Synthesis, Characterization, and Binding to the Translocator Protein (18 kDa, TSPO) of a New Rhenium Complex as a Model of Radiopharmaceutical Agents

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Abstract. A new tridentate 2-phenyl-imidazopyridin-dipropylacetamide ligand (CB239-H) with high (nanomolar) affinity for the TSPO protein was synthesized and its coordination compound with rhenium tricarbonyl, fac-[Re(CO)3(CB239-N,N,O)] was investigated. The procedure established for the synthesis of the 187/185Re complex can be also used for the synthesis of 99mTc and 188/186Re analogues, which find application in SPECT diagnosis and in therapy. Because of the tridentate coordination of CB239-H and the kinetic inertness of the carbonyl ligands, the new complex was expected to exhibit low reactivity towards plasma proteins and hence greater resistance to deactivation. Being TSPO overexpressed in numerous types of cancers and in activated microglial cells occurring in inflammatory neurodegenerative diseases, TSPO ligands can be exploited as carriers for receptor-mediated drug targeting and hence can be used in diagnosis as well as in therapy. Very surprisingly, fac-[Re(CO)3(CB239-N,N,O)] resulted to be not very stable in diluted human serum but maintained a good affinity towards TSPO.

Introduction

The 18 kDa translocator protein (TSPO, previously known as peripheral-type benzodiazepine receptor or PBR)[1] spans the mitochondrial membrane and has become an attractive target for therapeutic and imaging purposes.[2] TSPO is associated with a number of biological processes including cell proliferation, apoptosis, steroidogenesis, and immunomodulation.[3] Overexpression of TSPO has been found in numerous types of cancers including brain, breast, colon, prostate, and ovarian cancers, as well as in astrocytomas and in hepatocellular and endometrial carcinomas.[4–6] Furthermore, TSPO-specific ligands, such as 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isouquinoline carboxamide (PK11195),[7] 7-chloro-5-(4-chlorophenyl)-1-methyl-3H-1,4-benzodiazepin-2-one (Ro-5–4864),[8] and [2-(4-chlorophenyl)-8-amino-imidazo[1,2-a]-pyridin-3-yl]-N,N-di-n-propylacetamide (CB86)[9] induce apoptosis and cell cycle arrest in cancer cells (Figure 1).

For these reasons, TSPO ligands have been widely investigated for diagnostic purposes and explored as carriers for receptor-mediated drug delivery. Moreover, some of us have already prepared platinum complexes containing potent and selective TSPO ligands,[10,11] such as CB86 and 2-[6,8-dichloro-2-(1,3-thiazol-2-yl)-H-imidazo[1,2-a]pyridin-3-yl]-N,N-di-n-propylacetamide (TZ6).[12–14] The platinum complexes cis-[PtCl2(TZ6-N,N)] and cis-[PtX2(NH3)(CB86-N)] (X = Cl, I) (Figure 2) have demonstrated to keep the high affinity (nano-
molar concentration) and selectivity for TSPO characteristic of the free ligands.

TSPO expression is also increased in activated microglial cells occurring in inflammatory neurodegenerative diseases such as Alzheimer, Parkinson, Huntington, and multiple sclerosis.\[^{[15]}\] Therefore, TSPO-targeted metal complexes\[^{[6]}\] could be also explored for imaging purposes especially for the in vivo early diagnosis of neurodegenerative diseases.

To this end, we have recently prepared a $^{187/185}\text{Re}$ complex containing the bidentate TSPO ligand TZ6. This latter complex, fac-$\text{[ReBr(CO)$_3$(TZ6-$\eta^2$N,N)]}$, has been found to be endowed with high affinity for TSPO.\[^{[17]}\]

