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Synthesis, *in vitro* and *in silico* assessment of organometallic Rhenium(I) and Technetium(I) thymidine complexes

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Abstract

Thymidine kinases have been identified as suitable targets for non-invasive imaging of gene therapy and cancer. Thus, there is a high interest in new, reliable and inexpensive radiolabeled thymidine analogues for these applications. In this study we present the synthesis and *in vitro* evaluation of $M(CO)_3$-complexes of thymidine (M = ^{99m}Tc, Re) for potential use in SPECT tumor imaging. 5′-amino-5′-deoxythymidine was derivatized at position C5′ with spacers of various lengths (~0–30 Å) carrying tridentate metal chelating entities such as iminodiacetic acid and picolylamine-*N*-monoacetic acid. The nucleoside derivatives were reacted with the precursors $[ReBr_3(CO)_3]^{2-}$ and $[^{99m}Tc(OH_2)_3(CO)_3]^{+}$, respectively. The organometallic thymidine complexes have been fully characterized by means of IR, NMR and mass spectrometry. Enzyme kinetic studies revealed mixed inhibition of the human cytosolic thymidine kinase with K_i values ranging from 4.4 to 334 μM for all thymidine complexes. Competitive inhibition of herpes simplex virus type 1 thymidine kinase was only achieved when thymidine and the metal core were separated by a spacer of approximately 30 Å length. These findings were supported by *in silico* molecular docking and molecular dynamic experiments.

Keywords: Technetium; Rhenium; Enzyme inhibitors

1. Introduction

Thymidine kinases are key enzymes for metabolisms of viruses and mammals. The role of this group of enzymes is the phosphorylation of pyrimidines in the framework of the salvage pathway. Two of these enzymes, namely the human cytosolic thymidine kinase (hTK1) for assessment of cancer cells proliferation and the herpes simplex virus type 1 thymidine kinase (HSV1-TK) for gene therapy monitoring, have received attention for their potential use as targets in detection and therapy of cancerous diseases.

hTK1 activity is very low in G_1-phase and increased throughout *S*- and G_2-phase to reach maximum levels during mitosis [5]. This induces higher concentration and activity of hTK1 in the tumor cells compared to normal cells [18,5]. Based on this fact, thymidine derivatives have been developed for non-invasive *in vivo* imaging such as Positron Emission Tomography (PET). The thymidine analogue 3′-[^{18}F]Fluoro-3′-deoxythymidine ([^{18}F]FLT) has been lately considered as an alternative to 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG) as proliferation marker for breast and colorectal cancer [7,12]. HSV1-TK has become a target of interest in suicide gene therapy. When expressed in tumor cells via an adenovirus bearing the

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appropriated encoding gene, the HSV1-TK is able to transform e.g. ganciclovir into a highly cytotoxic phosphorylated form due to its broad substrate acceptance compared to, e.g. hTK1 [8–11,25]. An important factor in this methodology is to determine to which extent the cells have been transfected. Imaging and quantification of gene expression with PET-tracers has been shown to be helpful in this respect. So far, 9-[4-[^{18}F]fluoro-3-(hydroxymethyl)-butyl]guanine ([^{18}F]FHBG) and 5-[^{124}I]iodo-2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl-uracil ([^{124}I]FIAU) were found to be most suitable for monitoring the progress of gene therapy [22].

The low-cost single photon emission computed tomography (SPECT) isotope Technetium-99m shows almost ideal decay properties for diagnosis ($E_\gamma = 140$ keV and $t_{1/2} = 6.0$ h). Because ^{99m}Tc is readily available due to a ^{99}Mo/^{99m}Tc generator system it is still the mainstay in routine nuclear medicine [1,14,16]. Therefore, the development of new SPECT radiotracers based on this important isotope is still of highest interest.

The present work focuses on the synthesis of new organometallic thymidine complexes of technetium and rhenium, the quantitative evaluation of inhibition constants K_i and the determination of the type of the inhibition toward hTK1 and HSV1-TK of 5′-carboxamide thymidine derivatives [20]. For the (radio)labeling of the thymidine derivatives we employed the organometallic aqua ions $[M(OH_2)_3(CO)_3]^+$ ($M = {}^{99m}$Tc, ^{nat}Re) [4,2]. In addition molecular docking and molecular dynamics experiments were performed based on the X-ray structure of HSV1-TK with the most promising complex candidate.

