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Synthesis of tricarbonyl rhenium and technetium complexes of a 5′-carboxamide 5-ethyl-2′-deoxyuridine for selective inhibition of herpes simplex virus thymidine kinase 1

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Abstract

Herpes simplex virus thymidine kinase type 1 (HSV1-TK) is frequently used as reporter protein in gene therapy. Our aim is to produce single photon emitting reporter probe based on technetium-99m. The synthesis of organometallic technetium and rhenium complexes of a 5′-carboxamide 5-ethyl-2′-deoxyuridine derivative able to selectively inhibit HSV1-TK is presented. The 5-ethyl-2′-deoxyuridine functionalized with a suitable tridentate chelating system at position 5′ was synthesized from commercial 2′-deoxyuridine in seven steps. The 5-ethyl-2′-deoxyuridine derivative was labeled with the *fac*-$M(CO)_3$-core (M = Tc, Re). The resulting rhenium complex was found to be a selective competitive inhibitor of HSV1-TK ($K_i = 4.56\ \mu M$). Inhibition of the human cytosolic thymidine kinase (hTK1) previously reported with organometallic rhenium and technetium complexes of 5′-carboxamide thymidine derivative was not observed. The uptake of the technetium-99m complex in transfected cells expressing HSV1-TK has been evaluated to assess its possible use as reporter.
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1. Introduction

Gene therapy is characterized by the transfer of new genetic material encoding a therapeutic protein into the cells [1,2]. Often a reporter protein is co-expressed in order to control the level of expression of the therapeutic protein [3]. Herpes simplex virus thymidine kinase type 1 (HSV1-TK) has been used both as a reporter and/or suicide gene [4]. HSV1-TK is a multifunctional enzyme with a lower substrate specificity compared to the human cytosolic thymidine kinase (hTK1) [5]. Over the last decade, chemists and radiochemists have been trying to produce selective substrates and inhibitors of HSV1-TK acting as prodrug and reporter probes [6–9]. Currently, the best representatives of Single Photon Emission Tomography (SPET) or Positron Emission Tomography (PET) reporter probes are ^{131}I-labeled 2′fluoro-2′deoxy-1-β-D-arabinofuranosy-5-iodo-uracyl ([^{131}I]-FIAU) and ^{18}F-labeled 9-[4fluoro-3-(hydroxymethyl)butyl]-guanine ([^{18}F]FHBG), respectively [10].

The aim of the present work is the development of novel SPET reporter probes selective for HSV1-TK labeled with the low-cost radionuclide Technetium-99m. Herein the synthesis of the organometallic technetium/rhenium complexes of a 5′-carboxamide 5-ethyl-2′-deoxyuridine derivative **2** (Fig. 1) is described. The biological active entity was based on 5′-carboxamide 5-ethyl-2′-deoxyuridine derivatives functionalized with spacer and chelating system enabling the stable coordination of the metal center. The (radio)labeling was performed with the organometallic precursor *fac*-$[M(CO)_3(H_2O)_3]^+$ (M = ^{99m}Tc, ^{nat}Re), which has proven to form very inert and in vivo stable complexes [11–14]. Complex **2** has been evaluated in vitro for potency and selectivity toward both HSV1-TK and hTK1. The results are compared

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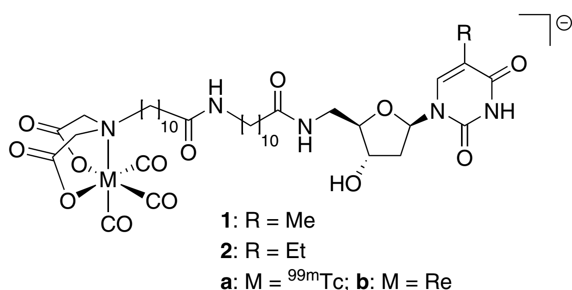


Fig. 1. General structures of the organometallic technetium/rhenium complexes of thymidine (**1**) and 5-ethyl-2′-deoxyuridine (**2**) used in this study.

with the data of the structurally related thymidine analogue **1** (Fig. 1) presented earlier by our group [15]. Moreover, the internalization of the radioactive complexes **1a** and **2a** into HSV-1 TK-expressing cell is reported.

2. Experimental

2.1. General

All chemicals and solvents were of reagent grade. They were used without further purification unless otherwise mentioned. The precursors *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^+$ and *fac*-$[ReBr_3(CO)_3]$ was prepared according to published protocols [11,12,16]. Nuclear magnetic resonance spectra were recorded on a 300 MHz Varian Gemini 2000 spectrometer. The ^1H and ^{13}C chemical shifts are reported relative to residual solvent peaks or water peak as a reference. Mass spectrometry was performed in a ESI-TOF waters micromass LCT premier spectrometer unless otherwise mentioned. The radioactivity was measured in a β-counter Packard 1900TR or a γ-counter Cobra II. HPLC analyses were performed on a Merck-Hitachi L-7000-system equipped with an L-7400 tuneable absorption detector and a Berthold LB 506 B radiometric detector. A Xterra® C-18 reverse phase column (5 μM 4.6 × 150 mm) was used. HPLC solvents consisted of a 0.05% TEAP in H_2O (pH = 2.25) (solvent A) and methanol (solvent B). Two different gradient systems were used. *System A*: The flow rate was 1 ml/min. 0–3 min: 100%A; 3–6 min: 75%A; 6–9 min: 67%A; 9–20 min: gradient to 100%B. 20–22 min: 100%B (2 ml/min). *System B*: The flow rate was 0.9 ml/min. HPLC system started with 95% A. 0–2 min: gradient to 75%A; 2–3 min: 75%A; 3–4 min: gradient to 66%A; 4–5 min: 66%A; 5–6 min: gradient to 50%A; 6–8 min: 50%B; 8–10 min: gradient to 80%B, 10–13 min: 80%B; 13–15 min: gradient to 95%B, 15–20 min: 95%B.