Pharmacokinetic experiments concerning complexes containing the M(CO)$_3$ core (M = Tc or Re) have addressed the question of minimal denticity of the chelate ligand for optimal clearance and stability in vivo.\[^{[18]}\] It has been observed that $^{99m}\text{Tc}$-tricarbonyl complexes containing a tridentate chelating ligand have good stability in human plasma and in the presence of excess cysteine, histidine, or glutathione.\[^{[19]}\] In contrast, complexes with bidentate ligands can be deactivated by plasma proteins both in vitro and in vivo, a phenomenon also observed in the case of platinum-based anticancer drugs.\[^{[20]}\] Therefore, tricarbonyl complexes with bidentate ligands are significantly retained in the blood and in the organs of excretion, such as liver and kidneys. Independently from their overall charge and lipophilicity, that appears to play only a limited role, the reason for such a behavior of tricarbonyl complexes with bidentate ligands can be identified in the presence of a coordination site occupied by a substitutionally labile aqua or chlorido ligand.\[^{[21,22]}\] Complexation of plasma proteins via this coordination site can explain the prolonged retention in the blood pool and, consequently, in all organs and tissues. Therefore, it is desirable to functionalize biomolecules with a tridentate chelate as this will result, hopefully, in a better pharmacokinetic behavior.

In this work we have used a new tridentate ligand with high selectivity and nanomolar affinity for TSPO, namely 2-(2-(4-chlorophenyl)-3-(2-(dipropylamino)-2-oxoethyl)imidazo[1,2-a]pyridin-8-ylamino)acetic acid (CB239-H, Figure 3), for the synthesis of the rhenium complex fac-$\text{[^{187/185}\text{Re(CO)}$_3$(CB239-N,N,O)]}$.

Besides the synthesis and complete characterization of the ligand and corresponding $^{187/185}\text{Re(CO)}$_3 complex, in vitro studies were performed to assess their affinity toward the mitochondrial TSPO. In addition, the inertness of the complex in diluted human serum was evaluated. The results are reported in this paper.

### Experimental Section

**Chemicals:** Commercial reagent grade chemicals, including [ReBr(CO)$_3$], and solvents were purchased from Sigma-Aldrich (Milan, Italy) and used without further purification.

HAM’S F12, PBS, trypsin-EDTA, penicillin (10,000 U·mL$^{-1}$), streptomycin (10 mg·mL$^{-1}$), l-glutamine solution (100×), and foetal bovine serum (FBS) were purchased from Euroclone (Italy). Disposable culture flasks and Petri dishes were from Corning, Glassworks (Corning, N.Y., USA). The radioligand $^{[3]H}$-PK11195 (85.7 Ci mmol$^{-1}$) was purchased from Perkin-Elmer Life Sciences, PK11195 was purchased from Sigma-Aldrich (Milan, Italy).

**Instrumental Measurements:** Mass spectrometry: electrospray ionization mass spectrometry (ESI-MS) was performed with an electrospray interface and an ion trap mass spectrometer (1100 Series LC/MSD Trap system Agilent, Palo Alto, CA). $^1$H 1D and 2D COSY, NOESY, and $^{[1]H-15N}$-HSQC (natural abundance $^{15}$N) spectra were recorded with Bruker Avance DPX 300 MHz and Avance II 600 MHz instruments. Standard Bruker pulse sequences were used for the NMR experiments using gradient selected versions when necessary. Chemical shifts are given in ppm. $^1$H chemical shifts were referenced by using the residual protic peak of the solvent as internal reference (δ = 3.31 ppm for [D$_4$]methanol, 2.50 ppm for [D$_6$]dimethylsulfoxide, 7.24 ppm for [D]chloroform). $^{15}$N chemical shifts were referenced to external $^{15}$NHCl (1 M in HCl 1 M). Elemental analyses were carried out with an Eurovector EA 3000 CHN.

