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Article

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^{99m}Tc-labeled Vasopressin Peptide as a Radiopharmaceutical for Small-Cell Lung Cancer (SCLC) Diagnosis

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ABSTRACT

The ^{99m}Tc-labeled conjugates of the vasopressin (AVP) peptide and of its analogue d$(CH_2)_5$[D-Tyr(Et^2)-Ile^4-Eda^9]AVP (AVP(an)) have been synthesized using the technetium complexes with tetradentate tripodal chelator – the tris(2-mercaptoethyl)-amine (NS_3) – and the monodentate isocyanide ligand (CN-peptide). The conjugates exhibit high stability in the presence of 100 times the molar excess of standard amino acids cysteine or histidine, and also satisfactory stability in human serum. The ^{99m}Tc(NS_3)(CN-AVP) and ^{99m}Tc(NS_3)(CN-AVP(an)) ability of binding to small-cell lung cancer (SCLC) cell line H69 was studied *in vitro*. The results suggest that the novel vasopressin conjugate ^{99m}Tc(NS_3)(CN-AVP(an)) is a desirable compound for imaging oncogene receptors overexpressed in SCLC cells and can be an important basis for further consideration the conjugate as a potential diagnostic radiopharmaceutical for patients suffering from the small-cell lung cancer.

Keywords: technetium, vasopressin, radiopharmaceutical, small cell lung cancer (SCLC), receptor, cell line H69

1. INTRODUCTION

The more and more important role in the medicine of present-days is played by radiopharmaceuticals, the compounds labeled with various short-lived radionuclides, which are introduced into the body for either diagnostic or therapeutic purposes. The introduced doses of radiopharmaceuticals are at so low level (nanomolar) that they do not influence the function of the biological organ. The radiopharmaceuticals, which selectively distribute within given tissues or organs, are usually coordination compounds with a central ion of a radiometal, firmly attached to a biologically active molecule (peptide). The measurement of space distribution of the intensity of the gamma rays emitted from the body makes possible to determine localization of the radiopharmaceuticals within the body and to define abnormalities in the tissue structures or in the functioning of the studied organs. Such methods are used to image receptors of the central nervous system, functions of heart, brain, kidneys, liver, bile-duct and skeleton, in order to determine and localize cancers.[1] The diagnostic methods of nuclear medicine make possible detection of diseases at their early stage, much earlier than the accompanying morphological changes could be detected by methods of classical medicine. Such early and apposite diagnosis strongly promotes the effectiveness of the consecutive therapy. Among the most important diagnostic radiopharmaceuticals are those labeled with technetium-99m – the radionuclide of nearly ideal nuclear properties and of rich coordination chemistry.[2-4]

The goal of this work was to label the vasopressin peptide Cys^1-Tyr^2-Phe^3-Gln^4-Asn^5-Cys^6-Pro^7-Arg^8-Gly^9 (AVP) and its analogue $d(CH_2)_5$[D-$Tyr(Et^2)$,Ile^4,Eda^9]AVP (AVP(an)) with technetium-99m radionuclide.

Vasopressin (arginine vasopressin, (Arg^8)-Vasopressin, **AVP**), also known as argipressin or antidiuretic hormone (ADH), is a cyclic peptide containing residues of nine amino acids. Two of them are the molecules of cysteine (Cys^1 and Cys^6). Because of the disulfide bond

between them, vasopressin forms a cyclic six-membered amino acid ring with three amino acid residues hanging out. Vasopressin is a peptide hormone found in most mammals, including humans. It is derived from a preprohormone precursor synthesized in the hypothalamus, from which it is liberated during transport to the posterior pituitary. AVP regulates the body retention of water, being released when the body is dehydrated (antiduretic action of AVP, mediated *via* V2 type receptors). It causes the kidneys to conserve water (but not salt) and to reduce urine volume. AVP also raises arteriar blood pressure by inducing moderate vasoconstriction (pressor action of AVP, mediated *via* V1 type receptor). In addition to its predominantly antidiuretic and pressor activities vasopressin, as a neuropeptide, generates a variety of neurological effects in the central nervous system (CNS). It is also involved, *via* different receptor subtypes, in higher brain functions, including cognitive abilities and emotionality. In recent years interest increased in the role of vasopressin in social behavior and in its participation in such diseases as schizophrenia and autism.[5-8] Normal plasma concentration of AVP is lower than 4 pg/ml and a half-life of AVP is about 10 ÷ 35 min (due to its biodegradation by vasopressinases which are found in the liver and kidney). [9,10] Decreased AVP level leads to diabetes insipidus, and therefore the increase of this period (to avoid recognition by degrading enzymes) is important and can be achieved by removing the N-terminal amine group of Cys^1 and using the non-natural D-arginine, instead of natural L-arginine at the position 8.[11]

The analogue of AVP, the $d(CH_2)_5[D\text{-}Tyr(Et^2)\text{-}Ile^4\text{-}Eda^9]AVP$ compound (**AVP(an)**), is one of the several effective antagonists to the antidiuretic arginine vasopressin V2-receptor.[5,6] Arginine vasopressin receptor 2 (AVPR2, also called V2 receptor) belongs to the seven-transmembrane, G-protein–coupled receptor family, and its intracellular signaling pathway involves a Gs/adenylyl cyclase-stimulating system that induces cAMP formation.[12] The V2 receptors are present in greatest amounts in the kidney, but recently, polymerase chain

reaction studies revealed that V2 receptor mRNA is also present in other tissues or cells such as brain, liver and lung.[13,14]

The overexpression of vasopressin receptor V2 has been found on small-cells of lung cancer (SCLC).[13,15-18] The SCLC accounts for about 20% of all lung cancers and usually involves a tumor of neuroendocrine origin with very low survival rate. The SCLC cells have a high proliferation rate and spread to lymph nodes and other vital organs, that is, brain, liver and bone. The 5-year survival rate is below 20%.[19]

The essential features of AVP affinity for V2 receptor are: the ring formed *via* disulfide bond between Cys^1 and Cys^6, the three amino acid "tail", the presence of tyrosine residue at position 2, and the C-terminal in glycine blocked by NH_2. The use of radiolabeled AVP targeting vectors to selectively target receptor-expressing tumors offers innovative diagnostic and treatment strategies for patients suffering from small-cell lung cancer.[20]

The aim of the experimental work was to synthesize and investigate novel conjugates of '4+1' mixed-ligand Tc(III)-99m complexes with AVP and its analogue AVP(an) (Figure 1).

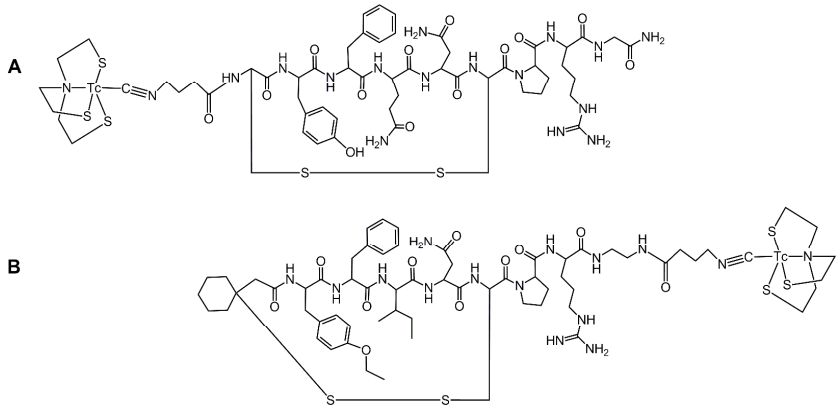


Figure 1. (A) ^{99m}Tc labeled **AVP**. (B) ^{99m}Tc labeled **AVP(an), AVP(an)**=d$(CH_2)_5$[D-Tyr(Et^2)-Ile^4-Eda^9]AVP.

These two conjugates are very stable not only in the thermodynamic sense, but also towards ligand exchange *in vivo*.[21-23] The conjugates ^{99m}Tc(RNS_3)(CN-peptide) consist of central metal ion Tc(III) coordinated by the tetradentate RNS_3 tripodal chelator (R=H, tris(2-mercaptoethyl)-amine) and a monodentate isocyanide species, previously coupled with the peptide. Introduction of a hydrophilic or hydrophobic group R at the periphery of the NS_3 ligand may change the lipophilicity of the complex molecule in such a way that the lipophilicity of the whole peptide-radiometal conjugate will be close to that of the free peptide.[21,24,25]

To verify the identity of the ^{99m}Tc-labeled peptides, the non-radioactive 'cold' rhenium reference compounds Re(RNS_3)(CN-peptide) have been synthesized and characterized by elemental analysis and mass spectrometry.

2. RESULTS AND DISCUSSION

2.1. AVP and AVP(an) labeling, stability, electrophoresis and lipophilicity studies

The HPLC chromatograms (using System 2) of the compounds Re(NS_3)(CN-AVP), ^{99m}Tc(NS_3)(CN-AVP), Re(NS_3)(CN-AVP(an)) and ^{99m}Tc(NS_3)(CN-AVP(an)) are shown in Figure 2. The retention times (R_T) determined for the studied conjugates are 11.7 min, 12.0 min, 15.9 and 16.3 min, respectively. Practically the same positions of the peaks of Re(NS_3)(CN-AVP) and ^{99m}Tc(NS_3)(CN-AVP) as well as of Re(NS_3)(CN-AVP(an)) and ^{99m}Tc(NS_3)(CN-AVP(an)) can be a proof of the existence of the species ^{99m}Tc(NS_3)(CN-AVP) and ^{99m}Tc(NS_3)(CN-AVP(an)) synthesized in n.c.a. (no carrier added) scale. The small peaks recorded at R_T = 3.4 min and 7.9 min correspond to the intermediate complexes ^{99m}Tc-EDTA/mannitol and ^{99m}Tc(NS_3), respectively.

