Synthesis, physicochemical and biological evaluation of tacrine derivative labeled with technetium-99m and gallium-68 as a prospective diagnostic tool for early diagnosis of Alzheimer's disease

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A R T I C L E   I N F O
Keywords: Tacrine Alzheimer’s disease Technetium-99m Gallium-68 Radiopharmaceuticals SPECT/CT PET/CT

A B S T R A C T
Design, physicochemical and biological studies of novel radioconjugates for the early diagnosis of Alzheimer’s disease, based on the newly synthesized tacrine derivatives were performed. Novel tacrine analogues were labeled with technetium-99m and gallium-68. For all obtained radioconjugates ([99mTc]Tc-Hynic-(tricine)2NH(CH2)n)Tacrine and [68Ga]Ga-DOTA-NH(CH2)n)Tacrine, where n = 2–9 denotes the number of methylene groups CH2) the studies of physicochemical properties (lipophilicity, stability in the presence of an excess of standard amino acids cysteine or histidine, human serum and in cerebrospinal fluid) were performed. For two selected radioconjugates [99mTc]Tc-Hynic-(tricine)2NH(CH2)9)Tac and [68Ga]Ga-DOTA-NH(CH2)9)Tacrine (characterized with the highest lipophilicity values) the biological tests (inhibition of cholinesterases action, molecular docking and biodistribution studies) have been performed. All novel radioconjugates showed high stability in biological solutions used. Both selected radioconjugates proved to be good inhibitors of cholinesterases and be able to cross the blood-brain barrier. Radioconjugates [99mTc]Tc-Hynic-(tricine)2NH(CH2)9)Tacrine and [68Ga]Ga-DOTA-NH(CH2)9)Tacrine fulfill the conditions for application in nuclear medicine. Radiopharmaceutical [68Ga]Ga-DOTA-NH(CH2)9)Tacrine, due to increased accuracy and improved sensitivity in PET imaging, may be better potential diagnostic tool for early diagnosis of Alzheimer’s disease.

1. Introduction
Alzheimer’s disease (AD), diagnosed by Alois Alzheimer in 1907, is the most common age-related brain disorder with the symptoms of memory loss and dementia. Tacrine (Tac) in the form of monohydrochloride was the first drug approved by the United States Food and Drug Administration in 1993 for palliative treatment of AD. Tacrine belongs to the cholinesterase inhibitors – it inhibits the action of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) which are the enzymes responsible for the degeneration of neurotransmitter acetylcholine (ACh) [1,2]. However, the use of tacrine is limited due to significant appearance of hepatotoxicity and cardiovascular system impairment, in spite of its mild cognitive benefits, which do not alter the course of the disease [3]. Therefore, the search for new tacrine analogues is still of interest for scientists involved in AD research [4–8]. Due to high biological activity of tacrine towards AChE and BuChE, the radiopharmaceuticals containing diagnostic radionuclides and based on tacrine or its analogues can image the areas of high concentration of cholinesterases (in the cholinergic system) and thereby can become a diagnostic probe able to determine indirectly the level of the ACh in specific brain areas and as the result the areas of neurodegeneration [2,9–13]. Moreover, due to presence of AChE also in liver and intestines, they can also play a role of markers to determine the physiological condition of these organs.

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The goals of this work were syntheses and investigations of the newly designed tacrine analogues labeled with diagnostic radionuclides technetium-99m, \(^{99m}\)Tc-Hynic-(tricine)\(_n\)NH(CH\(_2\))\(_n\)Tac (in the reaction of 6-(Boc-hydrazino)nicotic acid hydrazinonicotic acid and di-tert-butyl dicarbonate) and finally Bo-
hydrazinonicotic acid and di-tert-butyl dicarbonate) and finally Bo-

2. Materials and methods

All solvents and commercially available substances were reagent grade and used without further purification. Deionized water was prepared in the Hydrolab water purification system (Hydrolab, Poland). The 5,5′-dithiobisnitrobenzoic acid (DTNB), acetylthiocholine iodide (ATChI) (enzyme substrate) and tacrine were obtained from Sigma-Aldrich (Munich, Germany).

The gradient and HPLC conditions were as follows: solvent A, 0.1% (v/v) trifluoroacetic acid (TFA) in water; solvent B, 0.1% (v/v) TFA in acetonitrile.

System 1: analytical Phenomenex Aeris Peptide XB-C18 column, 3.6 μm, 150 × 4.6 mm, UV/Vis detection at 220 nm, gradient elution: 0–20 min: 5–70% solvent B, 20–25 min: 70% to 95% solvent B; 1.2 mL/min.

System 2: semi-preparative Phenomenex Jupiter Proteo column, 4 μm, 90 Å, 250 × 10 mm, UV/Vis detection at 220 nm, gradient elution: 0–20 min 20–80% solvent B, 20–40 min 80% solvent B; 2 mL/min.

Mass Spectrometry (MS): Mass spectra were measured on the Bruker 3000 Esquire mass spectrometer equipped with ESI.

Infrared (IR): IR spectra in solid KBr pellets (investigated species amount to about 1% of the pellet) were recorded in the range 4000–600 cm\(^{-1}\) using Bruker Equinox 55 FT-IR spectrophotometer. All spectra were registered independently at least three times with 50 scans each and with spectral resolution of 1 cm\(^{-1}\).

\(^1\)H NMR and \(^{13}\)C NMR spectra were obtained on a 400 MHz Varian Mercury spectrometer at room temperature. In the case of \(^1\)H NMR chemical shifts were reported as δ values relative to the internal TMS.

2.1. Syntheses of conjugates

2.1.1. Syntheses of tacrine derivatives, NH\(_2\)(CH\(_2\))\(_n\)Tac

Tc-Hynic-NH(CH\(_2\))\(_n\)Tac compounds recorded in HPLC analyses in system 1.