**Synthesis of Ethyl 2-(2-(4-Chlorophenyl)-3-(2-(dipropylamino)-2-oxoethyl)imidazo[1,2-a]pyridin-8-ylamino)acetic acid (CB238):** To a stirred solution of CB86 (0.30 g, 0.78 mmol) in anhydrous THF were added, in the order, ethyl bromoacetate (860 μL, 7.8 mmol) and K$_2$CO$_3$ (0.54 g, 3.9 mmol). Stirring was continued for additional 24 h at 40 °C, then the solvent was evaporated under reduced pressure and the residue was dissolved in 20 mL of water and extracted with ether (3 × 20 mL), and dried (Na$_2$SO$_4$). Evaporation of the solvent gave a residue, which was purified by silica gel chromatography (light petroleum ether/ethyl acetate 6/4 (v/v) as eluant). Obtained 0.27 g (73.6% yield). IR (KBr): ν = 3400, 1745, 1643 cm$^{-1}$. $^1$H NMR (CDCl$_3$): δ = 0.74 (t, 3 H, J = 7.4 Hz, CH$_3$), 0.84 (t, 3 H, J = 7.4 Hz, CH$_3$), 1.28 (t, 3 H, J = 7.1 Hz, CH$_3$), 1.4–1.6 (m, 4 H, CH$_2$), 3.11 (t, 2 H, J = 7.7 Hz, CH$_2$NCO), 3.27 (t, J = 7.7 Hz, 2 H, CH$_2$NCO), 4.05 (s, 2 H, CH$_2$CON), 4.0–4.1 (m, 2 H, CH$_2$COO), 4.23 (q, J = 7.1 Hz, 2 H, CH$_2$), 6.15 (d, J = 7.4 Hz, 1 H, Ar), 6.5 (br., 1 H, NH), 6.77 (t, J = 7.1 Hz, 1 H, Ar), 7.44 (d, J = 8.2 Hz, 2 H, Ar), 7.6–7.7 (m, 3 H, Ar) ppm. ESI-MS: calculated for [CB238 + Na]$^+$ = 493. Found: m/z (% relative to the base peak) = 493.1 (100) [M + Na]$^+$. Synthesis of 2-(2-(4-Chlorophenyl)-3-(2-(dipropylamino)-2-oxoethyl)imidazo[1,2-a]pyridin-8-ylamino)acetic Acid (CB239-H): To a solution of CB238 (0.20 g, 0.42 mmol) in EtOH (5 mL), NaOH (1 M, 5 mL) was added dropwise. The mixture was stirred at room temperature for 2 h. Afterwards the solvent was evaporated under reduced pressure and the residue was dissolved in 20 mL of water and extracted with ethyl acetate (3 × 20 mL). The cooled water phase was acidified...
with 0.1 M HCl and the resulting precipitate of the pure acid was collected by filtration and dried in a vacuum. Obtained 158 mg (85.1 % yield). IR (KBr): ν = 3398, 1639, 1566 cm⁻¹; \(^1H\) NMR (CDCl₃): δ = 0.77 (t, 3 H, J = 7.4 Hz, CH₃), 0.85 (t, 3 H, J = 7.4 Hz, CH₃), 1.4–1.6 (m, 4 H, CH₂), 3.11 (t, 2 H, J = 7.7 Hz, CH₂NCO). 3.28 (t, J = 7.7 Hz, 2 H, CH₂NCO), 3.93 (s, 2 H, CH₂CO), 3.98 (s, 2 H, CH₂CON), 6.25 (d, J = 7.4 Hz, 1 H, Ar), 6.86 (t, J = 7.1 Hz, 1 H, Ar), 7.41 (d, J = 8.2 Hz, 2 H, Ar), 7.53 (d, J = 6.6 Hz, 1 H, Ar), 7.67 (d, J = 8.2 Hz, 2 H, Ar) ppm. ESI-MS: calculated for [CB239]+ = 441. Found: m/z (% relative to the base peak) = 440.9 (100) [M – H].

**Synthesis of fac-[ReBr(CO)₃(OH₂)]²⁺**: This compound was obtained by applying the procedure reported for the synthesis of the compound fac-[Re(H₂O)₅(CO)₃]Br[23] [ReBr(CO)₃] (900 mg, 2.21 mmol) was suspended in water (40 mL) in a 250 mL round-bottom flask and heated to reflux for 24 h under gentle magnetic stirring. Periodic rinsing of the reflux condenser allowed unreacted [ReBr(CO)₃] deposited on the condenser to be brought back into the reaction solution. The crude mixture was cooled to room temperature and filtered through celite to remove small amounts of impurities. The colorless solution was dried under reduced pressure to give a white powder of the desired compound. Obtained 665 mg (76.9 % yield). [ReBr(CO)₃(OH₂)] (C₇H₅O₂BrRe): calc. C 9.33; H 1.04 %; found: C 9.42; H 1.10 %. ESI-MS: calculated for [Re(CO)₅(OH₂)]⁺ (C₁₆H₁₇O₄Re) = 325; found: m/z (% relative to the base peak) = 324.9 (100).