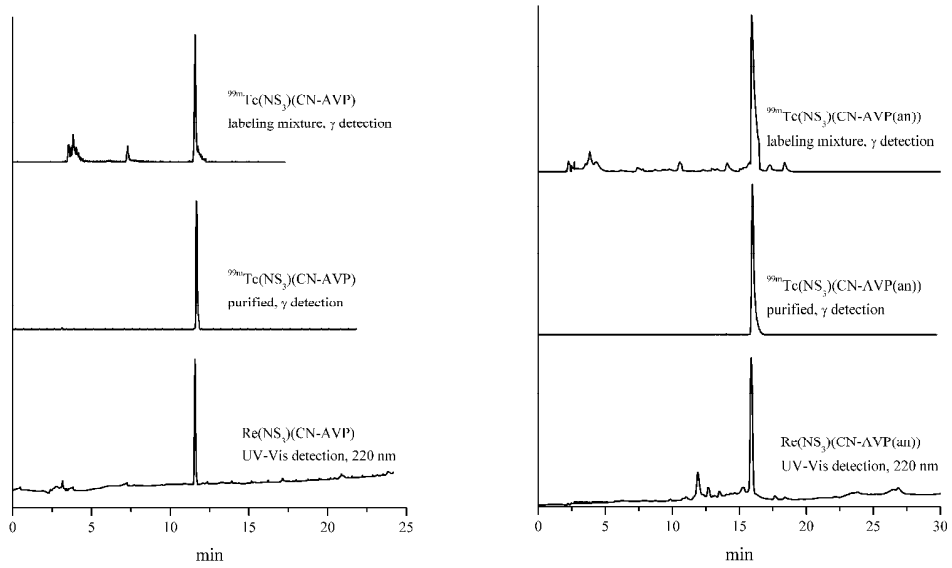


Figure 2. The HPLC chromatograms of the Tc/Re conjugates prepared in this study (system 2).

The ^{99m}Tc-labeled vasopressin conjugates ^{99m}Tc(NS$_3$)(CN-AVP) and ^{99m}Tc(NS$_3$)(CN-AVP(an)) were synthesized with radiochemical yield higher than 95% and specific activity of about 25 GBq/μmol of the conjugate.

Stability of ^{99m}Tc-labeled vasopressin conjugates ^{99m}Tc(NS$_3$)(CN-AVP) and ^{99m}Tc(NS$_3$)(CN-AVP(an)) is shown in Figure 3. Vertical axis presents the percentage of intact conjugates in the challenge experiments with an excess (10 mM) of histidine or cysteine. Both conjugates are sufficiently stable for up to 24 h. In both cases the HPLC chromatograms showed presence of only one radioactive species in the solution, with the retention time characteristic for either the conjugate ^{99m}Tc(NS$_3$)(CN-AVP) or ^{99m}Tc(NS$_3$)(CN-AVP(an)), respectively. Thus, we can consider that the conjugates *in vitro* will not undergo the ligand exchange reaction with amino acids or other strongly competing natural ligands containing SH or NH groups.

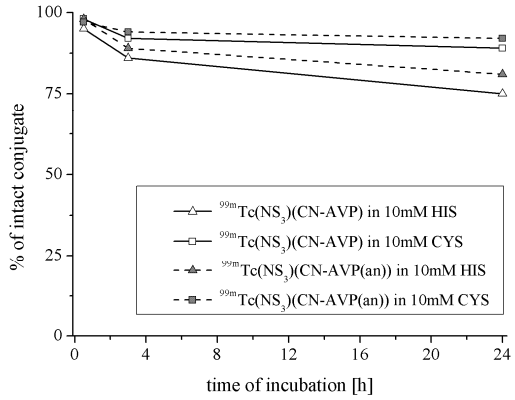


Figure 3. Histidine and cysteine challenge experiments showing the percentage of intact $^{99m}Tc(NS_3)$(CN-AVP) and $^{99m}Tc(NS_3)$(CN-AVP(an)) conjugates remaining after different periods of incubation at 37 ^{o}C.

Stability of $^{99m}Tc(NS_3)$(CN-AVP) and $^{99m}Tc(NS_3)$(CN-AVP(an)) conjugates in human and rat serum is shown in Figures 4 and 5, respectively. Investigations of the behavior of the isolated $^{99m}Tc(NS_3)$(CN-AVP) conjugate in human serum have shown that the studied complex is stable within about 10-12 minutes. After this period, corresponding to the half-live of vasopressin peptide *in vivo*, a new peak appeared on the HPLC chromatograms as the result of the enzymatic vasopressin degradation (Figure 4 left). According to the literature data there are two major sites of cleavage of vasopressin: the Tyr-Phe and Arg-Gly bonds.[26] The vasopressin derivative, which lacks free N-terminal amine group (in our case the N-terminal amine group of vasopressin is blocked by bonding with the isocyanide linker CN-BFCA) is more stable and in this case the main biodegradation product results from the cleavage of the Arg-Gly bond.

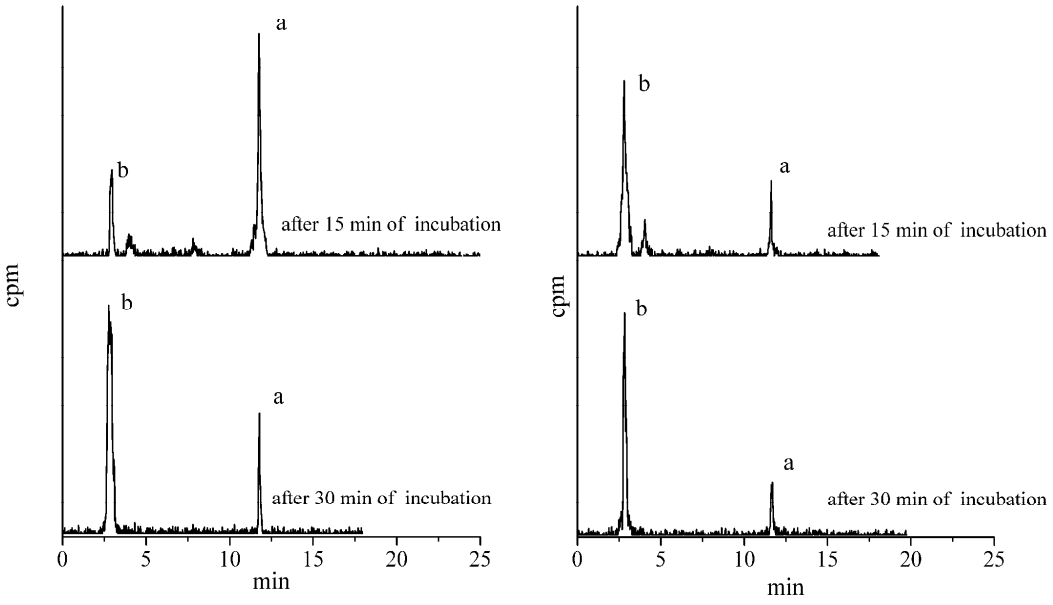


Figure 4. HPLC chromatograms of $^{99m}Tc(NS_3)$(CN-AVP) conjugate after incubation at 37 ^{o}C in human (left) and rat serum (right).

The HPLC chromatograms obtained after incubation of $^{99m}Tc(NS_3)$(CN-AVP) conjugate in human or in rat serum (Figure 4) show two peaks: (**a**) at R_T=11.8 min, corresponding to the $^{99m}Tc(NS_3)$(CN-AVP) conjugate (AVP = Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly), and (**b**) at R_T=2.8 min, corresponding, most probably, to the $^{99m}Tc(NS_3)$(CN-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg) analogue. Comparing the results of incubation obtained in the case of human (Figure 4 left) and rat (Figure 4 right) serum, one can see that biodegradation of vasopressin in rat serum is more rapid and the peak **b** at R_T=2.8 min appears already after about fifth minute of incubation. The removal of the C-terminal glycine from the vasopressin "tail" results in formation of significantly less lipophilic species and, according to the literature[26], is also responsible for the inactivation of the AVP peptide towards its V2 receptor. To avoid the cleavage of the Arg-Gly bond in the vasopressin peptide, the (D-Arg^{8})-Vasopressin (with the

non-natural D-arginine instead of naturally existing L-arginine in the position 8) can be used.[11]

The HPLC chromatograms obtained after incubation of $^{99m}Tc(NS_3)$(CN-AVP(an)) conjugate in human or rat serum are shown in Figure 5. The results show that the AVP(an) biomolecule, being the antagonist to the V2 vasopressin receptor, is stable in both human and rat serum.

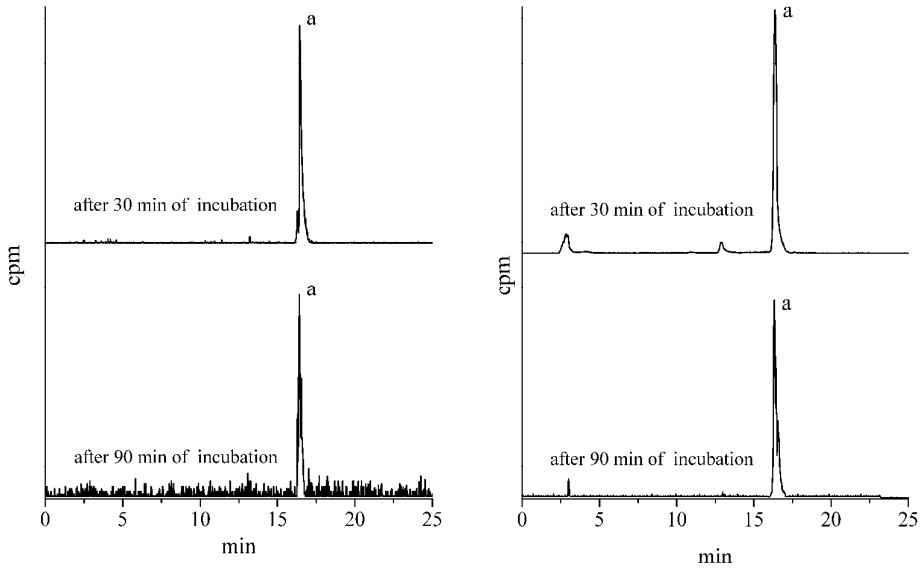


Figure 5. HPLC chromatograms of $^{99m}Tc(NS_3)$(CN-AVP(an)) conjugate after incubation at 37 ^{o}C in human (left) and rat (right) serum.