2.2. Enzymatic kinetics

Herpes simplex virus thymidine kinase of type 1 (HSV1-TK) and human thymidine kinase (hTK1) used in our experiments were expressed as bacterial fusion proteins and were purified according to published procedures [17,18]. The kinetic constants of both enzymes were determined by measurement of initial velocities according to a published procedure [19]. Reactions mixtures containing 50 mM Tris pH 7.4, 5 mM $MgCl_2$, 2 mM ATP, 2 mg/ml BSA, 0.1 ng of enzymes, various concentration of $[^3H]$thymidine (0–1.2 μM) and various concentration of our complexes (0–200 μM) in a final volume of 30 μL. Samples of 5 μl were taken every 5 min and inactivated by transferring them immediately onto DEAE-cellulose papers (Multi-Screen®HTS Assay System from Millipore). The DEAE-cellulose papers were washed four times with 200 μl ammonium acetate (4 mM) and two times with 200 μl MeOH. The papers were dried. Each paper was digested with 4 ml of a solution of cellulase from aspergillus niger in acetate buffer 75 mM pH = 5 (10 U/ml) at 37 °C for 1 h. Afterwards 4 ml of scintillation were added and the samples were measured in a β-counter. The counts were plotted against time. The slope resulting from the linear regression gave the initial velocity in count/min. By linear regression of the reciprocal initial velocity against reciprocal concentration of thymidine a Lineweaver–Burk equation was determined for each analogue, which allowed the determination of the type of inhibition as well as the K_i-values.

2.3. Cell culture

For in vitro cell experiments the 143B-HSV1-TK cell line (143-TK+) was used. 143-TK^+ cells were grown in MEM (AMIMED) containing 10% FCS, 1% non essential amino acids (AMIMED), 1% sodium pyruvate (AMIMED), 2% HAT-additive (Hypoxanthine, Aminopterine and Thymidine) and 1% penicillin.

The cell line was grown in a 7.5% CO_2-humidified atmosphere at 37 °C. The cells were released from the culture plates by treatment with trypsin/PBS for 2–5 min, re-suspended in the growth media to neutralize the trypsin, and passaged.

2.4. In vitro cell experiment

The cells were first trypsinized, re-suspended in the growth media to neutralize the trypsin, and counted. The cells were centrifuged and the supernatant was discarded. The cells were re-suspended in the growth media (without HAT) so that the concentration was ~350 000 cells/ml. The cells were then seeded into six well plates (~700 000 cells/well). The plates were incubated during 16 h in a 7.5% CO_2-humidified atmosphere at 37 °C. Cells were 70–90% confluent. The media were removed; the cells were washed with PBS and covered with 1450 μL of fresh medium. Afterwards, 50 μL of medium containing ~6 kBq of technetium complex (or $[^3H]$Thymidine) was added and time was counted. Experiments were performed in triplicate. When time was over, the medium was removed. The cells were washed with 1 × 500 μL cold PBS (4 °C). The cells were then detached by adding 2 × 500 μL 1M NaOH. The collected cells and the medium were then counted in a γ-counter (or β-counter). The protein content was determined spectrophotometrically using Micro BCA protein

assay reagent kit (Pierce, Socochim). The data were expressed as collected activity per gram of protein: medium concentration ratio (cpm/g cells)/(cpm/ml medium). The unit is %ID/(g/ml) with ID = injected dose.

2.5. Ligand synthesis

2.5.1. 5-Ethynyltrimethylsilyl-3′,5′-di-O-p-toluyl-2′-deoxyuridine (4)

