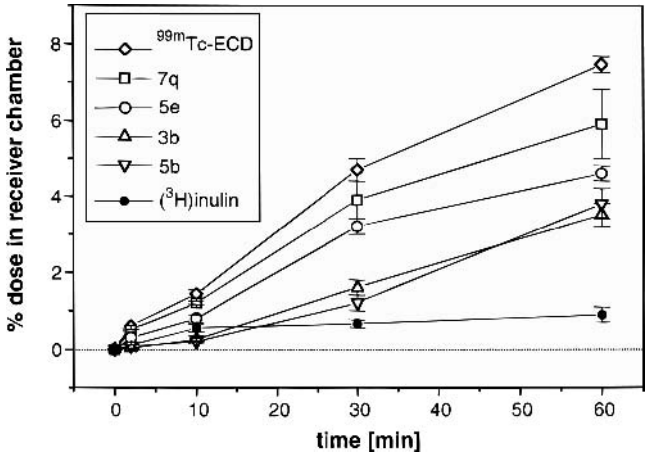


Fig. 6. Permeation studies *in vitro* with the ECV304/C6 supernatant cell culture model. Transport experiments with representative compounds (as indicated) were performed as described (see Methods). Error bars represent standard deviations (n = 6).

Simple alkyl amines at the monodentate ligand (**class I**) led to almost completely charged compounds with a very low brain uptake (<0.5%), whereas the introduction of a morpholine amine group in the same position (**class II**) resulted in a significantly increased brain uptake. Based on the higher ratio of neutral to charged molecules at pH 7.4 in **class II** as compared to **class I**, and based on the higher molecular weight of **class II** compounds—although with the same number of heteroatoms as **class I**, we would expect higher lipophilicity and brain uptake for **class II** than for **class I** molecules (see, e.g., (12)). This is true for brain uptake data but is not significant for the log $D_{pH\ 7.4}$ values. This finding might be related with the different basicities of **class I** and **class II** compounds, which might have a higher influence on the passage of lipid membranes than on the retention on the PRP polystyrene HPLC column material. The brain uptake of **3e–6e** (**class II**) is directly correlated with their log $D_{pH\ 7.4}$ values with the exception of **5e** (0.95%) which would be expected between 1.32% (**4e**) and 2.04% (**6e**). It remains open whether the slightly increased pK_a and therewith the difference in basicity is related with this behaviour. The relatively low brain uptake of **2e** in **class II** may be due to its instability in the presence of glutathion or rat blood, which has been reported for [SOS] co-ordinate compounds (16). The reason for the relatively high brain uptake of **3d** (1.7%) compared with other **class I** compounds remains unclear.

The higher brain uptake of **7p**, **7q** and **7s** as compared to **7o** and **7t** is not reflected in their log $D_{pH\ 7.4}$ values. However,

the log $D_{pH\ 7.4}$ value of **7p** (1.78), which has the highest brain uptake of the whole set, fits perfectly well into the proposed optimal lipophilicity range of log $D_{pH\ 7.4}$ equal to 2 ± 0.5 (17). It is striking that **7p** (**III**) shows significantly higher brain uptake than **6e** (**II**) although both have the same log $D_{pH\ 7.4}$ value of 1.78, and although for **6e** (**II**) more molecules are neutral under experimental conditions than for **7p** (**III**).

A direct correlation between the molecular structures and the physicochemical parameters, respectively, and the permeation through the BBB is difficult. Actual brain uptake may be the result of simultaneous passive permeation, carrier-mediated transport and metabolic degradation with species-related variations. In general, species-specific esterase activity is a common phenomenon. A classical example is ^{99m}Tc -ECD that is degraded in rat blood and thus shows low brain uptake, whereas in baboons and humans a reduced esterase activity seems to permit high brain uptake of this compound (18). As we could show by cross check of compounds **3b**, **3c** and **5c** in rats and mice, species-related difference in BBB passage can be excluded in our case. This is in accordance to published data for several ^{99m}Tc compounds (10).

As an alternative to brain uptake experiments in animals, permeation studies were performed with ECV304 cells grown with C6 supernatant. With one or two representative molecules of each class we saw the same tendencies for permeation as found for brain uptake. At 30 min highest permeation was found for **7q** (**class III**), namely 4% dose, followed by **5e** (**class II**) with about 3.3%. **3b** and **5b** (**class I**) remained below 1.5%. ^{99m}Tc -ECD, included as a reference compound, showed 5% dose permeation up to 30 min and about 7% up to 60 min. For the poorly permeating compounds **3b** and **5b** a linear increase up to 60 min was found in the receiver chamber, whereas for those showing better permeation up to 30 min, i.e., **5e** and **7q**, the rate of permeation seemed to slow down between 30 and 60 min. The significance of this observation remains to be substantiated with extended time series. As we could show the difference in the permeation behaviour at times later than 30 min is not due to chemical instability. Control of the tightness of cell layers with [^{3}H]inulin were run to exclude enhanced permeation due to leakiness.

Though only a small selection of compounds was tested, data from the ECV304 cell culture model are quite encouraging because they open an alternative for a first screening of new candidates for BBB passage with the consequence that experiments in animals can be reduced. Enzymatic degradation cannot be excluded *per se* in cell culture models, however, the activity of esterases and other enzymes remains to be determined, but seems to be very low in the ECV304 cells.

The availability of the cell culture model and a check of the complete test set in further studies may help to better understand passive permeation and partition processes at the BBB. It will be very interesting to see whether the cell permeability data for compounds of one single class better reflect the *in vivo* brain uptake than the log $D_{pH7.4}$ values in this work did. Crucial parameters such as the H-bonding capacity of the molecules, the flexibility and the molecular volume as well as polarity (12–14) should then be correlated with those permeation data. Non-metallic compounds with a low H-bonding capacity and low polarity but high molar volume are postulated to show high brain uptake (14).

To sum up, specific substitution at the amine group modulates log $D_{pH\ 7.4}$ and pK_a values and can lead to a significantly increased brain uptake of otherwise barely permeable molecules. Molecular size and flexibility as well as intramolecular interactions are probably responsible for this structural influence.

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