The electropherograms recorded during paper electrophoresis experiments for ^{99m}Tc-labeled AVP and ^{99m}Tc-labeled AVP(an) showed only one peak located at the starting position (a shift neither towards cathode nor anode was observed). These results confirmed the expected lack of charge on the studied conjugates.

The *log D* values obtained for ^{99m}Tc-labeled AVP and ^{99m}Tc-labeled AVP(an) are close to each other and are equal to -0.48±0.02 and -0.44±0.03, respectively (average values from five

independent measurements). These values, significantly higher than the lipophilicity of the free peptide, for which *log D* is equal to -2.15,[27] may be decreased by introducing a hydrophilic group, R, at the periphery of the NS_3 ligand[24,25,28], with no changes in the stability of the whole conjugate.

2.2. Binding studies

Affinity of different ^{99m}Tc-species to the V2 receptor of H69 cells is shown in Figure 6. Vertical axis presents affinity of the studied ^{99m}Tc-species, evaluated as a ratio of activity bound by cells to that of sum of activity bound by cells and that which remained in the solution above the cells. As one can see only ^{99m}Tc-species containing vasopressin peptide or its analogue interact with V2 receptors, and the interaction of ^{99m}Tc(NS_3)(CN-AVP(an)) is more effective than that of ^{99m}Tc(NS_3)(CN-AVP). Basing on these results the conjugate ^{99m}Tc(NS_3)(CN-AVP(an)) has been selected for the subsequent biological studies.

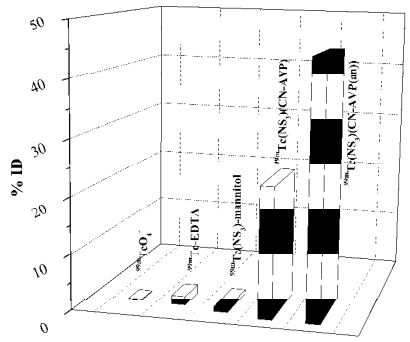


Figure 6. *In vitro* cell binding of different ^{99m}Tc-species synthesized in this work. Cell binding levels are expressed as the percentage of added doses (%ID).

Binding of the conjugate ^{99m}Tc(NS_3)(CN-AVP(an)) to V2 receptor of H69 cells was found to be saturable and specific in the predominant degree (Figure 7A). The non-specific binding determined in the presence of 0.1 μM solution of AVP(an) accounts in general for 6%

of specific binding. The B_{max} value was found to be 0.0058 ± 0.0003 nM (n=3), which corresponds to an approximate number of 7,000 binding sites per cell. The dissociation constant K_d, usually used to describe the affinity of the conjugate to its receptor (Figure 7B), was found to be 0.13 ± 0.04 nM (n=3) and the K_i value, calculated according to the Cheng-Prusoff equation[29] was found to be 0.11 ± 0.03 nM. Based on literature data concerning affinity of different peptidic and non-peptidic agonists and antagonists to arginine vasopressin receptors one can say that generally both non-peptide compounds and agonists are characterized with lower K_d values (higher binding affinity) than peptide compounds and antagonists, respectively.[13,18,30] Nevertheless the binding reaction is temperature dependent and tissue is temperature sensitive, as well as the same type of receptor expressed on cell surface of different cell lines can exhibit different biological behavior, the experimentally measured values (obtained by scientific teams usually under different conditions) can not be consider as 'absolute' and their comparison need to be qualitative only. Comparing our results with those presented in literature one can see that $^{99m}Tc(NS_3)$(CN-AVP(an)) conjugate is characterized with significantly low values of K_d and K_i which indicate high binding of the $^{99m}Tc(NS_3)$(CN-AVP(an)) conjugate to the V2 receptor of H69 cells.

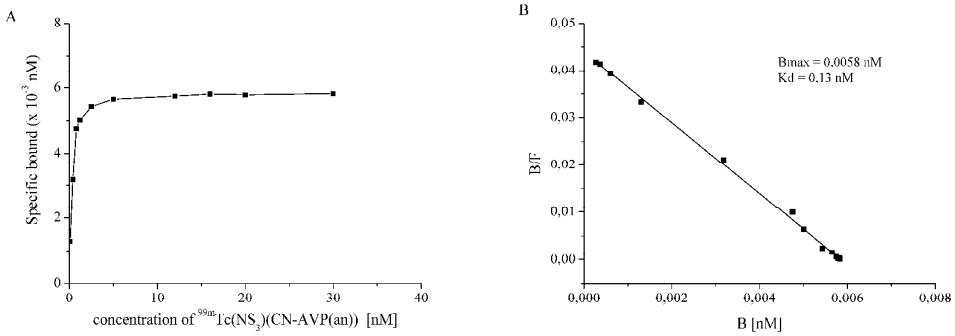


Figure 7. (A) Saturation curve for $^{99m}Tc(NS_3)$(CN-AVP(an)). The specifically bound radioligand is plotted as a function of increasing concentration of $^{99m}Tc(NS_3)$(CN-AVP(an)). (B) Scatchard plot.

In a competitive binding experiment AVP(an) inhibited the binding of $^{99m}Tc(NS_3)$(CN-AVP(an)) to V2 receptor and the inhibitory concentration of 50% (IC_{50}) was found to be 29 ± 1.3 nM (Figure 8). This value also confirms satisfactorily high binding of $^{99m}Tc(NS_3)$(CN-AVP(an)) conjugate to V2 receptor in SCLC cells H69.

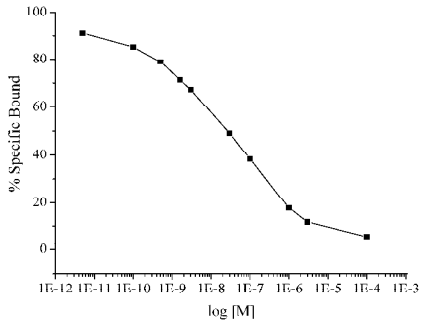


Figure 8. Displacement of $^{99m}Tc(NS_3)$(CN-AVP(an)) by increasing concentration of unlabeled AVP(an).

3. EXPERIMENTAL PROCEDURES

3.1. Materials and methods

(Arg^8)-Vasopressin peptide was a commercially available product, Bachem, Cat. Number: H-1780. The analogue ***d(CH$_2$)$_5$[D-Tyr(Et^2)-Ile^4-Eda^9]AVP*** was a gift from Professor Maurice Manning, University of Toledo, Toledo, Ohio, United States. Human male SCLC (Small Cell Lung Cancer) ***cell line NCI-H69*** was obtained from Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Poland, Wrocław). ***ATCC-formulated RPMI-1640 Medium*** (Catalog No. 30-2001) was obtained from ATCC (USA). ***Cell Dissociation Solution Non-enzymatic 1x*** (Catalog No. C5914) was obtained from Sigma-Aidrich. All solvents and commercially available substances were of reagent grade and used without further purification. Twice distilled water was used throughout.

The gradient and HPLC conditions were as follows: solvent A, 0.1% (v/v) TFA in water; solvent B, 0.1% (v/v) trifluoroacetic acid (TFA) in acetonitrile; System 1: 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 to 80 % solvent B, 20 min 80 % solvent B; 2 ml/min.; System 2: analytical Phenomenex Jupiter Proteo column, 4 μm, 90 Å, 250 × 4,6 mm, γ-detection, gradient elution: 0-20 min 20 to 80 % solvent B, 20 min 80 % solvent B; 1 ml/min.

$Na^{99m}TcO_4$ was eluted from a commercial $^{99}Mo/^{99m}Tc$ generator (Radioisotope Centre POLATOM, Institute of Atomic Energy, Poland).

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

Infrared (IR): IR spectra in solid KBr pellets (investigated species being about 1% of the pellet) were recorded in the range 4.000-600 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.

The purity of the synthesized compounds was determined using analytical HPLC and was found to be more than 95%.

3.2. Syntheses

The tetradentate NS_3 ligand (tris(2-mercaptoethyl)-amine; 2,2',2''-nitrilotriethanethiol) was prepared by two steps reaction according to the procedure described in ref.[31]. In the first step 850 mg (3.52 mmol) of tris(2-chloroethyl)amin hydrochloride and 2.04 g (17.85 mmol) of potassium thio-acetate in 15 ml of ethanol were refluxed (at 75°C) for 5 hours. After the

solvent removing the residue was dissolved in 4 mL of 1 M aqueous solution of KOH and extracted three times with diethyl ether. The product of this step – tris(thioacetate) was a yellow oil. Yield ≅ 68%. In the second step tris(thioacetate), 500 mg (1.54 mmol) dissolved in 5 ml of dry THF, was reduced at 0 °C with $LiAlH_4$, 250 mg (7.15 mmol) dissolved in 10 ml of dry THF. The final product, yield ≈ 45%, was precipitated as the oxalate salt and applied as such in further reactions.

EA: Calcd.: C 36.51; H 7.66; N 7.10; Found: C 36.52; H 7.27; N 7.62

^{1}H NMR ($CDCl_3$) δ (ppm): 1.80 (s, 3H, S**H**); 2.72 (m, 12H, NC**H**$_2$ and SC**H**$_2$)

^{13}C NMR ($CDCl_3$) δ (ppm): 822.81 (H_2**C**S); 57.01 (N**C**H_2)

IR (KBr) (cm^{-1}): 2987 (**CH**); 2425 (**SH**)

The aliphatic linker CN-BFCA (BFCA = Bifunctional Coupling Agent, isocyanobutyric succinimidyl ester) was synthesized in a three step according to procedure described in ref.[32] In the first step 4-aminobutanoic acid, 30 g (290.92 mmol), dissolved in 1.5 mL of acetic anhydride was refluxed with 225 mL of formic acid at 100 -115°C for 8 h. After the solvent was removed and the residue was stirred in a mixture of diethyl ether, acetonitrile, and ethyl acetate (30/5/5, v/v/v), the product was collected and dried. Yield ≈ 30%. In the second step equal molar quantities of 4-(formylamino)-butanoic acid, N-hydroxysuccinimide and dicyclohexylcarbodiimide were dissolved in 25 mL of dimethylformamide (DMF) and the reaction was stirred at room temperature overnight. The reaction mixture was filtered, the solvent was evaporated *in vacuo* and the residue was dissolved in solvent mixture (diethyl ether/acetonitrile/ethyl acetate 20/5/5, v/v/v) to crystallize out the product - 4-(formylamino)-butanoic succinimidyl ester. Yield≈ 50%. In the last step to the suspension of 1 g (4.4 mmol) of 4-(formylamino)-butanoic succinimidyl ester and 1.37 g (5.24 mmol) of triphenylphosphine in 6 mL of trichloromethane 214 μL (2.2 mmol) of tetrachloromethane and 305 μL (2.2 mmol) of triethylamine were added and the mixture was allowed to stirred

for 3 h at 50 °C. The solvent was evaporated *in vacuo* and the main product - isocyanobutyric succinimidyl ester was separated by column chromatography (SiO_2, acetonitryle/trichloromethane/n-hexane 15/10/5, v/v/v). Yield ≈ 30%.