Compound **3** (1.89 g, 3.21 mmol) was put in suspension in 100 ml NEt_3. Ethynyltrimethylsilane (0.89 ml, 6.42 mmol) as well as 55 mg CuI and 55 mg $(PPh_3)_2PdCl_2$ were added. The suspension was stirred at 60 °C for 7 h. NEt_3 was removed under reduced pressure. The residue is taken up in 150 ml $CHCl_3$ and washed with 3 × 80 ml 5% EDTA disodium/H_2O followed with 1 × 80 ml water. The organic phase was dried over Na_2SO_4 and after filtration, the solvent was evaporated in vacuo washed over Na_2SO_4 and evaporated in vacuo. The residue is dissolved in 40 ml hot $CHCl_3$ and 200 ml of MeOH were added. After filtration 1.36 g (76%) of **4** were collected as a white powder. ^{1}H NMR ($CDCl_3$) δ 8.43 (s, 1H), 7.93 (m, 4H), 7.27 (m, 4H), 6.36 (dd, J = 8.7, 5.3 Hz, 1H), 5.57 (dt, J = 6.4, 1.4 Hz, 1H), 4.82 (dd, J = 12.3, 3.5 Hz, 1H), 4.64 (dd, J = 12.3, 3.0 Hz, 1H), 4.57 (q, J = 3.2 Hz, 1H), 2.76 (ddd, J = 15.0, 5.4, 1.3 Hz, 1H), 2.43 (s, 3H), 2.41 (s, 3H), 2.24 (ddd, J = 15.0, 8.7, 6.4 Hz, 1H), 0.13 (s, 1H); ^{13}C NMR ($CDCl_3$)δ 166.0, 165.9, 160.7, 148.9, 144.6, 144.4, 142.0, 130.0, 129.5, 129.4, 129.3, 126.3, 126.1, 101.0, 99.7, 94.6, 85.9, 85.8, 83.5, 83.4, 74.9, 74.8, 64.1, 38.6, 21.7, −0.3; MS *m/z* 583.2 (MNa^+); Anal. Calc. for $C_{30}H_{32}N_2O_7Si$: C, 64.27; H, 5.75; N, 5.00. Found: C, 63.84; H, 5.34, N, 4.98%.

2.5.2. 5-Ethynyl-3′,5′-di-O-p-toluyl-2′-deoxyuridine (5)

Compound **4** (0.87 g, 1.55 mmol), NEt_4Br (0.65 g, 3.10 mmol) and KF (0.18 g, 3.10 mmol) were put in suspension in 100 ml dry CH_3CN. The mixture was stirred at reflux for 12 h. CH_3CN is removed in vacuo. The residue was dissolved in 150 ml $CHCl_3$. This organic phase was washed with 4 × 100 ml of water, dried over Na_2SO_4 and concentrated under reduced pressure to give 0.66 g of **5** (87%) as a yellowish powder. ^{1}H NMR ($CDCl_3$)δ 8.54 (s, 1H), 7.94 (m, 4H), 7.27 (m, 4H), 6.36 (dd, J = 8.4, 5.5 Hz, 1H), 5.60 (dt, J = 6.3, 1.6 Hz, 1H), 4.78 (dd, J = 12.6, 3.4 Hz, 1H), 4.73 (dd, J = 12.6, 2.9 Hz, 1H), 4.57 (q, J = 3.1 Hz, 1H), 3.04 (s, 1H), 2.78 (ddd, J = 15.0, 5.4, 1.5 Hz, 1H), 2.43 (s, 3H), 2.41 (s, 3H), 2.26 (ddd, J = 15.0, 8.4, 6.4 Hz, 1H); ^{13}C NMR ($CDCl_3$)δ 166.1, 166.0, 160.8, 148.8, 144.7, 144.5, 142.7, 129.8, 129.6, 129.4, 129.2, 126.1, 99.7, 85.9, 85.8, 83.5, 83.4, 82.2, 82.0, 74.9, 74.7, 64.0, 38.7, 21.8; MS (ESI+Q1MS) 511.1 $[M+Na]^+$ 527.1 $[M+K]^+$; Anal. Calc. for $C_{27}H_{24}N_2O_7$: C, 66.39; H, 4.95; N, 5.73. Found: C, 66.10; H, 5.02, N, 5.69%.

2.5.3. 5-Ethynyl-2′-deoxyuridine (6)

Compound **5** (0.66 g, 1.35 mmol) was stirred in a solution of sodium methylate in dry MeOH (40 ml) at 60 °C for 4 h. After completion the solution was carefully neutralized by addition of Dowex 50-X8 resin until pH ~6. The mixture was filtrated and the resin was washed with MeOH. The filtrates were grouped and the solvent was removed under vacuum. The residue was triturated in *n*-hexane. The resulting precipitate was filtered to afford 0.30 g (79%) of **6**. ^{1}H NMR (CD_3OD) δ 8.41 (s, 1H), 6.24 (t, 6.5 Hz, 1H), 4.40 (m, 1H), 3.94 (dd, J = 3.0, 3.4 Hz, 1H), 3.82 (dd, J = 12.0, 3.0 Hz, 1H), 3.73 (dd, J = 12.0, 3.4 Hz, 1H), 3.55 (s, 1H), 3.31 (m, 2H); ^{13}C NMR (CD_3OD) δ 146.4, 89.3, 87.2, 83.1, 72.0, 62.6, 41.8; MS *m/z* 251.3 (M^-); Anal. Calc. for $C_{11}H_{12}N_2O_5$: C, 52.38; H, 4.80; N, 11.11. Found: C, 51.80; H, 4.95, N, 10.94%.