2. Results and discussion

Martin and co-workers have reported a series of organic 5′-carboxamide thymidine and 5-ethyluridine derivatives bearing large aromatic entities. Several of these compounds have proved to be efficient and selective inhibitors of HSV1-TK and HSV2-TK revealing K_i-values in the nanomolar range [17]. These potent inhibitors have been selected as lead structures for the development of our organometallic nucleoside analogues for potential use in diagnostic nuclear medicine. We set out to exchange the aromatic residue at the position 5′ by chelating moieties suitable for the *fac*-[M(CO)$_3$]-fragment [20]. It was previously observed by our group that the complexes tested till today showed no inhibition of HSV1-TK while they exhibited inhibition of hTK1 [20]. We assumed that the lack of activity of the tested complexes against HSV1-TK was the result of steric clashes with the enzyme's ternary structure due to inappropriate lengths of the spacers between the tricarbonyl rhenium core and the thymidine moiety. Therefore, the synthesis of new thymidine derivatives with either significantly shorter **11**–**12** or longer **13** spacer entities were envisaged to avoid potential interferences between the metal core and the protein. The effect of the overall charge of the metal complex on the inhibitory capacity was also investigated by synthesizing the neutral complex **14** which is structurally related to the negatively charged complex (**16**) previously published [20]. The structures of the newly and previously synthesized complexes are depicted in Fig. 1.

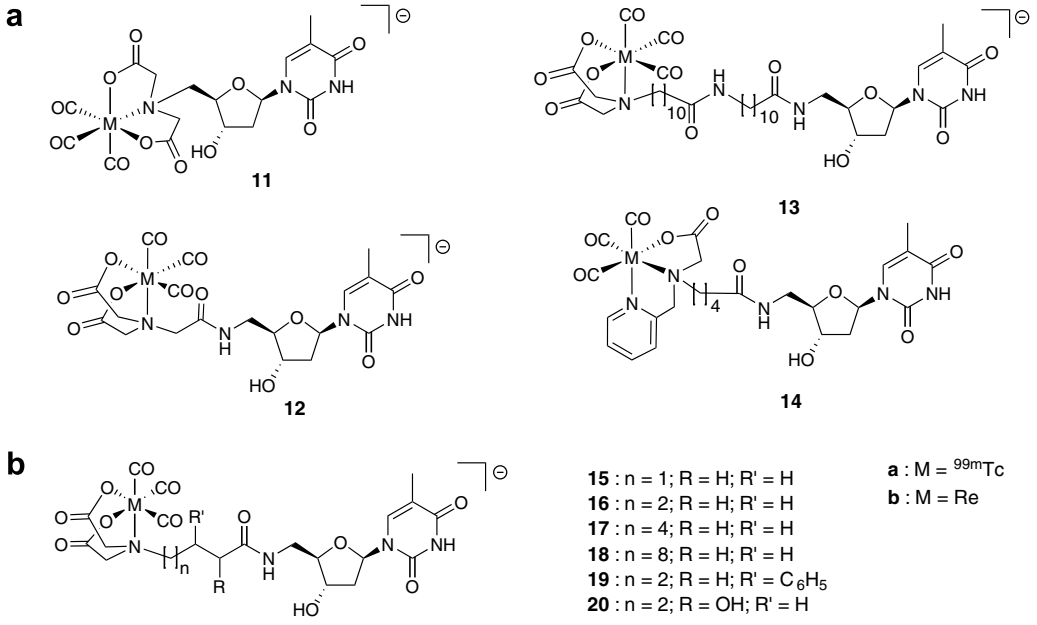
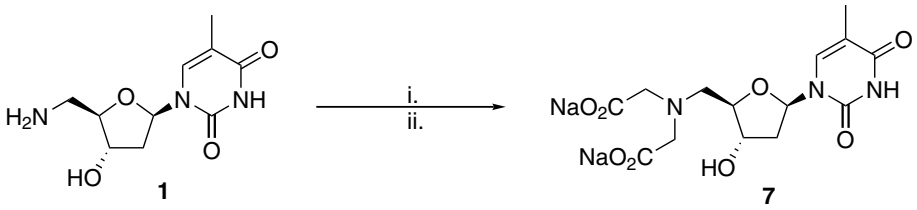


Fig. 1. (a) Structures of new complexes **11**–**14** and (b) previously synthesized complexes **15**–**20** [20].