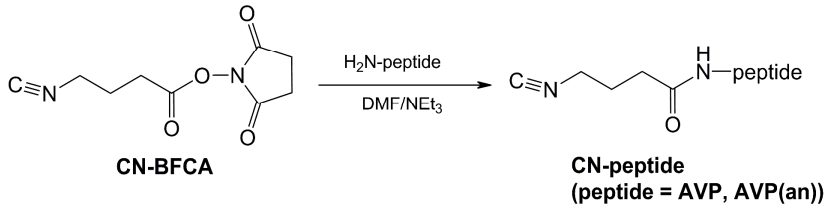
MS, m/z: Calcd. 212.21; Found: 213.1 [$M+H^+$]

^{1}H NMR (CD_3CN) δ (ppm): 2.07 (m, 2H, C**H**$_2$C**H**$_2$C**H**$_2$); 2.76 (t, 2H, C**H**$_2$CO); 3.56 (tt, 2H, C**H**$_2$NC)

^{13}C NMR (CD_3CN) δ (ppm): 25.6 (**C**H_2**C**H_2**C**H_2); 40.4 (**C**N); 170.2 (**C**O)

IR (KBr) (cm^{-1}): 2951, 2926 (**CH**); 2152 (**CN**); 1814, 1785, 1729 (**CO**)

Synthesis of CN-peptide (peptide=AVP, AVP(an)). The coupling reaction of the isocyanide linker CN-BFCA with AVP or AVP(an) peptide is shown in Scheme 1.



Scheme 1. Coupling of CN-BFCA linker with peptide.

The solution containing 0.47 mg (2.2 μmol) of CN-BFCA dissolved in 100 μL of DMF and 0.5 μL (3.6 μmol) of triethylamine was added to 2 mg (1.8 μmol) of AVP (in the synthesis with AVP(an) the same molar ratios of all components were used). The mixture was allowed to stay overnight at room temperature and then the solvent was removed under vacuum. The residue was dissolved in a mixture of 50 μL of acetonitrile and 100 μL of water. The crude product was purified by the semi-preparative HPLC (System 1), alkalized and lyophilized. Yield ≈ 38%.

MS of CN-AVP, (m/z): Calcd: 1179.35, Found: 1179.40 $[M+H]^+$

MS of CN-AVP(an), (m/z): Calcd: 1231.61, Found: 616.1 $[M+H]^+$, 1254.3 $[M+Na]^+$

The *"cold rhenium precursor" $Re(NS_3)(PMe_2Ph)$* was synthesized according to the procedure described in ref.[28] The mixture of 20 mL of methanol and 4 mL of EtOH/HCl containing 80.5 mg (0.41 mmol) of the tetradentate ligand NS_3, 98.90 mg (0.71 mmol) of the monodentate ligand PMe_2Ph and 317.78 mg (0.41 mmol) of the rhenium(III) precursor $Re[SC(NH_2)_2]_6Cl_3 \cdot H_2O$ was refluxed (at 62°C) under argon atmosphere for 3 hours. The crude product was purify by column chromatography (Silica Gel, 0.040-0.063 mm (230-400 mesh), Merck) using the eluent THF/n-Hexane (4/3, v/v). Yield ≈ 25

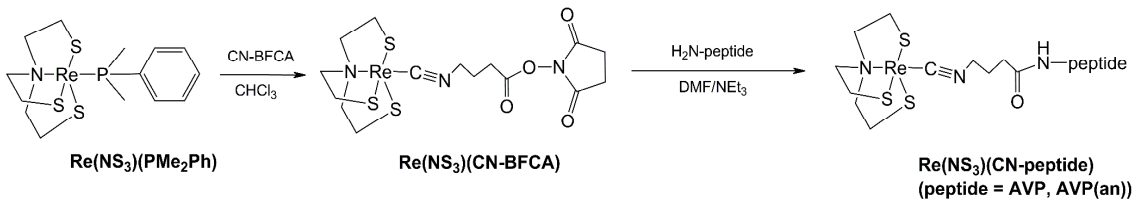
EA: Calcd.: C 32.42; H 4.47; N 2.70; S 18.54; Found: C 32.44; H 4.47; N 2.55; S 18.80

MS, m/z: Calcd.: 519.7; Found: 519.3 $[M+H^+]$

Synthesis of $Re(NS_3)$(CN-AVP) and $Re(NS_3)$(CN-AVP(an)). The non-radioactive rhenium reference compounds $Re(NS_3)$(CN-peptide) were prepared in two steps (Scheme 2). In the first step 10.1 mg (19.4 μmol) of 'cold rhenium precursor' $Re(NS_3)(PMe_2Ph)$ and 6.1 mg (28.7 μmol) of isocyanobutyric succinimidyl ester were dissolved in 1.5 mL of $CHCl_3$ and stirred at room temperature for 2h. In the second step to the mixture containing 1.02 mg (0.94 μmol) of AVP (in the case of AVP(an) the same molar amount was used) and 0.71 mg (1.2 μmol) of $Re(NS_3)$(CN-BFCA) dissolved in 100 μL of DMF we added 0.26 μL (1.85 μmol) of triethylamine. The mixture was allowed to stand overnight at room temperature. After completion of the reaction, DMF was removed under vacuum and the residue was dissolved in 200 μL of acetonitrile/water (1:1). The products of both steps were purified on a semipreparative HPLC column under the conditions described above (System 1) and lyophilized. Yield ≈ 45%.

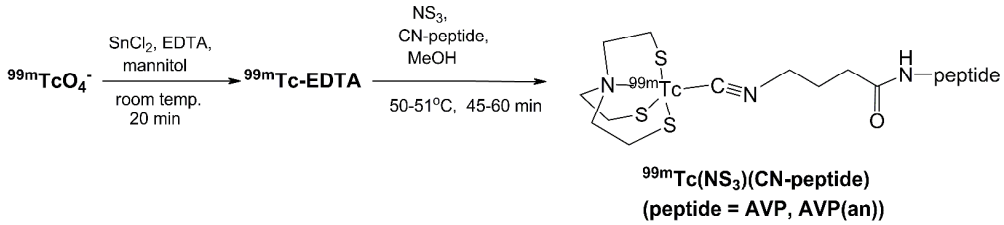
Re(NS_3)(CN-AVP): MS (m/z): Calcd: 1559.91, Found: 1560.08 $[M+H]^+$

Re(NS_3)(CN-(AVP(an)): MS (m/z): Calcd: 1612.1, Found: 806.8 $[M+H]^+$, 1634.5 $[M+Na]^+$



Scheme 2. Reaction route to Re(NS_3)(CN-peptide).

Preparation of ^{99m}Tc(NS_3)(CN-AVP) and ^{99m}Tc(NS_3)(CN-AVP(an)) For labeling AVP and AVP(an) with ^{99m}Tc a two-step procedure[21] was applied (Scheme 3).



Scheme 3. Labeling of AVP and AVP(an) with technetium-99m complex of the '4+1' type.

In the first step 1 mL of eluate from the ^{99}Mo/^{99m}Tc generator (200 – 1000 MBq) was added to a kit formulation containing 1 mg of Na_2EDTA, 5 mg of mannitol and 0.1 mg of $SnCl_2$ in freeze-dried form under nitrogen. The mixture was allowed to stand at room temperature for 20 min. The radiochemical purity was checked by HPLC (System 2) and TLC methods. When Merck 60 WF_{254} aluminum sheets are used the intermediate complex ^{99m}Tc-EDTA/mannitol migrates with the solvent front in water but remains at the origin in acetone. In the second step the intermediate ^{99m}Tc-EDTA/mannitol compound reacted with 300 µg of the NS_3 ligand and with about 50 µg of the isocyanide-modified peptide CN-AVP or CN-AVP(an). The

reaction progress and radiochemical purity were controlled by HPLC (System 2). The radiochemical yields of the ^{99m}Tc-labeled conjugates were approximately 95%.

3.3. In vitro stability studies

Stability studies of ^{99m}Tc(NS$_3$)(CN-AVP) and ^{99m}Tc(NS$_3$)(CN-AVP(an)). The conjugates isolated from the reaction mixture (using HPLC System 1), present in the solution in concentration no higher than 10^{-4} mM, were incubated at 37 ^{o}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.

Stability studies of ^{99m}Tc(NS$_3$)(CN-AVP) and ^{99m}Tc(NS$_3$)(CN-AVP(an)) in human and rat serum. For that purpose 0.1 mL of the solution of the isolated ^{99m}Tc(NS$_3$)(CN-AVP) or ^{99m}Tc(NS$_3$)(CN-AVP(an)) conjugate in the 0.1 M PB buffer, pH=7.4, was added to 0.9 mL of human or rat serum (obtained from the Centre of Radiobiology and Biological Dosimetry, INCT Warsaw and Sigma Aldrich, respectively) and incubated at 37 ^{o}C. At specified time intervals small samples (0.1 - 0.2 mL) of the mixture were withdrawn, mixed in an Eppendorf tube with ethanol (0.3 - 0.5 mL) and vigorously shaken to precipitate proteins. Then, the samples were centrifuged (14000 rpm, 5 min) and the supernatant was separated. The radioactivity of both supernatant and precipitate was measured using the well-type NaI(Tl) detector. To check if the conjugates did not convert into other water-soluble radioactive species, aliquots of the supernatant were analyzed by HPLC for the content of the ^{99m}Tc(NS$_3$)(CN-peptide) complex.