<table>
<thead>
<tr>
<th>(CH(_2))(_n)</th>
<th>Retention time, R(_t) [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BocHyinic-NH(CH(_2))(_n)Tac</td>
<td>10.03</td>
</tr>
<tr>
<td>Hyinic-NH(CH(_2))(_n)Tac</td>
<td>10.08</td>
</tr>
<tr>
<td>(^{99m})Tc-Tc-Hynic-NH(CH(_2))(_n)Tac</td>
<td>11.61</td>
</tr>
<tr>
<td>a: n = 2</td>
<td>12.30</td>
</tr>
<tr>
<td>b: n = 3</td>
<td>13.06</td>
</tr>
<tr>
<td>c: n = 4</td>
<td>13.80</td>
</tr>
</tbody>
</table>

2.1.3. Syntheses of Hynic-NH(CH\(_2\))\(_n\)Tac, (2a-h)

Deprotection of amine group was carried out using HCl/diethyl ether mixture. A solution of HCl in diethyl ether was prepared by bubbling HCl into ether at a moderate rate for 20 min. The BocHyinic-NH(CH\(_2\))\(_n\)Tac conjugate (1a-h) was dissolved in diethyl ether and next the mixture HCl/diethyl ether was added. The reaction mixture was stirred at room temperature. After 2 min the solution became cloudy and a precipitate formed. The precipitate was isolated by filtration and the solid was washed with diethyl ether and dried in vacuum. Retention time values of Hyinic-NH(CH\(_2\))\(_n\)Tac (2a-h) conjugates recorded in HPLC analyses in system 1 are presented in Table 1.

The analytical data for Hyinic-NH(CH\(_2\))\(_n\)Tac conjugates are presented in Ref. [13] (for conjugates containing 2, 3, 6 and 8 methylene groups in aliphatic hydrocarbon chain) and Ref. [14] (for conjugates containing 4, 5, 7 and 9 methylene groups in aliphatic hydrocarbon chain).

2.1.4. Syntheses of DOTA-NH(CH\(_2\))\(_n\)Tac, (5f-h)

The coupling reactions between DOTA-NHS (1,4,7,10-tetraazaacyclodocadecane-1,4,7,10-tetraacetic acid mono-N-hydroxysuccinimide ester) and the tacrine derivatives (4f-h) were performed in DMF, at 50 °C and in the presence of E\(_6\)N (Scheme 2). The molar ratio of the reagents used in the coupling reactions was 1.3:1:4, respectively. Crude products (Scheme 2, Table 2) were purified on a semi-preparative HPLC column in system 2 and lyophilized, yield = 85%. Bearing in mind that preparations able to cross the blood-tissue barrier should be characterized with relatively high lipophilicity, only three tacrine derivatives, containing 7, 8 and 9 methylene groups (n = 7–9) in an aliphatic hydrocarbon chain have been synthesized.

Retention time values of DOTA-NH(CH\(_2\))\(_n\)Tac (5f-h) conjugates recorded in HPLC analyses are presented in Table 2.

Analytical data for DOTA-NH(CH\(_2\))\(_n\)Tac, (5f, 1,4,7,10-Tetraazaacyclododecane-1,4,7,10-tetraacetic acid-10-[9-(1,2,3,4-tetrahydroacridin-9-ylamino)heptyl]acetamidate):

MS (FAB) m/z: Calculated for C\(_{36}\)H\(_{55}\)N\(_7\)O\(_7\): 697.88; Found 698.46 [M + H\(^+\)]

Analytical data for DOTA-NH(CH\(_2\))\(_n\)Tac, (5g, 1,4,7,10-Tetraazaacyclododecane-1,4,7,10-tetraacetic acid-10-[9-(1,2,3,4-tetrahydroacridin-9-ylamino)octyl]acetamidate):

MS (FAB) m/z: Calculated for C\(_{37}\)H\(_{57}\)N\(_7\)O\(_7\): 711.91; Found 712.49 [M + H\(^+\)]

Analytical data for DOTA-NH(CH\(_2\))\(_n\)Tac, (5h, 1,4,7,10-Tetraazaacyclododecane-1,4,7,10-tetraacetic acid-10-[9-(1,2,3,4-tetrahydroacridin-9-ylamino)nonyl]acetamidate):

Table 1

<table>
<thead>
<tr>
<th>(CH(_2))(_n)</th>
<th>Retention time, R(_t) [min]</th>
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<tbody>
<tr>
<td>BocHyinic-NH(CH(_2))(_n)Tac</td>
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<td>13.06</td>
</tr>
<tr>
<td>c: n = 4</td>
<td>13.80</td>
</tr>
</tbody>
</table>
MS (FAB) m/z: Calculated for C\textsubscript{38}H\textsubscript{59}N\textsubscript{7}O\textsubscript{7}: 725.93; Found 726.47 [M + H\textsuperscript{+}]

2.2. Preparation of radioconjugates

2.2.1. Preparation \([^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2\text{n)}\text{Tac, (3a-h)}\]

The \([^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2\text{n)}\text{Tac radioconjugates were synthesized according to the commonly used procedure: to the solution containing 20 μg (13.4 nmol) of Hynic-NH(CH\textsubscript{2})\text{n}TAC, 20 mg (0.11 mmol) of tricine (N-[Tris(hydroxymethyl)methyl]glycine) and 5 mg (28 μmol) of EDDA (ethylenediaminediacetic acid) in 500 μL of the 0.1 M PBS buffer was simultaneously added 500 μL of the \([^{99m}\text{Tc}]\) NaTcO\textsubscript{4} solution (0.9% NaCl eluate from the \([^{99m}\text{Mo/99m}\text{Tc generator, 500–1500 MBq}) and 5 μL of SnCl\textsubscript{2} (5.3 mM in 0.1 M HCl). The reaction mixture was incubated for 20 min at 90°C and the reaction progress was controlled by HPLC in system 1. The radiochemical yield of each \([^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2\text{n)}\text{Tac radioconjugate was determined to be approximately 98%. Retention time values of \([^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2\text{n)}\text{Tac radioconjugates recorded in HPLC analyses are presented in Table 1.}