2.5.4. 5-Ethynyl-5′-azido-2′,5′-dideoxyuridine (7)

Compound **6** (130 mg, 0.52 mmol) was taken up in 2 ml dry pyridine and evaporated. This was repeated with anhydrous toluene. The residue was dissolved in DMF (2 ml). PPh_3 (135 mg, 0.52 mmol) and NaN_3 (162 mg, 2.50 mmol) were added. The mixture was stirred at room temperature under N_2 flux for 15 min. CBr_4 (172 mg, 0.52 mmol) was added portion wise. After addition the reaction was further stirred at room temperature for 15 h. 0.15 ml of dry methanol was added and the reaction was further stirred for 1 h. The solvents were removed under N_2 flux. A flash chromatography was carried out with the residue using a mixture $MeOH/CH_2Cl_2$ (0.5:9.5) as eluent. The corresponding fractions were grouped and evaporated in vacuo to provide 100 mg (69%) of **7** as colorless powder. ^{1}H NMR (CD_3OD) δ 8.08 (s, 1H), 6.21 (t, J = 6.5 Hz, 1H), 4.34 (dd, J = 9.6, 5.5 Hz, 1H), 3.99 (dd, J = 8.7, 3.9 Hz, 1H), 3.68 (m, 2H), 3.59 (s, 1H), 2.31 (dd, J = 6.5, 5.5 Hz, 2H); ^{13}C NMR (CD_3OD) δ 145.9, 87.3, 86.6, 83.0, 72.2, 53.2, 40.8; MS *m/z* 276.3 $[M]^-$; Anal. Calc. for $C_{11}H_{11}N_5O_4$: C, 47.66; H, 4.00; N, 25.26. Found: C, 46.44; H, 4.15, N, 21.72%.

2.5.5. 5-Ethyl-5′-amino-2′,5′-dideoxyuridine (8)

The azide **7** (80 mg, 0.29 mmol) was stirred in 30 ml EtOH with 50 mg 10% Pd/C under H_2 pressure of 3 bars at room temperature for 5 h. The reaction was stopped and the catalyst was filtered over celite. After evaporation of the solvent, 64 mg (86.5%) of **8** were collected. ^{1}H NMR (CD_3OD) δ 7.42 (s, 1H), 6.19 (t, J = 6.9 Hz, 1H), 4.29 (m, 1H), 3.86 (m, 1H), 3.35 (s, impurity), 2.95 (m, 2H), 2.33 (m, 4H), 1.24 (t, J = 3.0 Hz, 3H); ^{13}C NMR (CD_3OD) δ 138.0, 117.6, 87.6, 87.3, 72.9, 44.3, 40.0, 21.1, 13.5. MS *m/z* 256.1 $[M+H]^+$.

2.5.6. {[10-(10-{[5-Ethyl-2′,5′-dideoxyuridine-5′-methyl]-carbamoyl}-decylcarbamoyl)-decyl]-methoxycarbonylmethyl-amino}-acetic acid methyl ester (10)

Compound **9** (41 mg, 0.078 mmol) was stirred with DCC (16 mg, 0.078 mmol) and NHS (10 mg, 0.078 mmol) in 5 ml DMF at 55 °C for 1 h. **8** (20 mg, 0.078 mmol) is added and the reaction is further stirred at 55 °C for 3 h.

The solvent was thoroughly evaporated. The residue was purified by preparative TLC. It was dissolved in the minimum amount of MeOH and spotted on Merck preparative layer chromatography silica gel 60 F_{254} (20×20 cm, 2 mm thick, 4 cm concentration zone). Elution was achieved with a mixture $NEt_3/MeOH/CH_2Cl_2$ (0.5:1.5:8). The line corresponding to $R_f = 0.63$ was scratched and the collected silica was put in suspension in 5 ml of a mixture $MeOH/CH_2Cl_2$ (1:1). The suspension was stirred for 5 min and the silica was filtered off. The filtrate was dried in vacuo to afford 20 mg of the product **10** (33%). ^{1}H NMR (CD_3OD) δ 7.45 (s, 1H), 6.19 (t, $J = 7.2$ Hz, 1H), 4.25 (q, $J = 4.5$ Hz, 1H), 3.93 (m, 1H), 3.69 (s, 6H), 3.52 (s, 4H), 3.46 (t, $J = 7.2$ Hz, 2H), 3.15 (t, $J = 6.6$ Hz, 2H), 2.65 (m, 2H), 2.36 (q, $J = 6.9$ Hz, 2H), 2.23 (m, 6H), 1.60 (m, 4H), 1.46 (m, 4H), 1.30 (s, 30H), 1.13 (t, $J = 7.5$ Hz, 3H); MS *m/z* 763.7 $[M]^-$.

*2.5.7. {[10-(10-{[5-Ethyl-2′,5′-dideoxyuridine-5′-methyl]-carbamoyl}-decylcarbamoyl)-decyl]-methoxycarbonylmethyl-amino}-acetic acid (**11**)*