3.4. Paper Electrophoresis

Paper electrophoresis experiments were performed on 20 x 1 cm chromatographic paper strips, Paper Chromedia GF 83 (Whatman), pre-treated with the phosphate buffer (0.1 M, pH 7.40), using a midi horizontal electrophoresis unit (Sigma-Aldrich) device. The experiments were carried out at 200 V (10 V cm^{-1}) for 60 min for both conjugates. The developed paper strips were left to dry, and then the distribution of radioactivity on the strips was determined using an automatic TLC analyzer SC-05 (home-made, INCT, Warsaw).

3.5. Lipophilicity studies

Lipophilicity of the $^{99m}Tc(NS_3)$(CN-AVP) and $^{99m}Tc(NS_3)$(CN-AVP(an)) conjugates, which is an important factor affecting the distribution of drug molecules in the organism, was characterized by their distribution coefficients, *D*, in the system *n*-octanol/PBS buffer pH=7.4. The activity of each layer (which shows concentration of the ^{99m}Tc species in the layer) was determined by measuring γ-radiation, using a well-type NaI(Tl) detector. Distribution coefficient D was calculated as the ratio of activity of organic to that 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 to check whether the studied conjugate had not decomposed during the experiment.

3.6. Cell culture

Human small cell lung cancer NCI-H69 cell line was maintained in ATCC-formulated RPMI-1640 Medium enriched in fetal bovine serum to a final concentration of 10% and supplemented with 0.1 IU/ml penicillin and 0.1 mg/ml streptomycin. Cells were cultured at 37^{o}C in a humidified incubator under an atmosphere containing 5% CO_2 and subcultured once a week. The cells grow in suspension, in the form of multicell aggregates (Figure 9), and

before cell counting as well as before affinity studies the addition of Cell Dissociation Solution Non-enzymatic 1x is required in order to disperse the H69 cells (the Cell Dissociation Solution Non-enzymatic 1x medium was applied according to protocol attached to the product).

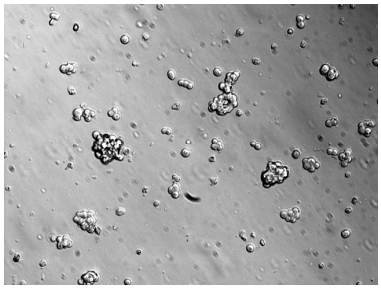


Figure 9. Human male cell line NCI-H69 under microscope (IX81 Motorized Inverted Microscope, Olympus). Besides single cells the multicell aggregates are visible.

3.7. Affinity studies

Affinity studies were carried out on the human SCLC cell line NCI-H69 seeded in 6-well plates in RPMI-1640 medium in amount of approximately $5x10^5$ cells/well. Cell count was determined using a hemocytometer and microscope. Preliminary affinity studies were performed by incubating H69 cells suspended in 1 mL of RPMI-1640 medium in the presence of conjugates $^{99m}Tc(NS_3)$(CN-AVP) or $^{99m}Tc(NS_3)$(CN-AVP(an)), as well as other ^{99m}Tc-species ($^{99m}TcO_4^-$, intermediate compounds ^{99m}Tc-EDTA/mannitol and $^{99m}Tc(NS_3)$). After 45 min of incubation the binding was stopped and suspended cells were centrifuged. Both phases, medium from above cells and the cells (after two washing steps in PBS in order to eliminate unbound activity) were measured in a gamma counter. Affinity of the studied ^{99m}Tc species was calculated as a ratio of activity bound by cells to the sum of activity bound by cells and remained in the solution aspirated from above the cells (Figure 6).

In order to determine K_d, NCI-H69 cells were grown in 6-well plates in RPMI-1640 medium (approximately $5x10^5$ cells in each well) and incubated at 37 ^oC for 45 min with different concentrations, in the range 0.1÷30 nmol/L, of selected conjugate $^{99m}Tc(NS_3)$(CN-AVP(an)). After washing twice the suspended cells with PBS at 37 ^oC, in order to eliminate unbound activity, the cells were centrifuged and counted in a gamma counter. Nonspecific binding was defined as the amount of activity still bound in the presence of 1 μM solution of AVP(an). Under these conditions virtually all receptors are occupied by the unlabeled conjugate, so that the radioconjugate can be bound with cells only *via* nonspecific interactions. In order to determine K_d value the data were analysed by the Scatchard plot (Figure 7) using the relationship between the ratio of Specific bound ligand/Free ligand (B/F) *vs*. Specific bound ligand (B). The slope of the straight line indicates the negative reciprocal of K_d ($-1/K_d$). The intercept of the straight line with the B axis represents the maximum concentration of peptide (B_{max}) bound to the V2 receptor of cells (represented by the number of binding sites per cell) at infinite peptide excess.

3.8. Competitive binding assays

Competitive binding assays were performed for AVP(an) using NCI-H69 cells line and $^{99m}Tc(NS_3)$(CN-AVP(an)) as the radioligand (Figure 8). Briefly, 20,000 cpm of $^{99m}Tc(NS_3)$(CN-AVP(an)) in 0.1 M PBS sterile buffer, pH 7.4, was added to each well in 6-well plates in RPMI-1640 medium (approximately $5x10^5$ cells in each well) and incubated at 37 ^oC for 45 min. Then, the incubation was stopped, the suspended cells were centrifuged and washed twice with PBS at 37 ^oC, in order to eliminate unbound activity. Next, the cells were incubated again at 37 ^oC for 45 min applying concentrations of AVP(an) in the range 0.005–1000 nM. After that time the incubation was stopped and the solution from above the cells was quantitatively separated and measured in a gamma counter. The suspended cells were

again washed twice with PBS at 37 ^{o}C, centrifuged and counted in a gamma counter. Nonspecific binding was defined, as before, as the amount of activity still bound by cells in the presence of 1 μM solution of AVP(an). The specific binding was obtained by subtracting nonspecific binding from total binding. IC_{50} value was determined from the relationship between the ratio of the activity of the cells to that of quantitatively separated solution from above the cells and concentration of AVP(an). Inhibition constant K_i value was calculated from the IC_{50} value using the Cheng-Prusoff equation.[29]

4. Conclusions

The ^{99m}Tc-labeled vasopressin conjugates show high stability in the presence of an excess of standard amino acids cysteine or histidine. The proof of their stability is that no transchelation reactions have been observed in the challenge experiments. In human or rat serum the $^{99m}Tc(NS_3)$(CN-AVP) conjugate is cut into two species as the result of enzymatic degradation of the peptide, while the $^{99m}Tc(NS_3)$(CN-AVP(an)) conjugate, where AVP(an) is an antagonist to the V2 vasopressin receptor, is quite stable even in rat serum.

The B_{max} value obtained for $^{99m}Tc(NS_3)$(CN-AVP(an)) conjugate is equal to 0.0058 which corresponds to an approximate number of 7,000 binding sites per cell. The dissociation constant K_d equal to 0.13 nM, inhibition constant K_i value equal to 0.09 nM and the IC_{50} value equal to 29 nM, indicate satisfactorily high binding of the $^{99m}Tc(NS_3)$(CN-AVP(an)) conjugate to V2 vasopressin receptor in SCLC cells H69.

In the conclusion one can say that the physicochemical and biological properties of $^{99m}Tc(NS_3)$(CN-AVP(an)) conjugate can be an important basis for further consideration the conjugate as a potential diagnostic radiopharmaceutical for patients suffering from the small-cell lung cancer.

Acknowledgements and notes

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Abbreviations used: AVP, arginine vasopressin(1-9); AVP(an), analogue d$(CH_2)_5$[D-Tyr(Et^2)-Ile^4-Eda^9]AVP; V2, arginine vasopressin receptor 2; SCLC, small-cell lung cancer; ADH, antidiuretic hormone; CNS, central nervous system; HPLC, high-performance liquid chromatography; n.c.a., no carrier added; RCP, radiochemical purity; RCY, radiochemical yield; NS_3, tris(2-mercaptoethyl)-amine; CN-BFCA, isocyanobutyric acid succinimidyl ester; PBS, Phosphate buffered saline; TFA, trifluoroacetic acid

ANCILLIARY INFORMATION

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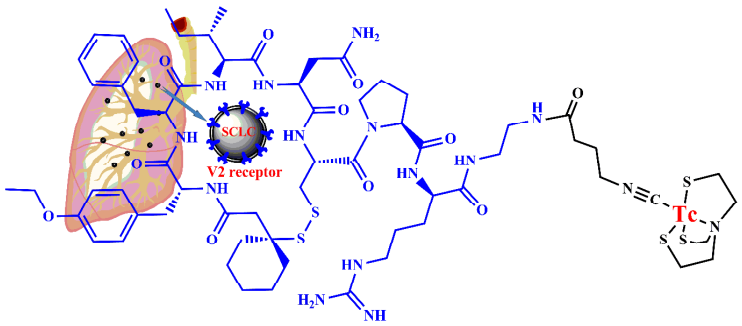
TABLE OF CONTENTS GRAPHICS



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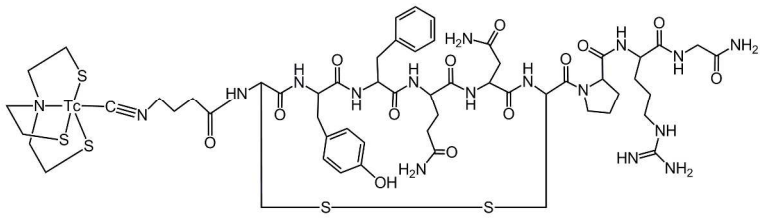

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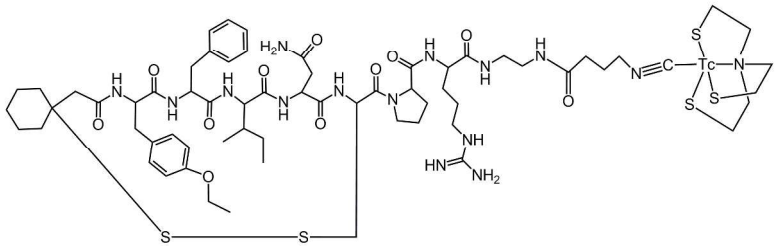