Additionally, we have also performed the synthesis of \([^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2\text{n)}\text{Tac radioconjugate using only tricine as coligand. On HPLC chromatogram of reaction mixture we recorded again one peak of \(R\textsubscript{T} = 12.71 \text{ min. We have also determined the lipophilicity parameter for this compound and we obtained the log D value equals to −1.36 ± 0.01. Based on these results we concluded that both in the presence of a small amount of EDDA or without EDDA in reaction mixture the same radioconjugate \([^{99m}\text{Tc}]\text{Tc-Hynic-(tricine)}\text{NH(CH}_2\text{n)}\text{tacrine is formed (containing two molecules of tricine as coligands).}

2.2.2. Preparation \([^{68}\text{Ga}]\text{Ga-DOTA-NH(CH}_2\text{n)}\text{Tac radioconjugates (6f-h)}\]

The \([^{68}\text{Ga}]\text{Ga-DOTA-NH(CH}_2\text{n)}\text{Tac radioconjugates were synthesized according to the following procedure: to the vial containing about 50 μg of lyophilized DOTA-NH(CH\textsubscript{2})\text{n}TAC, 300 μL of acetate buffer (pH = 5.89) and 50 + 100 μL of concentrated solution of \([^{68}\text{Ga}]\)GaCl\textsubscript{3} from the \([^{68}\text{Ge/68}\text{Ga generator (100–280 MBq}) were added. The reaction mixture was heated for 30 min at 95°C and the reaction progress was checked by HPLC method in system 2. Bearing in mind that preparations able to cross the blood-tissue barrier should be characterized with

Table 2

<table>
<thead>
<tr>
<th>(CH\textsubscript{2})\text{n}</th>
<th>Retention, (R\textsubscript{T} [\text{min}])</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH\textsubscript{2}(CH\textsubscript{2})\text{n}TAC</td>
<td>(n=7) 11.78 11.45 12.17</td>
</tr>
<tr>
<td>DOTA-NH(CH\textsubscript{2})\text{n}TAC</td>
<td>(n=8) 12.36 12.04 12.62</td>
</tr>
<tr>
<td>([^{68}\text{Ga}])Ga-DOTA-NH(CH\textsubscript{2})\text{n}TAC</td>
<td>(n=9) 13.01 12.60 13.20</td>
</tr>
</tbody>
</table>

Scheme 1. Synthesis route of Hynic-NH(CH\textsubscript{2})\text{n}TAC conjugates.

Scheme 2. Coupling reaction of DOTA-NHS chelator with tacrine derivatives.
relatively high lipophilicity only three
radioconjugates, containing 7, 8 and 9 methylene groups (n = 7–9) have been synthesized. The radiochemical yield of the synthesized radioconjugates was higher than 98%. Retention time values of \([\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}\) radioconjugates recorded in HPLC analyses are presented in Table 2.

2.3. Synthesis of Ga-DOTA-NH(CH_2)_9Tac

In order to verify the identity of the \([\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}\) radioconjugates synthesized in n.c.a. scale, for the selected compound \(n = 9\) the non-radioactive reference compound Ga-DOTA-NH(CH_2)_9Tac conjugate was prepared in milligram scale (using GaCl_3 solution and according to the procedure described above), isolated by HPLC method and characterized by MS analysis.

Analytical data for Ga-DOTA-NH(CH_2)_9Tac (‘cold’ reference compound):

MS (FAB) m/z: Calculated for C_{38}H_{57}N_{7}O_{7}Ga: 793.64; Found 793.97

[M + H]^+:

2.4. Physicochemical and biological properties studies of novel radioconjugates

All radioconjugates were isolated by HPLC (system 1 or 2) method and then used in further experiments such as: lipophilicity or stability studies. In case of \(\text{in vitro}\), in \(\text{in vivo}\) test Sep-Pak purification method was performed (according to manufacturer recommended procedure; Sep-Pak® classic short C-18 cartridge, WATERS), previously validated by HPLC (system 1 or 2).

2.4.1. Lipophilicity studies (in vitro)

Lipophilicity (expressed as Log D) of \([\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}\) and \([\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}\) radioconjugates, which is an important factor affecting the distribution of drug molecules in the organism, was characterized by partition coefficients, \(P\), in the system n-octanol/PBS buffer (pH = 7.4). The activity of each layer (which shows concentration of the radionuclide containing species in the layer) was determined by measuring \(\gamma\) radiation, with a well-type Na(Tl) detector. Partition coefficient \(P\) was calculated as the ratio of radioactivity of organic to radioactivity of aqueous phase (as an average value from at least three independent measurements). Immediately after the distribution experiments the aqueous phases were analyzed by HPLC in system 2 to check whether the studied radioconjugate has not decomposed during the experiment.

2.4.2. Stability studies in challenging solutions (in vitro)

Stability studies of \([\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}\) and \([\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}\) radioconjugates were carried out as follows: the radioconjugate isolated from the reaction mixture using HPLC system 1 or system 2, respectively, present in the solution in concentration no higher than \(10^{-4}\) mM, was incubated at \(37°C\) with \(10\) mM solutions of histidine or cysteine in the PBS buffer (pH = 7.4). HPLC analyses of the incubated solutions were performed at different time periods from 0.5 h up to 24 h, since staring the incubation.