Compound **10** (50 mg, 0.02 mmol) was dissolved in 3 ml $MeOH/H_2O$ (2:1). NaOH (191.8 mg, 4.97 mmol) was added and the mixture was stirred at room temperature for 1h. Then the methanol was removed under vacuum. A precipitation occurred. The precipitate, supposedly the urea of DCC, was filtered off. The pH of the filtrate was brought down to 7 by careful addition of HCl 6 M followed by HCl 0.06 M. At pH 7 a precipitation occurred. After filtration 20 mg (42%) of **11** were isolated. ^{1}H NMR (CD_3OD) δ 7.45 (s, 1H), 6.19 (t, $J = 7.2$ Hz, 1H), 4.26 (q, $J = 4.8$ Hz, 1H), 3.92 (m, 1H), 3.65 (s, 4H), 3.53 (m, 2H), 3.15 (t, $J = 6.9$ Hz, 4H), 2.36 (q, $J = 7.5$ Hz, 2H), 2.19 (m, 6H), 1.48 (m, 32H), 1.13 (t, $J = 7.5$ Hz, 3H). ^{13}C NMR (CD_3OD) δ 176.6, 176.2, 165.9, 152.2, 137.8, 137.6, 117.6, 87.1, 87.0, 86.6, 86.4, 73.1, 58.7, 57.1, 42.4, 40.3, 40.1, 37.1, 30.6, 30.4, 30.2, 28.0, 27.6, 27.1, 27.0, 25.8, 21.3, 13.9, 13.7; MS *m/z* 369.7 $(M(2H)^{2+})$ 738.4 $[M+H]^+$ 760.4 $[M+Na]^+$; Anal. Calc. for $[C_{37}H_{63}N_5O_{10}]_{0.15}[H_2O]_{0.85}$: C, 52.90; H, 8.92; N, 8.34. Found: C, 52.50; H, 8.21, N, 8.06%.

2.6. Syntheses of metal complexes

*2.6.1. Technetium complex **2a***

Three hundred microliters of a 10^{-3}M aqueous solution of **11** (5% DMSO was added to dissolve **11**) and 100 μl of a $[^{99m}Tc(CO)_3(H_2O)_3]^+$ solution were stirred for 30 min at 75 °C. The radioactive product was separated from unlabeled ligand **11** by HPLC using the solvent gradient system A.

*2.6.2. Rhenium complex **2b***

11 (10 mg, 0.013 mmol) and $(NEt_4)_2[ReBr_3(CO)_3]$ (10.4 mg, 0.013 mmol) were stirred in 5 ml MeOH at 60 °C for 1 h. The solvent was removed under vacuum and the residue was purified onto a Sep-Pak® cartridge. The sample was first desalted by eluting with 20 ml water. Then side products were eluted with 10 ml $MeOH/H_2O$ (3:7) followed by 10 ml $MeOH/H_2O$ (1:1). Finally the expected product **2 b** was eluted with 20 ml $MeOH/H_2O$ (7:3). After evaporation of the solvents 7 mg (52%) of **2b** as white powder were collected. ^{1}H NMR (CD_3OD) 8.17 (m, 1H), 7.95 (m,1H), 7.45 (s, 1H), 6.19 (t, $J = 6.9$ Hz, 1H), 4.25 (q, $J = 4.5$ Hz, 1H), 3.90 (m, 1H), 3.71 (d, $J = 15.9$ Hz, 2H), 3.52 (d, $J = 15.9$ Hz, 2H), 3.44 (m, 2H), 3.15 (t, $J = 6.9$ Hz, 2H), 2.36 (q, $J = 7.2$ Hz, 2H), 2.24 (m, 6H), 1.40 (m, 40H), 1.13 (t, $J = 7.5$ Hz, 3H); ^{13}C NMR (CD_3OD) δ 137.8, 87.1, 86.5, 73.1, 70.8, 64.2, 42.4, 40.3, 40.1, 37.1, 30.6, 30.6, 30.5, 30.4, 27.9, 27.1, 27.0, 26.2, 21.3, 13.8; HRMS *m/z* calcd for $C_{40}H_{61}N_5O_{13}Re$ 1006.3826, found 1006.3823 $[M]^-$. Anal. Calc. for $C_{40}H_{61}N_5O_{13}Re[Na]_{0.9}[C_8H_{20}N]_{0.1}$: C, 46.91; H, 6.04; N, 6.84. Found: C, 46.25; H, 6.75, N, 6.66%.

*2.6.3. Technetium complex **12a***

Compound **9** (20 mg, 0.038 mmol) was hydrolyzed in situ with NaOH (200 mg, 5 mmol) in 5 ml $H_2O/MeOH$ (4/1). The mixture was stirred at rt for 1 h. The pH was brought to 6 by addition of 6 M HCl (final concentration of the ligand 10^{-3} M). Three hundred microliters of this solution were added to 100 μl of freshly prepared $[^{99m}Tc(CO)_3(H_2O)_3]^+$. The mixture was stirred for 30 min at 75 °C. The radioactive product was separated from the unlabeled ligand by HPLC using system B ($t_r = 17.1$ min).

*2.6.4. Rhenium complex **12b***

Compound **9** (20 mg, 0.038 mmol) was hydrolyzed in situ with NaOH (200 mg, 5 mmol) in 5 ml $H_2O/MeOH$ (4/1). The mixture was stirred at rt for 1 h. The pH was brought to 6 by addition of 6 M HCl (final concentration of the ligand 10^{-3} M). $(NEt_4)_2[ReBr_3(CO)_3]$ (22.0 mg, 0.029 mmol) was added and the mixture was stirred at 60 °C for 3 h. The reaction was concentrated under vacuum and a Sep-Pak® cartridge was used to remove NaCl. The collected product revealed two peaks on the HPLC with system B ($t_r = 16.5$ min; $t_r = 17.8$ min). A LC-MS allowed the attribution of the mass corresponding to each peak. The peak at 16.5 min was identified to be the labeled free acid (M^- 768.9) and peak at 17.7 min was identified to be the esterified complex due to presence of methanol as solvent (M^- 783.0).