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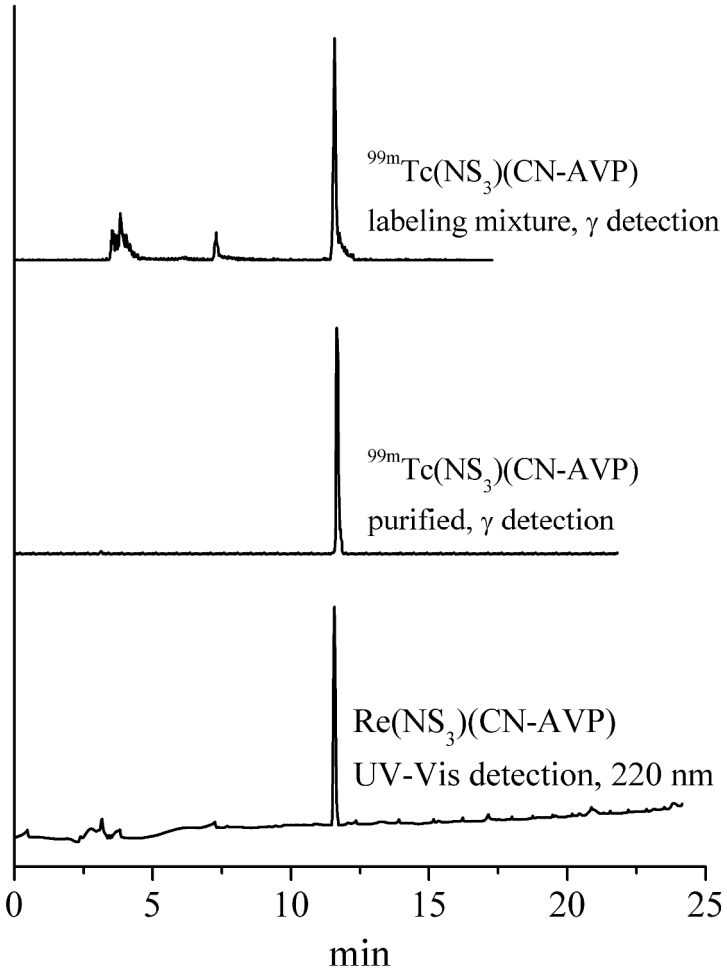

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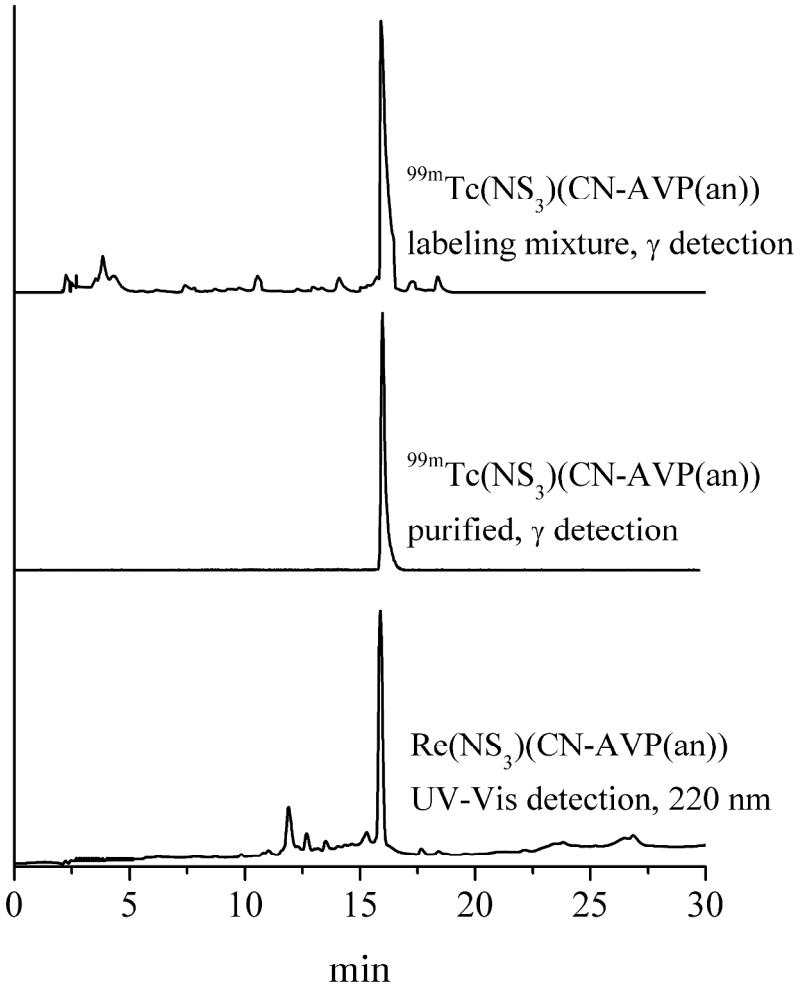

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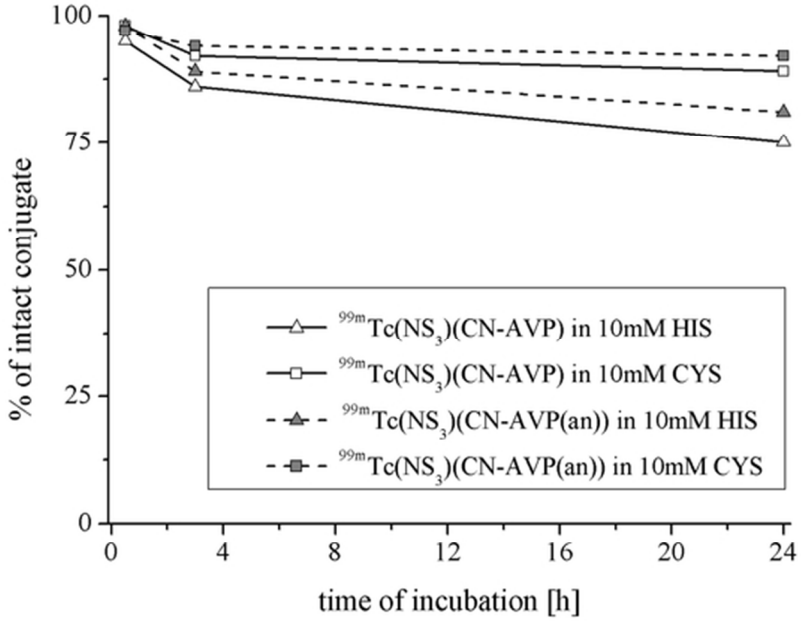

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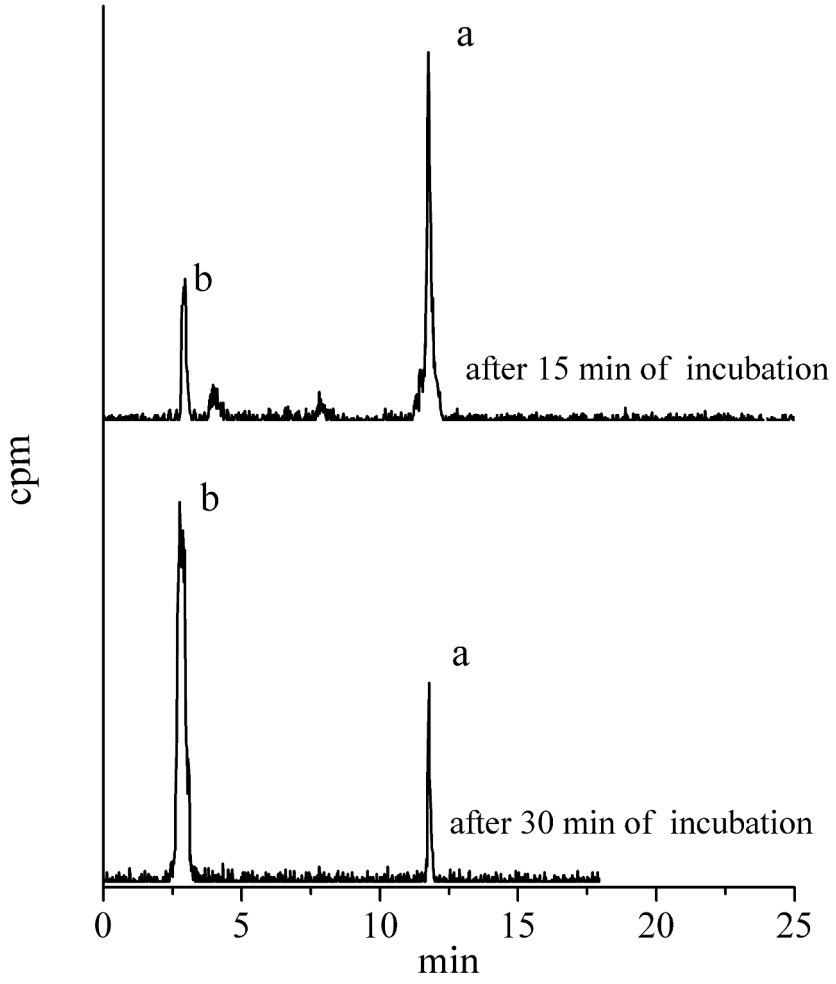