2.4.3. Stability studies of \([\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}\) and \([\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}\) in human serum and in cerebrospinal fluid (in vitro)

For that purpose 0.1 mL of solution of the isolated radioconjugate in the 0.1 M PBS buffer (pH = 7.4), was added to 0.9 mL of human serum (isolated and purified at the Centre of Radiobiology and Biological Dosimetry, INCT Warsaw) or 0.5 mL of cerebrospinal fluid (obtained from Warsaw hospital) and incubated at \(37°C\). At specified time intervals small sample (0.1–0.2 mL) of the mixture was withdrawn, mixed with ethanol (0.3–0.5 mL) and vigorously shaken to precipitate proteins. Then, the sample was centrifuged (14000 rpm, 5 min) and the supernatant was separated. The radioactivities of both supernatant and precipitate were measured using the well-type Na(Tl) detector. To check if the radioconjugate did not convert into other water-soluble radioactive species, aliquots of the supernatant were analyzed by HPLC for the content of the studied radioconjugate.

2.4.4. Biological activity studies of \([\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}\) and \([\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}\) (in vitro)

2.4.4.1. AChE and BuChE inhibition assay. Ellman’s colorimetric method was used to determine the activity of tacrine and the most promising new radioconjugates \([\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}\) and \([\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}\) against both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Test was conducted on the stable gallium conjugate (Ga-DOTA-NH(CH_2)_9Tac) and long-lived technetium radioconjugate \((\text{Tc})\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}\) to minimize the radiation effect on assay for nine concentrations of each compound. Tacrine and tested compounds were dissolved in DPBS (Dulbecco's phosphate buffered saline) and diluted to obtain appropriate concentrations. Test was performed using 96-well plates. The reaction mixture consisted of 0.4 mg/ml Ellman’s reagent (DTNB), 2 U/ml AChE or 4 U/ml BuChE, different concentrations of the new inhibitor or tacrine and ATChI solutions 1 mM or 2 mM (40 μL) for AChE or BuChE, respectively in total amount of 140 μL/well. All the reagents were purchased from Sigma-Aldrich. Control wells were prepared without inhibitors. The absorbance at 412 nm was measured using microplate reader (Synergy H1, Bio-Tek) in time intervals at 30°C. Each measurement was made in triplicate. IC_{50} was evaluated for compounds to determine their inhibition activity (Table 4.).

2.4.4.2. Kinetic characterization of AChE inhibition. Ellman’s method was used for kinetic characterization of AChE inhibition. Each tested solution in one plate contains DTNB (0.4mg/ml), AChE (2U/ml), inhibitor in one concentration and 80–350 μM ATChI solution. Total volume was 140 μL/well. We tested solutions without addition of inhibitor and with three different concentrations for each inhibitor: \([\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac} – 50\) pM, 75 pM and 100 pM and Ga-DOTA-NH(CH_2)_9Tac – 173 nM, 260 nM and 346 nM. Absorbance was measured using microplate reader (Synergy H1, Bio-Tek) at a wavelength of 412 nm, at 1 min intervals at 30°C. The experiments were done in triplicates and averaged. The results are shown as Lineweaver–Burk plot to determine \(K_{in}\), \(V_{max}\) and type of enzyme inhibition (Figs. 5 and 6).

2.4.5. Molecular modeling studies (in silico)

Docking studies were performed for the most promising radioconjugates \([\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}\) and \([\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}\) and compared to the reference tacrine. The three-dimensional structures of studied compounds were drawn in Maestro 2016-2 and subsequently optimized by application of DFT-B3LYP method with LACV3P** basis set in Jaguar 9.2 software. Structures of both cholinesterases were obtained from Protein Data Bank. Human acetylcholinesterase of resolution 2.35Å (complex with donepezil, PDB code: 4EY7) was split into single monomers and chain A was utilized for docking. On the other hand, human butyrylcholinesterase of resolution 2.0 Å (complex with butryate, PDB code: 1POI) was used directly. Both proteins were prepared by Hermes 1.7.0, including setting histidine residues as Ne tautomers, adding missing hydrogen atoms and removing ligand molecules. The binding site was defined as amino acid residues within 10 Å from donepezil and 20 Å from glycerol present in the active site of AChE and BuChE, respectively. Tyr337 and Trp286 residues of AChE were treated as flexible because they could occur in the form of rotamers. Dockings were performed with Gold 5.3.0, using default settings of genetic algorithm. GoldScore function and visual inspection were applied for evaluation of the results. For each compound the final results involved 10 conformations, sorted according to the scoring function values. Docking of the tested inhibitors to both cholinesterases was validated on the basis of reference
compound. Gold Suite could reproduce original orientation of tacrine with low rmsd (root-mean square deviation) values below 2 Å. Since Gold software did not contain parameters for technetium and gallium, they were parameterized on the basis of manganese and aluminum. Results were visualized with PyMOL 0.99rc6.

2.4.6. Biodistribution studies of $[^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2\text{n}^8\text{Tac}$ and $[^{68}\text{Ga}]\text{Ga-DOTA-NH(CH}_2\text{n}^8\text{Tac}$ (in vivo and ex vivo)

Pharmacodynamics evaluation of the most promising radioconjugates were performed on animal model of Wistar rats in in vivo and ex vivo studies. 58 Wistar male rats in age of 4–5 weeks were obtained from the animal facility at Mossakowski Medical Research.
Centre, Polish Academy of Sciences (Warsaw, Poland). All the experimental procedures were performed according to the national legislation and were approved by the First Local Ethical Committee of the Warsaw University Biology Department (Permission No. 510/2018). Rats were kept under constant conditions of 12–12h light cycle, humidity at level of 55 ± 5%, and temperature of about 22 ± 2°C in individually ventilated cages with free access to drinking water and standard laboratory diet. Any procedures were performed after minimum 5 days of acclimation after travel and all efforts were made to minimize animals suffering.