3. Results and discussion

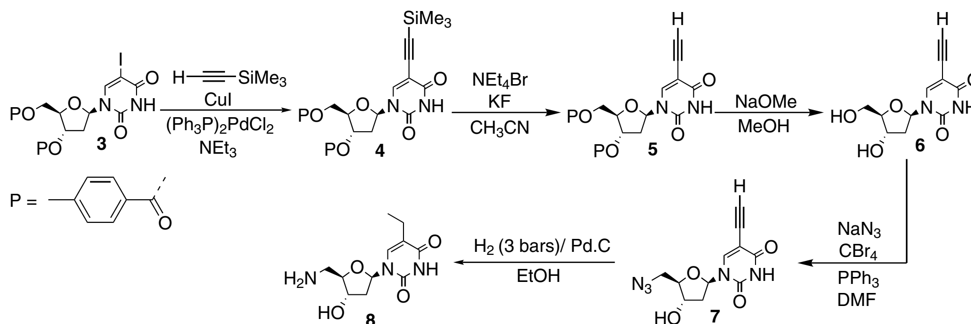
In a previous study we reported that organometallic Tc/$Re(CO)_3$ 5′-carboxamide thymidine complexes could inhibit the HSV1-TK if the pharmacophor and the metal core were separated via a spacer of about 30 Å [15]. The corresponding complex **1** was found to be a competitive inhibitor of HSV1-TK ($K_i = 16.3 \pm 4.6$ μM) but **1** revealed an undesired mixed inhibition toward hTK1 ($K_{ic} = 73 \pm 20$ μM; $K_{iu} = 52 \pm 35$ μM) as well. This activity towards hTK1 could eventually result in poor tumor-to-back-

ground ratio which prohibits detection of the targeted cells. By comparing a series of 5′-substituted thymidine and 5′-substituted 5-ethyl-2′-deoxyuridine derivatives, Martin et al. could clearly demonstrate that introduction of an ethyl group at position 5 improves the inhibition of HSV1-TK significantly and reduces the inhibition of hTK1 [20,21]. Therefore we reasoned to synthesize the organometallic rhenium/technetium complexes of 5′-carboxamide 5-ethyl-2′-deoxyuridine **2** to obtain an inhibitor with a high selectivity for HSV1-TK.

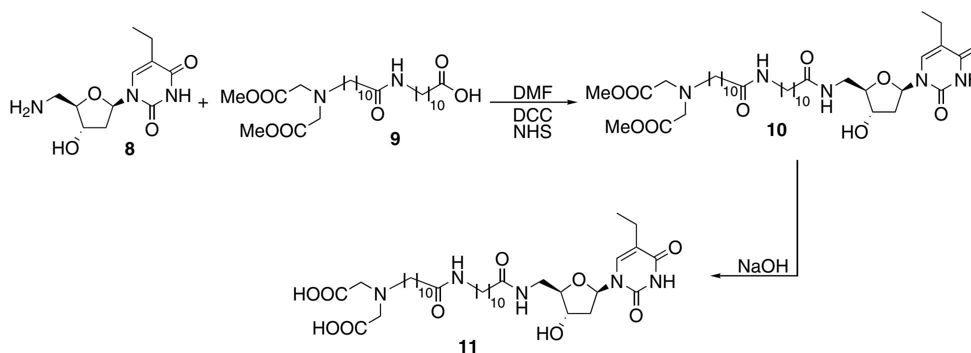
3.1. Chemical syntheses

The synthesis of the 5′-carboxamide 5-ethyl-2′-deoxyuridine **11** started from commercial 2′-deoxyuridine. The hydroxyl groups 3′ and 5′ of the ribose were protected as *p*-toluyl ester and the position 5 was iodinated to afford **3** by published procedures [22]. The iodine was substituted by a trimethylsilylethynyl group via a Sanagoshira coupling using a palladium catalyst in presence of CuI to yield **4**. Compound **5** comprising the terminal alkyne group was then obtained by removing the trimethylsilyl protection in presence of potassium fluoride/tetraethylammonium bromide. The *p*-toluyl esters at position 3′ and 5′ were removed via *trans*-esterification using sodium methylate in methanol to afford compound **6** [23]. The 5′-hydroxyl group was substituted by an azide via a double nucleophilic substitution using triphenylphoshine, tetrabromomethane and sodium azide (compound **7**) [24]. Elegantly, both, the azide and the ethynyl groups of intermediate **7** were simultaneously reduced to a primary amine and an ethyl group under pressurized condition in presence of hydrogen and 10% palladium on charcoal to yield the building block **8** (Scheme 1). The chelating system imine diacetic acid (IDA) including the spacer entity **9** was synthesized in two steps from commercial 11-aminoundecanoic acid as previously published by our group [15]. Amidic coupling of **9** to **8** gave the methyl ester protected intermediate **10**. Finally, the hydrolysis of the esters in basic conditions afforded the desired ligand **11** (Scheme 2).