**Synthesis of fac-[Re(CO)₃(CB239-N,N,O)]**: CB239-H (31.0 mg, 0.07 mmol) was dissolved in methanol (8 mL) and the solution was treated with fac-[ReBr(CO)₃(OH₂)] (29.9 mg, 0.07 mmol) dissolved in methanol (3 mL). The resulting solution was kept under magnetic stirring for 1 h at room temperature and treated with a solution of KOH in methanol (0.07 mmol in 300 mL). The reaction mixture was kept whilst stirring at room temperature for 1 h and filtered in order to separate the precipitated white solid (KBr). The filtrate was concentrated to a final volume of ca. 3 mL and addition of ca. 10 mL of water caused the precipitation of a brown solid. The crude product was suspended in water (40 mL) in a 250 mL round-bottom flask and treated with a solution of KOH in methanol (0.07 mmol in 300 mL). The mixture was filtered through celite to remove small amounts of impurities. The colorless solution was dried under reduced pressure to give a white powder of the desired compound. Obtained 656 mg (76.9 % yield). 

Radioligand Binding Assay at TSPO: Binding of [¹H]-PK11195 at TSPO was performed according to Denora et al. with minor modifications.[14] In 0.5 mL of incubation buffer (PBS, pH 7.2) were suspended 100 µg of C6 membranes, 0.7 nM [¹H]-PK11195, and the compound under investigation or the reference compound (six to nine different concentrations). The samples were incubated for 90 min at 25 °C. Subsequently the incubation was stopped by rapid filtration through Whatman GF/C glass microfiber filters (pre-soaked in 0.3 % polyethyleneimine for 20 min) and the filters washed with 3 × 1 mL of ice-cold buffer (PBS, pH 7.2). Nonspecific binding was determined in the presence of 10 µM PK11195. Approximately 90 % of specific binding was determined under these conditions.

**Results and Discussion**

Technetium and rhenium have very similar chemistry that enables the same experimental conditions to be applied to the preparation of radiopharmaceuticals for diagnostic (99mTc) and therapeutic applications [¹⁸⁶,¹⁸⁸Re] in nuclear medicine.[24] Therefore, nonradioactive rhenium complexes are often used as model compounds for the ⁹⁹mTc congeners.[25–30] Technetium and rhenium offer also the possibility of exploring a large variety of oxidation states in relation to different types of ligands and bifunctional and trifunctional chelators.[31–34] Following the introduction by Alberto and co-workers of a convenient and fully aqueous kit preparation method for the organometallic precursors fac-[M(OH₂)₃(CO)₃]⁺ (M = Tc, Re),[35,36] the so-called tricarbonyl approach has gained considerable attention.

Within the tricarbonyl approach, it is possible to coordinate to the metal core uni-, bi-, and tridentate ligands, the last chelator usually providing complexes with a higher chemical robustness and enhanced in vivo stability. For this reason, tridentate ligands are considered the most appropriate for stabilizing the fac-[M(CO)₅]⁺ (M = Tc, Re) core in biological media. We have recently prepared a ¹⁸⁷/¹⁸⁹Re complex containing a bidentate ligand, namely fac-[ReBr(CO)₃(TZ6-N,N)], endowed with high affinity for the TSPO.[17] Preliminary stability studies showed that fac-[ReBr(CO)₃(TZ6-N,N)] is completely degraded in diluted human serum (50 %) in about 3.6 h time. In the presented work, we have extended the investigation to a new TSPO ligand, also having high affinity (nanomolar) and
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Scheme 1. (a) THF anhydrous at 40 °C, ethyl bromoacetate, K₂CO₃; (b) EtOH and 1 M aqueous NaOH at 40 °C; (c) MeOH, fac-[ReBr(CO)₃(OH₂)₂], and KOH; room temperature.

selectivity for the protein, but which can act as tridentate chelator towards rhenium forming the complex fac-[¹⁸⁷/¹⁸⁵Re(CO)₃(CB239-N,N,O)], which has all coordination sites occupied and therefore should be endowed with higher inertness in biological medium.