First in vivo procedure was a dynamic PET/CT imaging performed on 6 rats using Albira PET/SPECT/CT Preclinical Imaging System (Bruker, Germany). Before each imaging, rat get anesthetized on the heating pad with 3.5–4% isoflurane (Aerrane, Baxter Polska Sp. z o.o., Poland) in oxygen applied through a nose cone. Prepared rat was placed in the prone position on the suitable bed place in the imager, afterwards 2–15 MBq $[^{68}\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}$ was administered intravenously through tail vein cannula and image acquisition was performed subsequently. Spatial resolution of PET measurements was 1.5 mm and scan parameters were set as follows: tube voltage – 45 kV, tube current – 400 μA, projections number – 400. Minimal resolution of CT was 90 μm. The scans from both tomographies were fused and coregistered using PMOD software, since rats remained in the same position on the bed for both PET and CT acquisitions. After acquisitions still anesthetized rats got a lethal dose of 5% isoflurane until respiratory system completely stops, then were decapitated and sacrificed for further radioactivity measurements of internal tissues. Based on six executed PET imaging, mean standardized uptake values (SUVs) for brain, kidneys, lungs, heart and liver were plotted in function of acquisition time.

Second in vivo procedure was a dynamic SPECT/CT imaging performed also on 6 rats using Albira PET/SPECT/CT Preclinical Imaging System. Before each imaging, rat get anesthetized and placed in Albira system in similar protocol as it is presented in previous procedure, afterwards 33–62 MBq $[^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}$ was administered intravenously through tail vein cannula and image acquisition was performed subsequently. Dynamic SPECT scans were fused and coregistered using PMOD software, since rats remained in the same position on the bed for both PET and CT acquisitions. After acquisitions still anesthetized rats got a lethal dose of 5% isoflurane until respiratory system completely stops, then were decapitated and sacrificed for further radioactivity measurements of internal tissues. Based on six executed SPECT imaging, mean standardized uptake values (SUVs) for brain, kidneys, lungs, heart and liver were plotted in function of acquisition time.

<table>
<thead>
<tr>
<th>n</th>
<th>LogD $[^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}$</th>
<th>LogD $[^{68}\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}$</th>
</tr>
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<tbody>
<tr>
<td>a: n = 2</td>
<td>–2.95 ± 0.06</td>
<td>–</td>
</tr>
<tr>
<td>b: n = 3</td>
<td>–2.80 ± 0.01</td>
<td>–</td>
</tr>
<tr>
<td>c: n = 4</td>
<td>–2.53 ± 0.02</td>
<td>–</td>
</tr>
<tr>
<td>d: n = 5</td>
<td>–2.41 ± 0.01</td>
<td>–</td>
</tr>
<tr>
<td>e: n = 6</td>
<td>–2.08 ± 0.01</td>
<td>–</td>
</tr>
<tr>
<td>f: n = 7</td>
<td>–1.86 ± 0.02</td>
<td>–2.52 ± 0.01</td>
</tr>
<tr>
<td>g: n = 8</td>
<td>–1.50 ± 0.01</td>
<td>–2.02 ± 0.01</td>
</tr>
<tr>
<td>h: n = 9</td>
<td>–1.38 ± 0.01</td>
<td>–1.52 ± 0.01</td>
</tr>
</tbody>
</table>

Table 3
LogD values of $[^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}$ and $[^{68}\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}$ radioconjugates.

Fig. 3. The HPLC radiochromatograms of $[^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}$ and $[^{68}\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}$ radioconjugates recorded, respectively, after 24 h and 5 h of incubation at 37 °C in human serum (left) and cerebrospinal fluid (right).
performed with sequence of 10 measurements, 60 projections with 10 and 20s time of acquisition and then CT scans were performed. After acquisitions still anesthetized rats got a lethal dose of 5% isoflurane until respiratory system completely stops, then were decapitated and sacrificed for further radioactivity measurements of internal tissues.

Ex vivo procedure was performed as static pharmacodynamics analysis for both radioconjugates by similarly done intravenous administrations of 2–15 MBq [68Ga]Ga-DOTA-NH(CH2)9Tacrine (20 rats) or 33–62 MBq [99mTc]Tc-Hynic-NH(CH2)9Tacrine (20 rats) under anesthesia. Then in 4 specific time points (5, 15, 30, 60 min) an euthanasia of animals were executed by reanesthetizing, application of isoflurane lethal dose and decapitation. Additional time points for [68Ga]Ga-DOTA-NH(CH2)9Tacrine in 135 min and for [99mTc]Tc-Hynic-NH(CH2)9Tacrine in 150 min were taken from rats after dynamic imaging procedures. From each sacrificed animal a whole brain, blood sample (about 5 mL), both kidneys, lungs, heart, spleen and whole liver were removed and weighted to perform radioactivity measurements of desired tissues. Each sample was analyzed on WIZARD2 2480 Automatic Gamma Counter (PerkinElmer, Inc., USA) in 60 s triple measurements with decay correlation protocol. Collected radioactivity

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**Table 4**
The activity of [99Tc]Tc-Hynic-NH(CH2)9Tac and Ga-DOTA-NH(CH2)9Tac compounds against two cholinesterases.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 ± SD (nM)</th>
<th>Selectivity for AChE</th>
<th>Selectivity for BuChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>[99Tc]Tc-Hynic-NH(CH2)9Tac</td>
<td>0.10 ± 0.01</td>
<td>1.20</td>
<td>0.83</td>
</tr>
<tr>
<td>Ga-DOTA-NH(CH2)9Tac</td>
<td>290 ± 20</td>
<td>0.57</td>
<td>1.75</td>
</tr>
<tr>
<td>Tacrine</td>
<td>107 ± 9</td>
<td>0.15</td>
<td>6.67</td>
</tr>
</tbody>
</table>

* Inhibitor concentration for 50% inactivation of AChE or BuChE effect, respectively. Results are the means of three independent experiments 3 times in triplicate ± standard deviation SD.

* Selectivity for AChE is defined as IC50(BuChE)/IC50(AChE).

* Selectivity for BuChE is defined as IC50(AChE)/IC50(BuChE).

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**Fig. 4.** Percentage of intact [99mTc]Tc-Hynic-NH(CH2)9Tac and [68Ga]Ga-DOTA-NH(CH2)9Tac radioconjugates remaining after certain periods of incubation at 37 °C in human serum (left) and cerebrospinal fluid (right).