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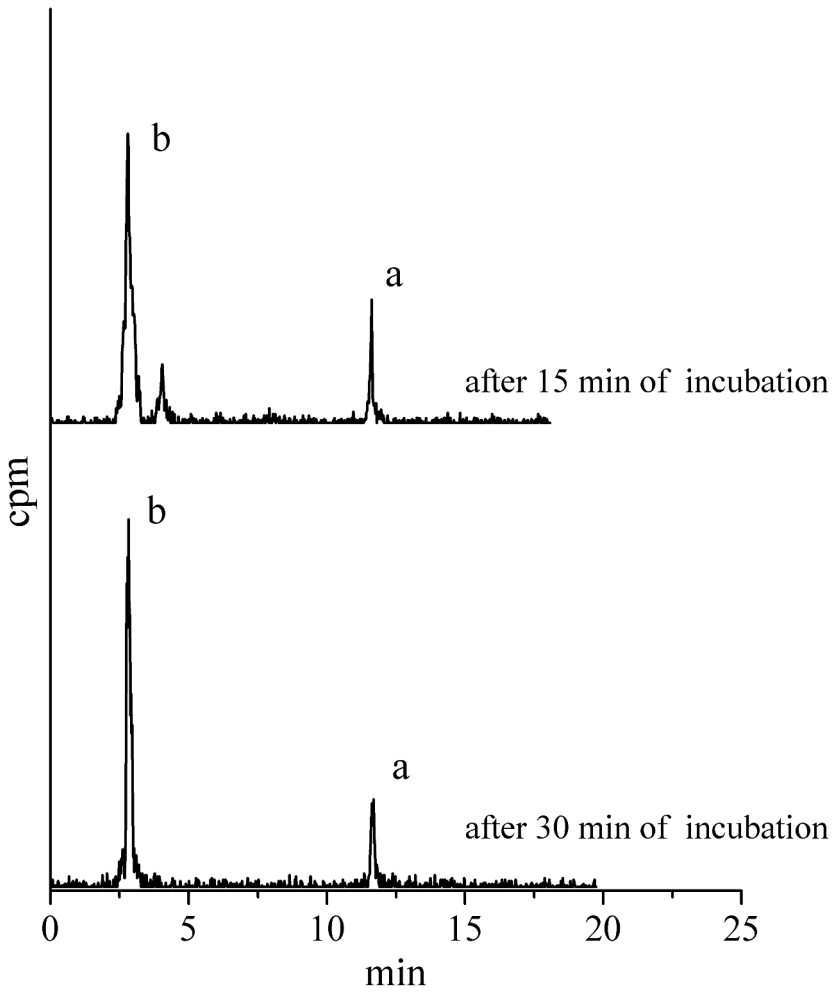

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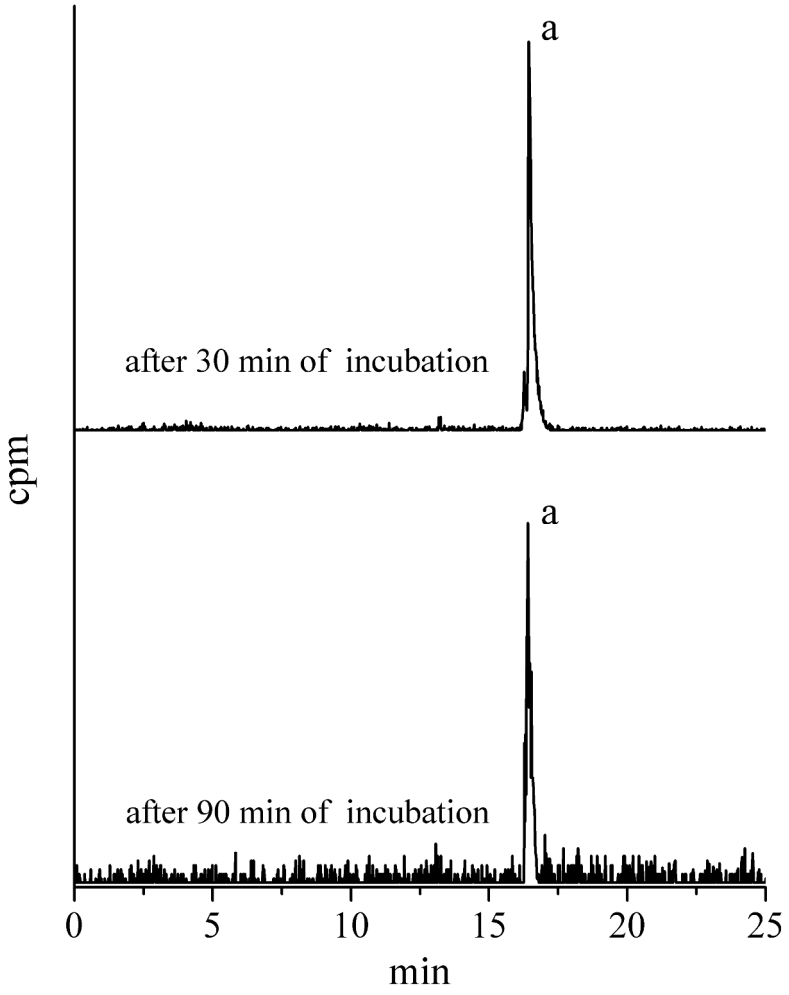

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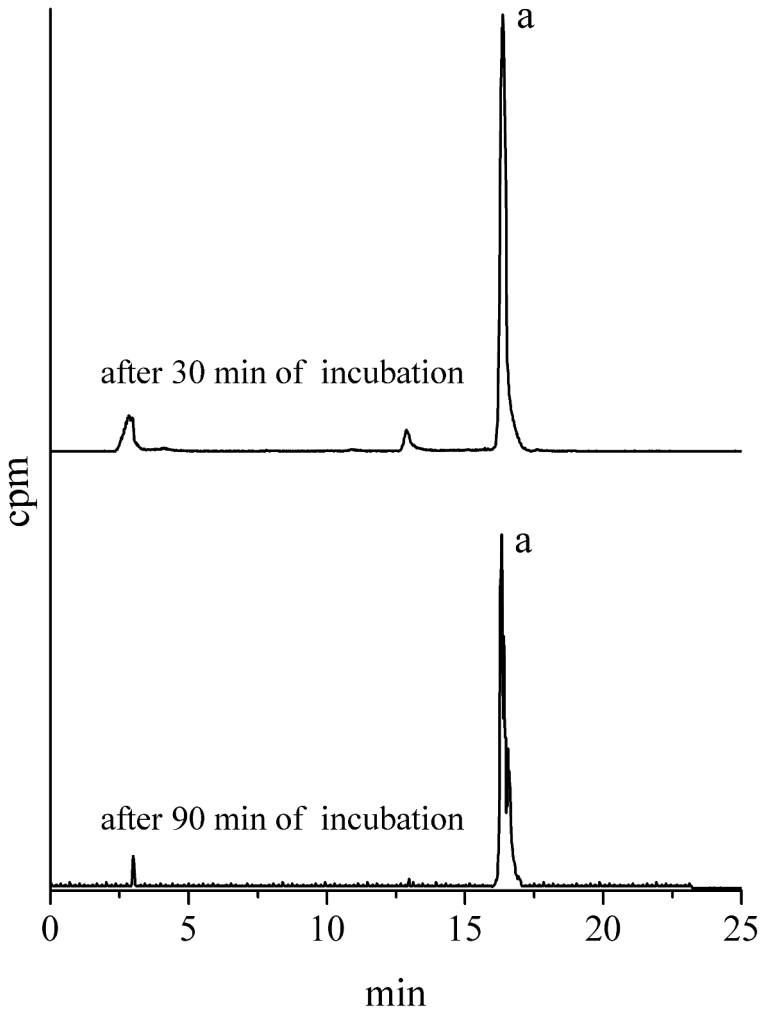

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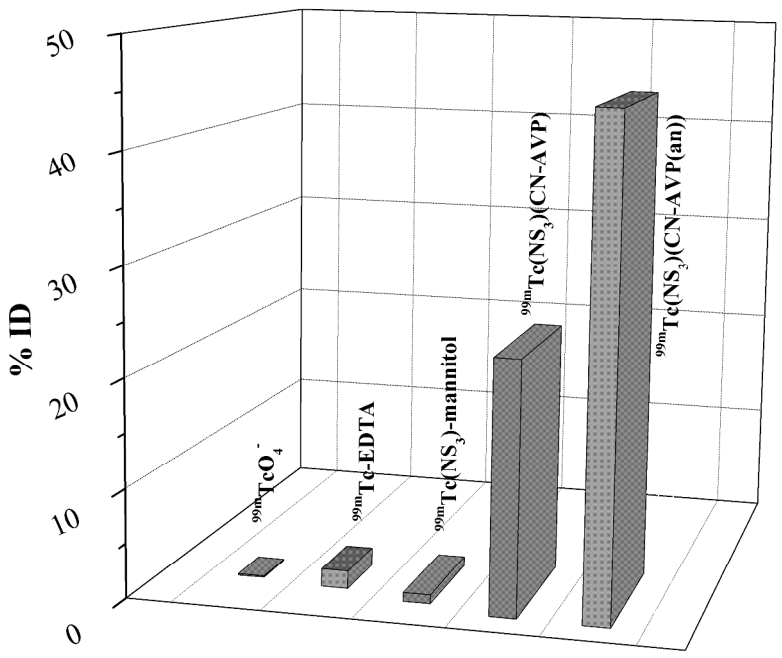