Synthesis and Characterization of the TSPO-ligand CB239-H

The synthesis of CB239-H was achieved by the procedure shown in Scheme 1. In particular, compound CB239-H was obtained by hydrolysis of the corresponding ester, CB238, in aqueous NaOH/ethanol (1:1 v:v) solution. In turn, CB238 was synthesized by reacting the TSPO ligand CB86[12] with ethyl bromoacetate in anhydrous THF. CB239-H was fully characterized by elemental analysis, ESI-MS, IR, and ¹H NMR spectroscopy as reported in the Experimental Section.

Synthesis and Characterization of Rhenium Compounds

Fac-[ReBr(CO)₃(OH₂)₂] was obtained by applying the procedure previously reported for the synthesis of fac-[Re(H₂O)₃(CO)₃]Br.[23] Differently from Zubieta et al.,[23] who isolated the light green complex fac-[Re(CO)₃(OH₂)₂]Br by concentration of the mother solution, we obtained the neutral white complex fac-[ReBr(CO)₃(OH₂)₂] by complete evaporation of the mother liquor. The neutral or slightly acidic pH of our solution prevented the formation of dimeric and oligomeric hydroxo-bridged rhenium compounds, which have been reported to form predominantly in basic conditions.[37,38]

Moreover, the ESI-MS spectra did not show any peak having patterns assignable to Re₂ or Re₃ clusters.

The synthesis of fac-[Re(CO)₃(CB239-N,N,O)] (Scheme 1) was carried out in methanol by reaction of CB239-H with 1.1 equivalents of fac-[ReBr(CO)₃(OH₂)₂]. Treatment with KOH allowed the deprotonation of the carboxylic group in position 8 of the imidazopyridine ring and its coordination to rhenium. The final, pure, fac-[Re(CO)₃(CB239-N,N,O)] complex was obtained in good yield by column chromatography on silica gel.

The complex fac-[Re(CO)₃(CB239-N,N,O)] was characterized by elemental analysis, ESI-MS, ¹H 1D NMR and 2D NOESY, COSY and [¹H-¹⁵N]-HSQC experiments. The ESI-MS analysis revealed the presence of a peak at m/z (% relative to the base peak) = 734.9 (100) corresponding to the species ([Re(CO)₃(CB239-N,N,O)] + Na⁺). The experimental isotopic pattern was coincident with the theoretical one.

The ¹H NMR spectrum (Figure 4) shows, in the range of 4.5–3.8 ppm, the presence of two overlapping AB systems, one each for the methylene groups 9 and 16 (see Figure 3 for numbering of protons). Because of the asymmetry of the central rhenium core, the two protons of each methylenic group become diastereotopic and generate an AB spin system.

The most deshielded AB system (centered at δ = 4.18 ppm) was assigned to methylene 9 because of its spatial correlations with the singlet at δ = 7.59 ppm (4 H) (Figure 5, cross-peak A), belonging to the protons of the 4-Cl-phenyl group (accidentally equivalent), and with the doublet at δ = 8.32 ppm (Figure 5, NOESY cross-peak B) assigned to proton 5 of the imidazopyridine ring. The characterization of the imidazopyri-
dine ring continued with the assignment of the triplet at δ = 7.23 ppm (1 H) to proton 6 (Figure 6, cross-peak C with proton 5) and of the doublet at δ = 7.65 ppm (1 H) to proton 7 (Figure 6, COSY cross-peak D with proton 6). Finally, proton 7 shows a NOESY cross-peak (E in Figure 5) with the most shielded of the above mentioned AB systems (δ = 4.09 ppm), which was assigned to methylene 16.