**Fig. 5.** Lineweaver – Burk plot illustrating mixed type of AChE inhibition for [99mTc]Tc-Hynic-NH(CH2)9Tac. The inverse of initial velocity (V−1) and inverse of the substrate concentrations ([ATCh]−1) are presented on y- and x-intercepts.
data were converted into the tissue per cent of initial dose applied into the rat divided by tissue mass in grams (%ID/g) and their averages were illustrated in the function of time points.

3. Results

In the frame of the work two series of potential diagnostic radiopharmaceuticals, based on tacrine derivatives, have been synthesized and studied. In the first series 8 tacrine derivatives (containing different number of methylene groups in aliphatic chain, n = 2–9, Fig. 1) were labeled with technetium-99m using bifunctional ligand Hynic in complex with tricine (3a-h). In the second series 3 tacrine derivatives were labeled with Ga-68 using macrocyclic ligand DOTA (6f-h). Bearing in mind that radiopharmaceuticals able to cross the blood-tissue barrier should be characterized with relatively high lipophilicity, for the second series, as it was mentioned in previous sections, only 3 tacrine derivatives, containing 7, 8 and 9 methylene groups (n = 7–9) have been synthesized. All obtained \[^{99mTc}\]Tc-Hynic-NH(CH\(_2\))\(_n\)Tac and \[^{68Ga}\]Ga-DOTA-NH(CH\(_2\))\(_n\)Tac radioconjugates were formed with high yield > 97% and high purity > 98%. The chemical formulas of \[^{99mTc}\]Tc-Hynic-NH(CH\(_2\))\(_n\)Tac and \[^{68Ga}\]Ga-DOTA-NH(CH\(_2\))\(_n\)Tac radioconjugates are presented in Fig. 1.

The conjugates Hynic-NH(CH\(_2\))\(_n\)Tac were synthesized according to the Scheme 1 and procedure described in reference [13,14]. The retention time (R\(_T\)) values of BocHynic-NH(CH\(_2\))\(_n\)Tac, Hynic-NH(CH\(_2\))\(_n\)Tac and \[^{99mTc}\]Tc-Hynic-NH(CH\(_2\))\(_n\)Tac compounds recorded in HPLC analyses in system 1 are presented in Table 1. The HPLC chromatogram of selected \[^{99mTc}\]Tc-Hynic-NH(CH\(_2\))\(_n\)Tac radioconjugate is presented in Fig. 2.

The conjugates DOTA-NH(CH\(_2\))\(_n\)Tac were synthesized according to the Scheme 2. The R\(_T\) values of NH\(_2\)(CH\(_2\))\(_n\)Tac, DOTA-NH(CH\(_2\))\(_n\)Tac and \[^{68Ga}\]Ga-DOTA-NH(CH\(_2\))\(_n\)Tac compounds recorded in HPLC analyses in system 2 are presented in Table 2. The HPLC chromatograms of selected \[^{68Ga}\]Ga-DOTA-NH(CH\(_2\))\(_n\)Tac radioconjugate as well as ‘cold’ reference compound Ga-DOTA-NH(CH\(_2\))\(_n\)Tac are presented in Fig. 2.

The determined lipophilicity values of \[^{99mTc}\]Tc-Hynic-NH(CH\(_2\))\(_n\)Tac and \[^{68Ga}\]Ga-DOTA-NH(CH\(_2\))\(_n\)Tac radioconjugates are presented in Table 3.

The HPLC chromatograms of \[^{99mTc}\]Tc-Hynic-NH(CH\(_2\))\(_n\)Tac and \[^{68Ga}\]Ga-DOTA-NH(CH\(_2\))\(_n\)Tac, presenting stability in the solutions containing about 1000 times molar excess of histidine or cysteine (the so called ‘challenge experiments’, recorded after 24 h of incubation in the case of \[^{99mTc}\]Tc-Hynic-NH(CH\(_2\))\(_n\)Tac and after 5 h of incubation in the case of \[^{68Ga}\]Ga-DOTA-NH(CH\(_2\))\(_n\)Tac, showed presence of only one radioactive species in each solution, with the retention time characteristic for each studied radioconjugate.

For the subsequent \textit{in vitro} and biodistribution studies (stability in human serum and cerebrospinal fluid, biological activity towards cholinesterases and multigorgan biodistribution) the following compounds, characterized by the highest LogD values (Table 3), have been selected: \[^{99mTc}\]Tc-Hynic-NH(CH\(_2\))\(_n\)Tac, 3h and \[^{68Ga}\]Ga-DOTA-NH(CH\(_2\))\(_n\)Tac, 6h. Both selected radioconjugates are based on the tacrine derivative containing nine methylene groups CH\(_2\) in the aliphatic chain (Fig. 1).

Stabilities of 3h and 6h radioconjugates in both biological fluids...
human serum and cerebrospinal fluid) are presented in Figs. 3 and 4. The biological activity of [99mTc]Tc-Hynic-NH(CH2)9Tac, 3h, and [68Ga]Ga-DOTA-NH(CH2)9Tac, 6h, radioconjugates were studied in vitro against acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in Ellman’s colorimetric assay. The IC50 values for the tested derivatives are presented in Table 4. Tacrine was used as reference inhibitor in various concentrations between 2 and 422 nM. Tests were done for different range concentrations of inhibitors (4 pM − 1 nM of [99Tc]Tc-Hynic-NH(CH2)9Tac and 7 nM − 1.733 μM of Ga-DOTA-NH(CH2)9Tac) with the constant substrate (ATChI) concentration.

Mechanism of action of the new derivatives were investigated, as well as AChE inhibition kinetic characterization. We made tests for zero and three other concentrations of the target compounds. Analyzing Lineweaver-Burk plot we revealed the type of AChE inhibition (Figs. 5 and 6). As the plotted lines crossed in the same point between both axis, we defined mixed type of the inhibition for both analysed compounds.