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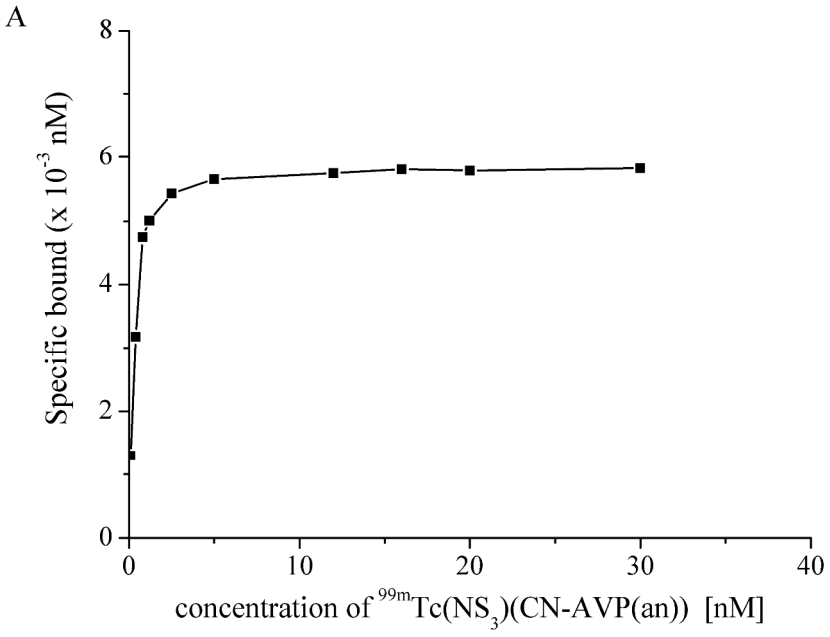
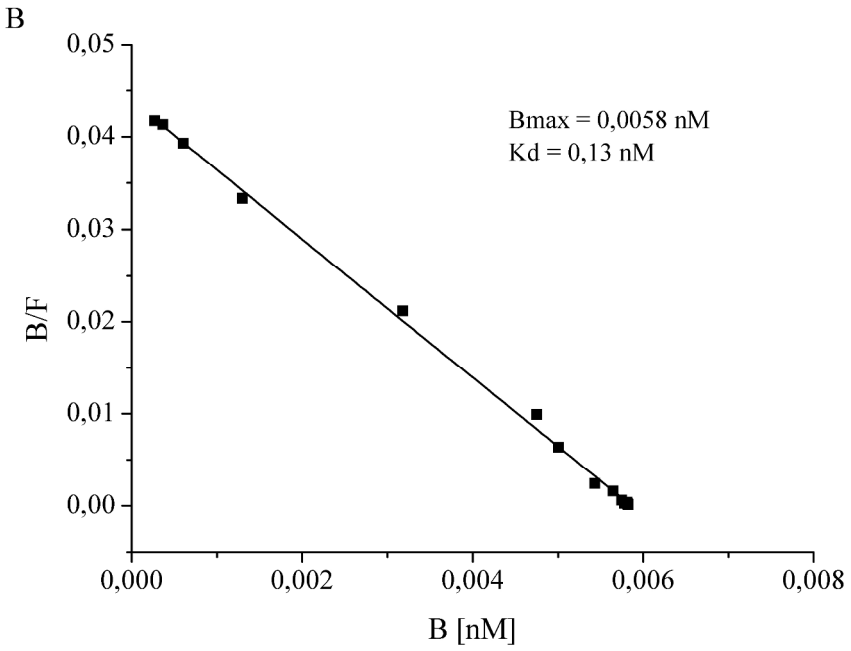
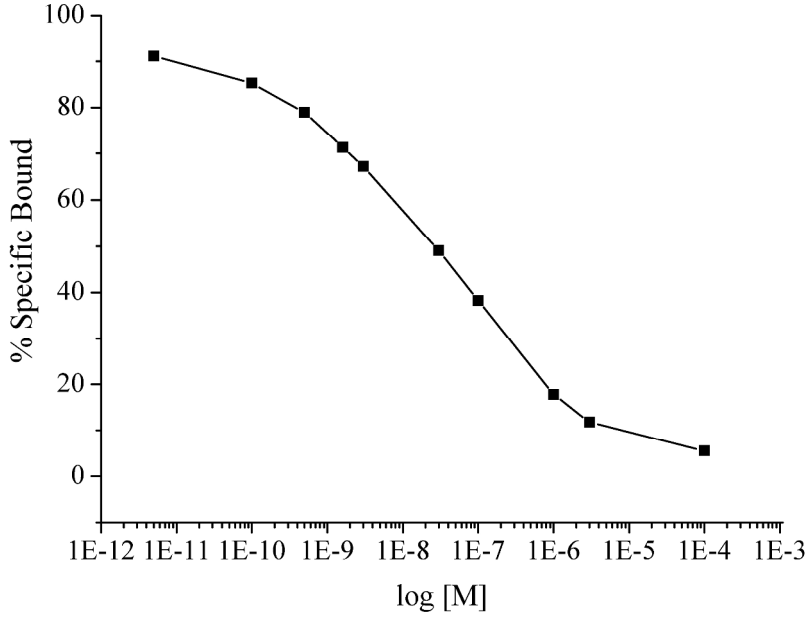


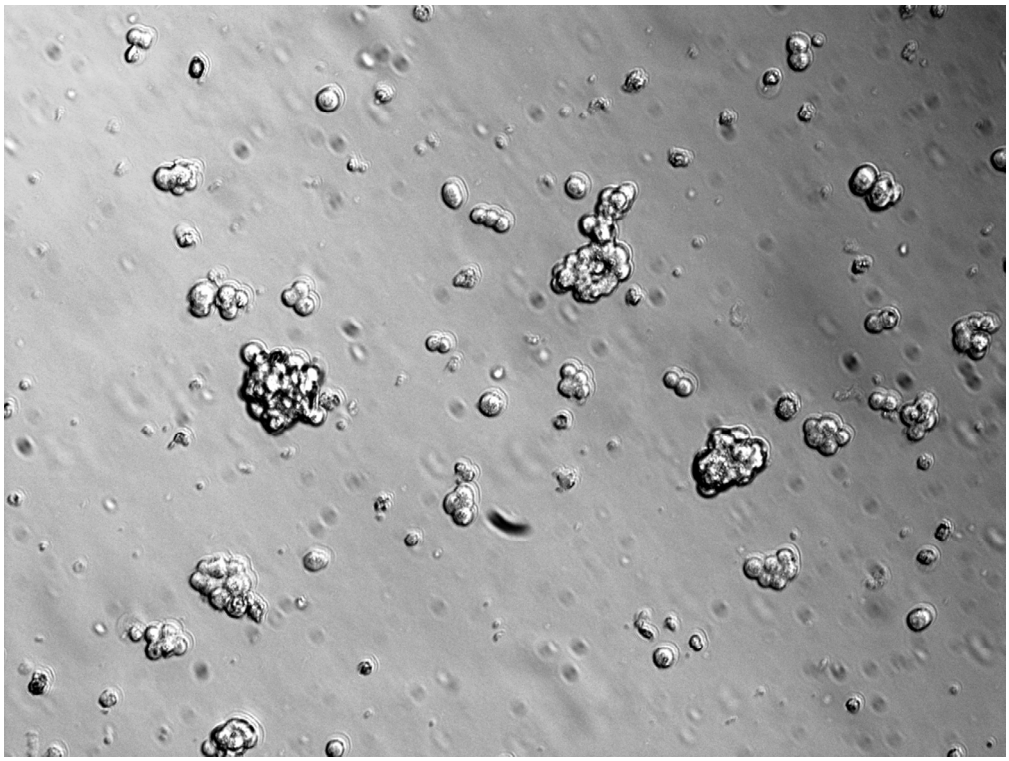
289x204mm (300 x 300 DPI)



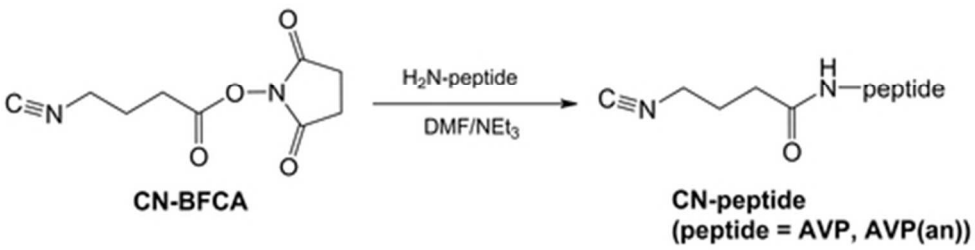
289x204mm (300 x 300 DPI)



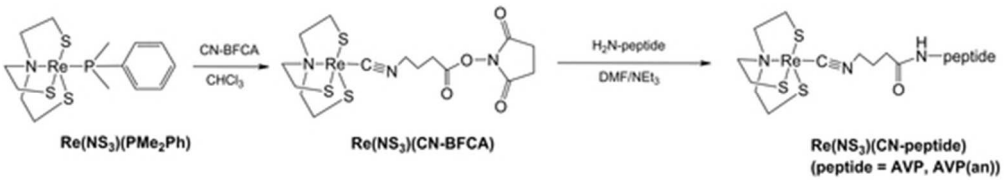
289x204mm (300 x 300 DPI)



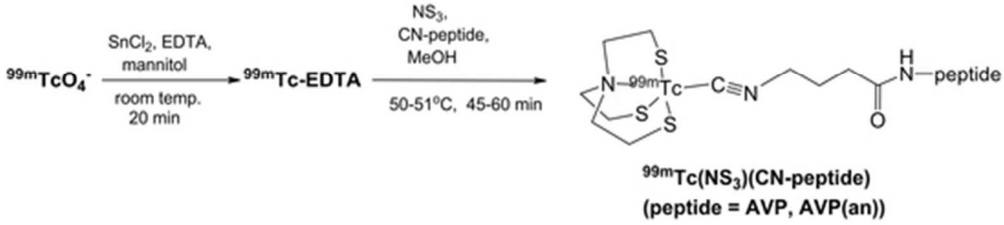
218x163mm (150 x 150 DPI)



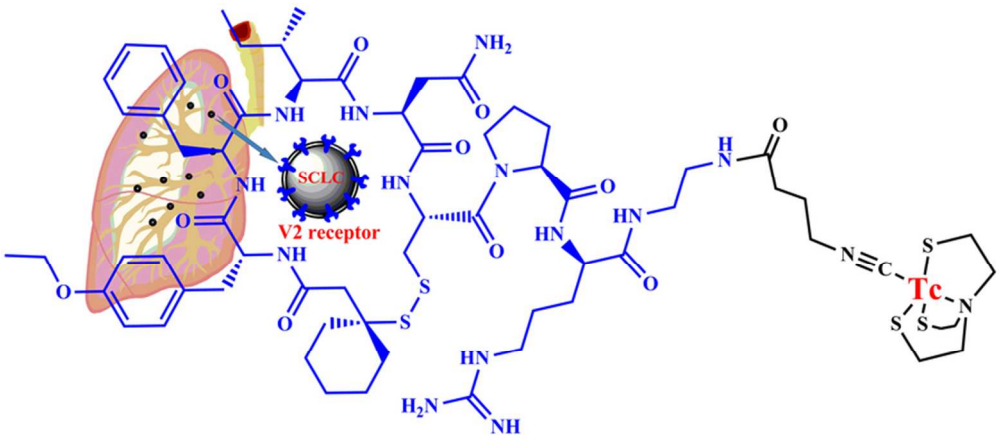
41x10mm (300 x 300 DPI)



47x8mm (300 x 300 DPI)



46x10mm (300 x 300 DPI)



75x32mm (300 x 300 DPI)