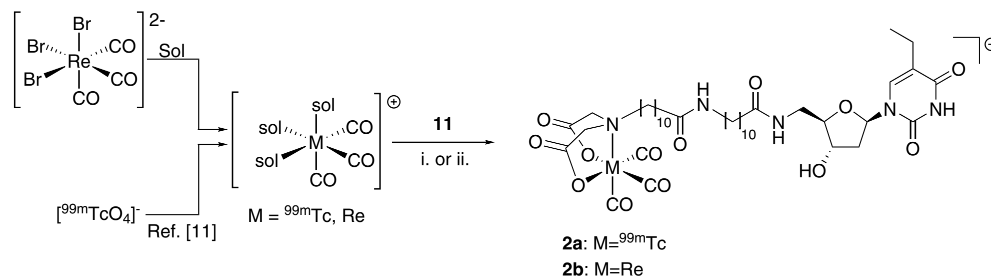
The rhenium analogue **2b** was synthesized starting from the organometallic precursor $(NEt_4)_2[ReBr_3(CO)_3]$ in presence of an equimolar amount of ligand **11** in methanol at 60 °C (Scheme 3). After purification via a Sep-Pak® cartridge, **2b** was analyzed by IR, MS and NMR. The high resolution mass spectrometry displayed the typical pattern of rhenium complexes due to the natural abundance of ^{185}Re and ^{187}Re with a peak at M = 1006.3823 (100%) and a peak at M = 1004.3831 (70%). In the ^{1}H NMR the typical pattern of the four protons of the coordinated IDA proved the rigid coordination of the metal center via the tridentate chelating moiety [25]. The Isolink™ kit was used to produce the precursor $[^{99m}Tc(CO)_3(H_2O)_3]^+$ for radiolabeling of the ligand **11** (Scheme 3). The resulting radiotracer **2a** (retention time on the reversed phase column, t_r = 20.48 min) was separated from unlabeled ligand **11** (t_r = 19.28 min) by HPLC for further in vitro testing (Fig. 2a and b). Since the radioactive complex **2a** revealed



Scheme 1.



Scheme 2.



Scheme 3. (i) $^{99m}Tc(CO)_3(H_2O)_3]^+$, pH 7.4, 75 °C, 30 min and (ii) $[ReBr_3(CO)_3]^{2-}$, MeOH/H_2O, 60 °C, 3 h.

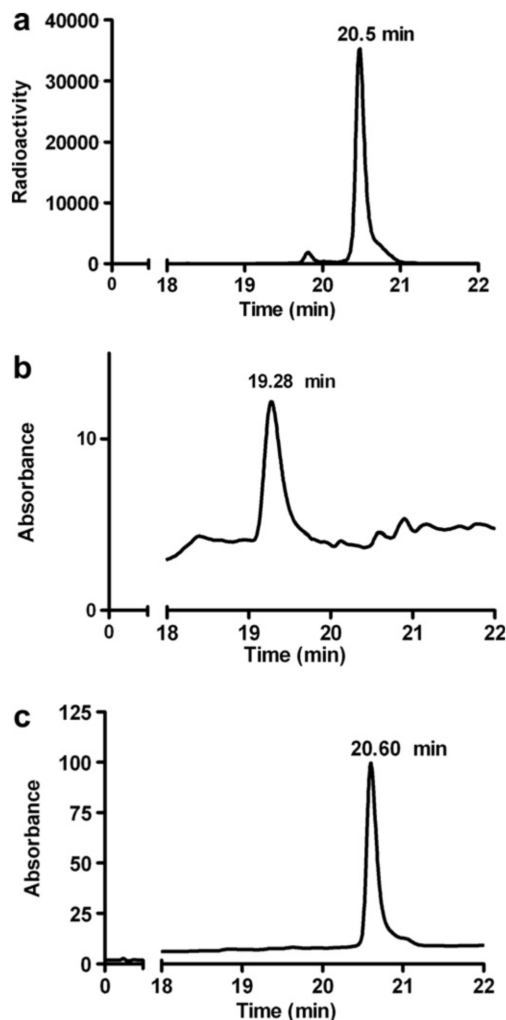


Fig. 2. (a) HPLC chromatogram of purified **2a** (radioactive trace), (b) HPLC chromatogram of **2a** (UV trace, 254 nm) and (c) HPLC chromatogram of **2b** (UV trace, 254 nm).

an almost identical retention times as the non-radioactive complex **2b** (respectively 20.48 and 20.60 min, Fig. 2a and c) the complexes are expected to be isostructural.