Insilico studied binding modes for novel radioconjugates [99mTc]Tc-Hynic-NH(CH2)9Tac, 3h, and [68Ga]Ga-DOTA-NH(CH2)9Tac, 6h, into the structures of cholinesterases are illustrated in Figs. 7 and 8.

In vivo pharmacodynamic study of 6h is presented in Fig. 9 and supplemented by scans in Fig. 10. All 6 rats have successfully passed through PET acquisition procedures, without any noticeable side effects after administration of radiopharmaceutical or even anesthesia agent. In vivo pharmacodynamic study of 3h is not presented, because activity of [99mTc]Tc-Hynic-NH(CH2)9Tac radiotracer in brain region was lower than detection threshold of applied gamma camera in Albira imaging system. Analysis of received images were pointless. All 40 rats have successfully passed through SPECT acquisition procedures, without any noticeable side effects after administration of radio-pharmaceutical or even anesthesia agent.

Static pharmacodynamic analyses of 3h and 6h are presented in Tables 5 and 6 and in Figs. 11 and 12, respectively. Based on the obtained results, an effective half-lifes of both radioconjugates have been graphically determined from the charts (Fig. 13). All 40 rats have

---

**Table 5**

<table>
<thead>
<tr>
<th>Time period organ</th>
<th>[99mTc]Tc-Hynic-NH(CH2)9Tac (mean ± SD) [%ID/g]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>Brain</td>
<td>0.98 ± 0.16</td>
</tr>
<tr>
<td>Blood</td>
<td>6.1 ± 2.3</td>
</tr>
<tr>
<td>Kidneys</td>
<td>9.4 ± 1.6</td>
</tr>
<tr>
<td>Lungs</td>
<td>9.6 ± 0.5</td>
</tr>
<tr>
<td>Heart</td>
<td>6.9 ± 1.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Liver</td>
<td>32 ± 5</td>
</tr>
</tbody>
</table>

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**Table 6**

<table>
<thead>
<tr>
<th>Time period organ</th>
<th>[68Ga]Ga-DOTA-NH(CH2)9Tac (mean ± SD) [%ID/g]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>Brain</td>
<td>0.21 ± 0.07</td>
</tr>
<tr>
<td>Blood</td>
<td>4.3 ± 1.2</td>
</tr>
<tr>
<td>Kidneys</td>
<td>28 ± 11</td>
</tr>
<tr>
<td>Lungs</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td>Heart</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Liver</td>
<td>43 ± 30</td>
</tr>
</tbody>
</table>
successfully passed through radiopharmaceutical application procedures without any noticeable side effects.

4. Discussion

All $[^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}$ and $[^{68}\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}$ radioconjugates are obtained with high yield and high radiochemical purity. Lipophilicity studies showed that all $[^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}$ and $[^{68}\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}$ radioconjugates are definitely hydrophilic compounds (Table 3). Moreover, the $\text{LogD}$ values determined for tacrine derivatives coupled with $[^{68}\text{Ga}]\text{Ga-DOTA}$ complex are remarkably lower than that of the same tacrine derivatives coupled with $[^{99m}\text{Tc}]\text{Tc-Hynic}$ complex. In both series the $\text{LogD}$ values of radioconjugates increase with the number of methylene groups in aliphatic chain.

Stability studies showed that all synthesized radioconjugates do not undergo the ligand exchange reaction with strongly competing natural ligands containing SH or NH reactive groups in challenging solutions containing about 1000 times molar excess of competitive SH or NH groups. $[^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}$ and $[^{68}\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}$ showed almost complete stability in human serum and cerebrospinal fluid (Figs. 3 and 4). The HPLC chromatograms, recorded after 24 h of incubation in the case of $[^{99m}\text{Tc}]\text{Tc-Hynic-NH(CH}_2)_n\text{Tac}$ and after 5 h in the case of $[^{68}\text{Ga}]\text{Ga-DOTA-NH(CH}_2)_n\text{Tac}$, showed presence of only one radioactive species in the solutions, with the retention times characteristic for the studied radioconjugates (Fig. 3). Stability studies of $[^{3h}]$ and $[^{6h}]$ radioconjugates in human serum showed also that the obtained solid residue which precipitated after ethanol...
addition and sample centrifugation, contained about 5–12% of studied radioconjugate bound by serum protein components (Fig. 4). In the case of incubation in cerebrospinal fluid the solid residue contained less than 8% of initial radioactivity (Fig. 4). This difference is caused by a different content of protein in human serum and cerebrospinal fluid (the protein content in cerebrospinal fluid is about 200 times less than that of human serum) [15]. As one can see, both [99mTc]Tc-Hynic-NH(CH2)9Tac and [68Ga]Ga-DOTA-NH(CH2)9Tac radioconjugates best fulfill the requirements for radiopharmaceuticals [16]. Both radioconjugates, in a time interval corresponding to four radionuclide half-lives, are almost completely stable in challenging solutions, human serum and cerebrospinal fluid.

Biological activity studies of obtained compounds show that new tacrine derivatives are satisfactorily active cholinesterase inhibitors. Especially [99mTc]Tc-Hynic-NH(CH2)9Tac with IC50 values of 0.10 nM and 0.12 nM for AChE and BuChE, respectively, shows much more higher activity than the reference compound tacrine (IC50 = 107 nM and 16 nM for AChE and BuChE, respectively), while [68Ga]Ga-DOTA-NH(CH2)9Tac proved to be slightly worse inhibitor (with IC50 values of 290 nM and 167 nM for AChE and BuChE, respectively) than tacrine. Moreover, [99mTc]Tc-Hynic radioconjugate showed more selective inhibition activity for AChE, with selectivity index 1.20, but in comparison, the [68Ga]Ga-DOTA radioconjugate showed more selective inhibition activity for BuChE, with selectivity index 1.75. However, tacrine selective inhibition index for BuChE proved to be the highest one from the analyzed inhibitors and it is equal to 6.67.