Scheme 1. (i) $BrCH_2CO_2Me$, NEt_3, MeOH, reflux, 12 h and (ii) 1 M NaOH, RT, 2 h.

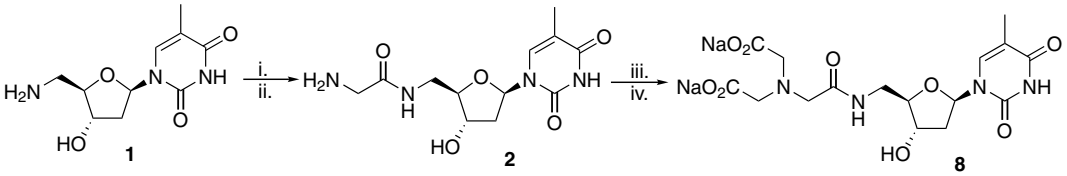
2.1. Ligand syntheses

The versatile precursor 5′-amino-5′-deoxythymidine **1** was synthesized according to a published procedure [6] and used as the starting material for the synthesis of 5′-carboxamide thymidine derivatives. Two tridentate chelating systems iminediacetic acid (IDA) in case of ligands **7**–**9** and picolylamine-*N*-monoacetatic acid (PAMA) in case of ligand **10** have been used to stably coordinate the organometallic metal core. Compound **7** was obtained in a two steps synthesis. First the primary amine of 5′-amino-5′-deoxythymidine **1** was double alkylated with two equivalents of methyl bromoacetate in presence of triethylamine in methanol. Then the methyl esters were hydrolyzed at room temperature with aqueous NaOH to yield the ligand **7** as the disodium salt (Scheme 1).

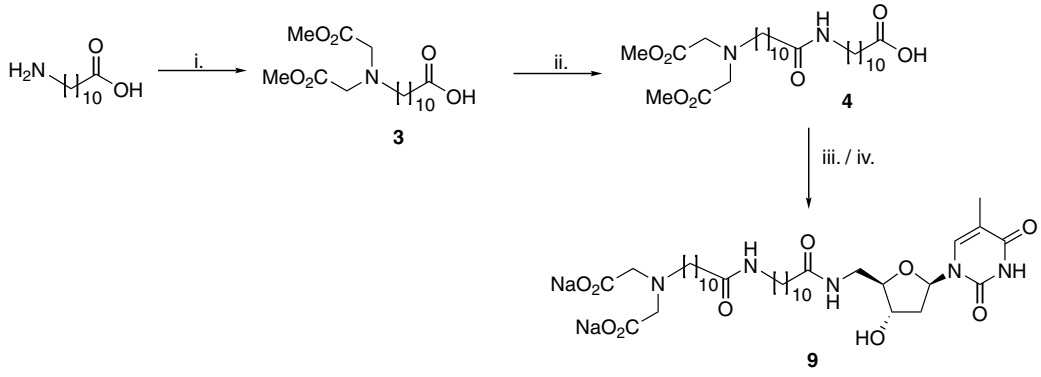
Compound **8** was synthesized in three steps (Scheme 2). Commercially available Boc-glycine was reacted with **1** using a coupling protocol, employing *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) in DMF. Purification of the intermediate was performed by flash chromatography on silica gel. After removal of the Boc protecting group in presence of boron trifluoride-ethyl etherate compound **2** was isolated. The N-terminus of **2** was double alkylated with two equivalents of methyl bromoacetate. After hydrolysis of the methyl ester, the thymidine derivative **8** was obtained (yield: 67%).

For the preparation of ligand **9** the strategy was slightly different (Scheme 3). First commercial 11-aminoundecanoic acid was reacted with two equivalents of methyl bromoacetate chelate in presence of triethylamine in methanol to afford 11-[bis(carboxymethyl)amino]undecanoic acid **3**. This intermediate was then coupled to another molecule of 11-aminoundecanoic acid to give intermediate **4**. Then compounds **4** and **1** were coupled. After deprotection of the acid functionalities, ligand **9** was obtained in an overall yield of 68%.