3.2. Enzyme kinetics and in vitro cellular experiments

Compound **2b** was tested in vitro against HSV1-TK and hTK1, both expressed as bacterial fusion proteins. Compound **2b** revealed a competitive inhibition of HSV1-TK with $K_i = 4.56 \pm 0.11$ μM (Fig. 3a). This represents an enhancement of four fold compared to the thymidine analogue **1b** ($K_i = 16.3 \pm 4.6$ μM). Another important observation was that **2b** exhibits no inhibition of hTK1

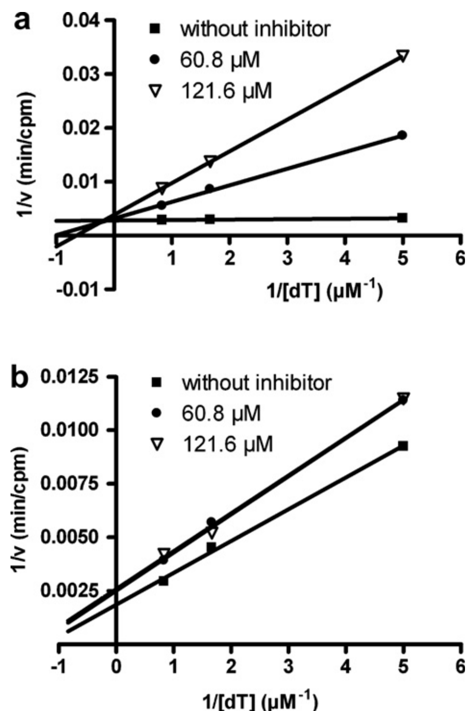


Fig. 3. (a) Lineweaver–Burk plot at two different concentrations of **2b** with HSV1-TK and (b) Lineweaver–Burk plot at two different concentrations of **2b** with hTK1.

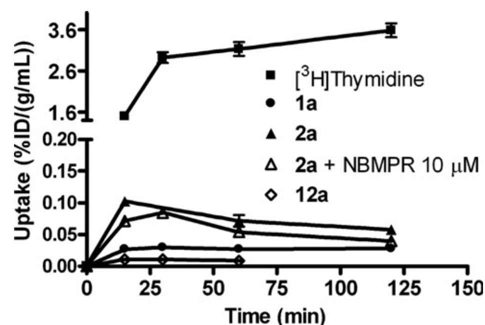
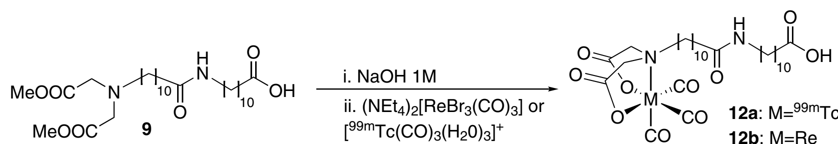


Fig. 4. Uptake of [^{3}H]thymidine, **1a** and **2a** in 143B-TK$^+$ transfected cells.



Scheme 4.

(Fig. 3b) whereas **1b** exhibits a mixed inhibition with only a slight competitive component ($K_{ic} = 73 \pm 20\ \mu M$). It is known from the X-ray structure of hTK1 that the methyl group of the feedback inhibitor dTTP is facing the β-carbon of threonine 163 [26]. The loss of activity against hTK1 observed for **2b** can be attributed to steric clashes between the 5-ethyl group and this residue, which prevents **2b** to be hosted in the active site.

In vitro cell uptake experiment with transfected cells (143-TK$^+$) were carried out with our tricarbonyl technetium complexes **1a** and **2a**. The results are presented in Fig. 4. The maximal uptake of **1a** was 0.030%ID/(g/ml) after 30 min and the maximal uptake of **2a** was found to be 0.103%ID/(g/ml) after 15 min. Under the same conditions we observed for [^{3}H]thymidine an uptake of up to 1.5%ID/(g/ml) after 15 min. The uptake increases to reach a plateau of 3.7%ID/(g/ml) after 60 min. The low uptake of complex **2a** (and **1a**) compared to [^{3}H]thymidine indicated the absence of an active transport. This hypothesis could be verified with blocking experiment using the known nucleoside transporter inhibitor nitrobenzylmercaptopurineriboside (NBMPR) [27,28]. We observed no significant effect on the cellular uptake of **1a/2a** in the presence of NBMPR, whereas e.g. the uptake of [^{3}H]thymidine was significantly reduced. Thus, radioactivity found in cells incubated with **1a/2a** presumably entered the cells via passive diffusion only. On the other hand, control experiments performed with the tricarbonyl technetium complex **12a** lacking the thymidine/uridine moiety (Scheme 4) revealed an even lower internalization into143-TK$^+$ cells than **1a/2a** (0.011%ID/(g/ml)). This observation evidences that the presence of the pharmacophor has a positive influence on the (passive) uptake in cells. The fact that **2a** bears a negative charge probably prevents good solubility in the lipid membranes and consequently prohibits a better diffusion into the cells.

4. Conclusion

The organometallic 5′ carboxamide 5-ethyl-2′-deoxyuridine derivatives of technetium-99m and rhenium **2a/b** have been successfully prepared. 5-Ethyl-2′-deoxyuridine derivative **2** inhibits HSV1-TK four-fold better than its thymidine analogue **1**. Moreover, introduction of the ethyl group at position 5 lead to an increased selectivity of the complex for the targeted enzyme HSV1-TK. However, the experiments revealed a low cellular uptake of the technetium 2′-deoxyuridine complex **2a** due to the absence of an active transport and inefficient passive diffusion due to the negative overall charge of the complex.

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