Molecular docking studies show that both radioconjugates interact with AChE and BuChE in a similar manner. They could interact with both catalytic and peripheral active sites of the enzymes. Even though the whole molecules of inhibitors were engaged in the interactions with cholinesterases, the most important fragment was tacrine moiety. This fragment of both inhibitors always adopted the same arrangement as tacrine in its complexes with AChE and BuChE. Tacrine moiety was each time located in the anionic subsite near the catalytic triad. In case of AChE it formed a characteristic sandwich by π–π stacking interaction with Trp286 and Tyr337 (Fig. 7). Additionally, it could be engaged in hydrogen bond with the carbonyl group of His438 and cation–π interaction with Trp86 and Tyr337 due to the protonation of nitrogen atom in the cyclic system. The alkyl chain was engaged in hydrophobic interactions with Phe329, Tyr332 and Pro285. Additionally, compound [99mTc]Tc-Hynic-NH(CH2)9Tac created π–π stacking interaction only with Trp82 and cation–π interaction with Trp82 could be formed. The alkyl chain was engaged in hydrophobic interactions with Phe329, Tyr332 and Pro285. The radionuclide complex fragments with 99mTc and 68Ga cations were located at the entry to the gorge of butyrylcholinesterase and interacted mainly with Tyr282.

In vivo study of biodistribution shows only a qualitative view about [68Ga]Ga-DOTA-NH(CH2)9Tac radioconjugate pharmacodynamics in tissues of interest (Fig. 9). Assessment of the radiopharmaceutical ability of to cross the BBB is inconclusive and difficult to evaluate (Fig. 10), due to observed low brain uptake. Ex vivo radioactivity measurements for both radioconjugates show an expected and statistically significant relationship of brain uptake in function of acquisition time (Figs. 11–13). This enable to assume that both radioconjugates demonstrate the ability of BBB penetration. Based on these results it is possible to provide simple comparison between both radioconjugates level of activity. Crucial differences are presented in uptake levels of radioconjugates in certain regions of interest and in the way of elimination. [99mTc]Tc-Hynic-NH(CH2)9Tac shown markedly higher uptake in tissues of interest (Figs. 11–13). To each plot an initial point of measurements for both radioconjugates show an expected and statistically significant relationship of brain uptake in function of acquisition time (Figs. 11–13). This enable to assume that both radioconjugates demonstrate the ability of BBB penetration. Based on these results it is possible to provide simple comparison between both radioconjugates level of activity. Crucial differences are presented in uptake levels of radioconjugates in certain regions of interest and in the way of elimination. [99mTc]Tc-Hynic-NH(CH2)9Tac shown markedly higher uptake in all analyzed organs, and had a 4-fold higher brain uptake than gallium radioconjugate. Similarly the blood concentration of first radioconjugate was higher than the second one, due to longer effective half-life of [99mTc]Tc-Hynic-NH(CH2)9Tac in rats. Both values of effective half-life were determined theoretically, based on plotted relation of per cent of administrated activity in blood in function of time. Excretion of radioconjugates gone most likely through kidneys-liver mixed involvement clearance route in noticeable higher degree through hepatic way of elimination. Generally, the changes in radioactivity of both organs were similar in time and after 30 min were maintained.

![Fig. 13. Results of [99mTc]Tc-Hynic-NH(CH2)9Tac and [68Ga]Ga-DOTA-NH(CH2)9Tac pharmacokinetics in blood samples determined by ex vivo radioactivity measurements, with an effective (real) half-lives of radioconjugates determination from the charts. To each plot an initial point of the coordinate system was added representing an absence of radioconjugate at 0 min time point.](image-url)
5. Conclusion

Obtained radioconjugates 3h and 6h fulfill many crucial aspects for the potential diagnostic radiopharmaceuticals strictly required from the clinical application point of view. Additionally, all biodistribution studies were performed using more than one year old conjugate kits (prepared sets dedicated for a hospital use, containing the lyophilized form of appropriate amounts of reagents needed for the synthesis of a given radiopharmaceuticals) reaching labeling yields more than 95% in every case. It can be truly said that performed studies enable confirmation of analyzed radioconjugates ability to cross the BBB into the brains of rats, but in insufficient level. In our opinion, the main reason is relatively low lipophilicity of studied tracers mainly due to hydrophilic character of Hynic and DOTA chelators used (application of more lipophilic one would probably improve the effectiveness of BBB crossing). Enhanced utility of PET imaging with $[^{68}\text{Ga}]$Ga-DOTA-NH$_2$-fatty one would probably improve the effectiveness of BBB crossing. Obtained radioconjugates 3h and 6h fulfills many crucial aspects for the potential diagnostic radiopharmaceuticals strictly required from the clinical application point of view. Additionally, all biodistribution studies were performed using more than one year old conjugate kits (prepared sets dedicated for a hospital use, containing lyophilized forms of appropriate amounts of reagents needed for the synthesis of a given radiopharmaceuticals) reaching labeling yields more than 95% in every case. It can be truly said that performed studies enable confirmation of analyzed radioconjugates ability to cross the BBB into the brains of rats, but in insufficient level. In our opinion, the main reason is relatively low lipophilicity of studied tracers mainly due to hydrophilic character of Hynic and DOTA chelators used (application of more lipophilic one would probably improve the effectiveness of BBB crossing). Enhanced utility of PET imaging with $[^{68}\text{Ga}]$Ga-DOTA-NH$_2$-fatty one would probably improve the effectiveness of BBB crossing.

Both tacrine derivative radioconjugates may show an usefulness in diagnosis of cholinergic system changes and proved to be a helpful reference in further development of neuroradiodiagnosis solutions, especially for Alzheimer's disease.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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References