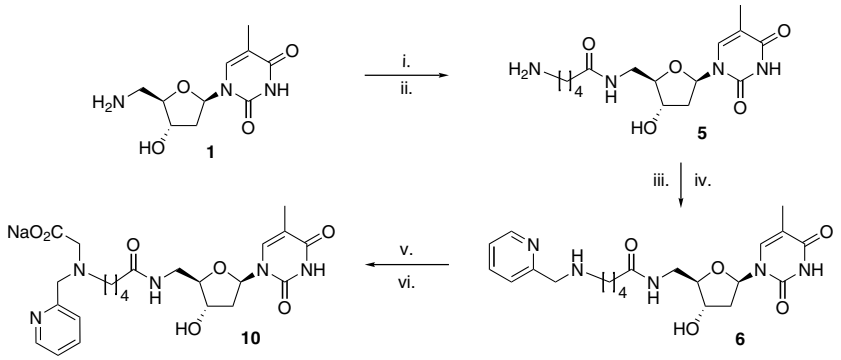
Ligand **10** with a picolylamine-*N*-monoacetic acid chelating entity was synthesized in a step by step approach as outlined in Scheme 4. *N*-Boc-4-aminovaleric acid was coupled to 5′-amino-5′-deoxythymidine **1**. Purification of the intermediate was achieved by chromatography on silica gel (yield: 77%). After deprotection of the amine functionality, compound **5** was isolated and further reacted with



Scheme 2. (i) BocGly, DCC, NHS, DMF, 50 °C, 90 min; (ii) 20% boron trifluoride-ethyletherate; (iii) $BrCH_2CO_2Me$, NEt_3, MeOH, reflux, 12 h; and (iv) 1 M NaOH, 50 °C, 12 h.



Scheme 3. (i) Methyl bromoacetate, NEt_3, MeOH, reflux, 12 h; (ii) 11-aminoundecanoic acid, DCC, NHS, DMF, 55 °C, 90 min; (iii) **1**, DCC, NHS, DMF, 55 °C, 90 min; and (iv) 1 M NaOH, 50 °C, 12 h.