In order to check if the aminic nitrogen in position 8 of the imidazopyridine ring loses its proton after coordination to rhenium, a 2D [1H-15N]-HSQC spectrum in [D₆]DMSO (Figure 7) was recorded. The spectrum shows the presence of a cross-peak falling at 8.99/28.2 ppm (1H/15N) consistent with the presence of an aminic proton that was not detectable in [D₄]methanol as a result of the exchange with the deuterium of the solvent.

Stability Studies

Compound fac-[Re(CO)₃(CB239-N,N,O)] was challenged in diluted (50%) human serum/phosphate buffer (0.02 M, pH 7.4, isotonized with NaCl) at 37 °C to determine its inertness. An almost immediate reaction of the rhenium complex with the serum proteins was observed. This result was quite unexpected and does not comply with the general trend that complexes, in which the M(CO)₃ core (M = ⁹⁹ᵐTc or ¹⁸⁶/¹⁸⁸Re) is...
bound to a tridentate chelating system have good stability in human plasma.\textsuperscript{188} We are currently performing other stability studies in order to unravel the cause of such an unexpected reactivity and, consequently, make the required changes in the metal-coordinating groups.

**Radioligand Binding Assays**

The affinity of fac-[Re(CO)\textsubscript{3}(CB239-N,N,O)] was evaluated by measuring its ability to displace the reference compound \(^{3}H\)-PK 11195 from binding to TSPOs in membrane of C6 glioma cells, a tumour cell line, which is well known to over-express TSPO.\textsuperscript{15}

The affinity (Table 1) of the free ligand CB239-H and that of fac-[Re(CO)\textsubscript{3}(CB239-N,N,O)] for the TSPO protein is quite good, namely 2.43 and 190 nM, respectively. While the affinity of CB239-H is comparable to that of the reference compound PK11195, that of fac-[Re(CO)\textsubscript{3}(CB239-N,N,O)] is slightly lower but still good for biological applications.

**Table 1. Affinities (Ki \(\pm \text{SEM}\)) of fac-[Re(CO)\textsubscript{3}(CB239-N,N,O)] for TSPO from rat cerebral cortex. Corresponding values for PK11195 and CB239-H are also reported for comparison.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ki (nM) ± SEM</th>
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<tbody>
<tr>
<td>PK11195</td>
<td>1.05 ± 0.05</td>
</tr>
<tr>
<td>CB239-H</td>
<td>2.43 ± 0.06</td>
</tr>
<tr>
<td>fac-[Re(CO)\textsubscript{3}(CB239-N,N,O)]</td>
<td>190 ± 43</td>
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</tbody>
</table>

**Conclusions**

In this work, a new 2-phenyl-imidazopyridin-dipropylacetamide ligand (CB239-H), with high affinity for the TSPO protein, was synthesized and the corresponding \(^{187}/^{188}\text{Re} \) complex fac-[Re(CO)\textsubscript{3}(CB239-N,N,O)] prepared. NMR techniques (\(^{1}H\) 1D and 2D COSY, NOESY, and \(^{1}H-^{15}N\)-HSQC experiments) were used to fully characterize both the free ligand and the new rhenium complex. The ligand appears to be tri-coordinated to the metal by the imidazopyridine nitrogen, the secondary amine nitrogen, and the carboxylate group.

The affinity of the new compounds for TSPO was evaluated in vitro on membrane extracts from C6 rat glioma cells. Fac-[Re(CO)\textsubscript{3}(CB239-N,N,O)] and CB239-H have demonstrated to be endowed with good affinity for the target protein, namely 190 and 2.43 nM, respectively.

Even though the chemical robustness of fac-[Re(CO)\textsubscript{3}(CB239-N,N,O)] in diluted human serum was lower than expected for a Re(CO)\textsubscript{3} core bound to a tridentate ligand, CM1105, Metallo-Drug Design and Action), and the Inter-University Consortium for Research on the Chemistry of Metal Ions in Biological Systems (C.I.R.C.M.S.B.) are gratefully acknowledged.

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