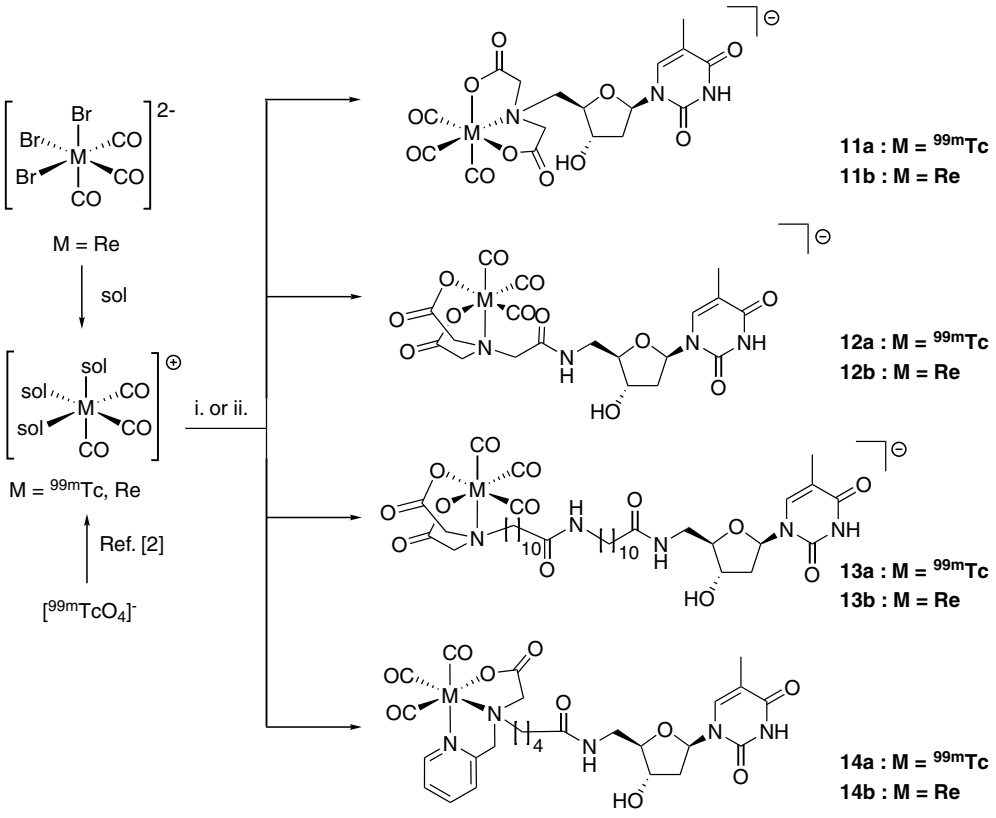


Scheme 4. (i) *N*-Boc-4-aminovaleric acid, DCC, NHS, DMF, 55 °C, 2 h; (ii) 3 M HCl, r.t., 1 h; (iii) pyridine carbaldehyde, MeOH; (iv) $Na[BH(CH_3CO_2)_3]$, MeOH, 5 h; (v) methyl bromoacetate, NEt_3, MeOH, reflux, 12 h; and (vi) 1 M NaOH, r.t., 2 h.

2-pyridinecarboxaldehyde to form the Schiff base, which was reduced in situ to give intermediate **6**. Alkylation of **6** was accomplished with methyl bromoacetate under basic conditions. The intermediate was purified over silica gel and finally deprotected with NaOH to yield ligand **10** (overall yield: 22%).

2.2. Syntheses of organometallic thymidine complexes

The organorhenium complexes **11b-14b** have been prepared in a mixture of methanol/water (1/1) at 50 °C using the precursor $(NEt_4)_2[ReBr_3(CO)_3]$ and stoichiometric amount of the corresponding ligand (Scheme 5). The reactions were monitored by mean of HPLC ($\lambda = 254$ nm) until completion (after 2–4 h). The crude products were purified over SepPak® reversed phase columns using a water–methanol gradient. The corresponding anionic complexes **11b**, **12b**, and **13b** were obtained as single species in good yields with a mixture of Na^+ and NEt_4^+ as counter ions as evident from elemental and NMR analyses. The IR spectra of the complexes **11b**–**14b**, revealed the typical *fac*-$M(CO)_3$ pattern with significantly blue-shifted CO stretch frequencies (around 2020 cm^{-1} and 1880 cm^{-1}) compared to the starting material $(NEt_4)_2[ReBr_3(CO)_3]$ (2000 cm^{-1} and 1868 cm^{-1}). The ^1H NMR spectra revealed the frequently observed metal-induced low-field shifts of protons of the



Scheme 5. (i): M = ^{99m}Tc: PBS buffer, pH 7.4, 75 °C, 1 h and (ii) M = Re: MeOH/H_2O, 50 °C, 5 h.

chelator due to the rigid coordination of the metal center. No significant chemical shifts of other protons were observed, which excluded unspecific coordination of the *fac*-[M(CO)$_3$]-core via other functional groups present in the thymidine derivatives. The formation of isomers in case of complex **14b** is likely. However, we were unable to distinguish them neither be NMR nor by HPLC. Complexes **11b**, **12b**, and **14b** are readily soluble in water and only complex **13b** had to be dissolved by addition of 0.05% Tween 80 a hydrophilic non-ionic surfactant.

The corresponding radioactive Technetium-99m complexes **11a**–**14a** have been almost quantitatively prepared in physiological phosphate buffer (PBS; pH 7.4) at ligand concentrations of 10^{-4} M after 60 min at 75 °C using the IsoLink™ kit. Single species have been produced with all nucleoside derivatives. The characterization of the complexes was accomplished by comparison of the retention times observed in the γ-trace with those of the UV-trace of the corresponding rhenium complexes. The ^{99m}Tc-complexes were purified via HPLC for stability testing. The organometallic ^{99m}Tc-complexes were challenged in PBS buffer and human serum albumin for 24 h at 37 °C. Decomposition or dissociation of the complexes to either [$^{99m}TcO_4$]$^-$ or other side products was less than 8% for all complexes under these conditions, which is tolerable for potential nuclear medical applications.

2.3. In vitro experiments

The rhenium complexes **11b**–**20b** were evaluated for inhibition of HSV-1 TK and hTK1 with a protocol using ^{3}H-thymidine [13]. Lineweaver–Burk plots allowed the determination of the type of inhibition as well as the evaluation of the K_i-values for all synthesized complexes. The kinetics experiments confirmed our earlier observations: Derivatives **15**–**20** act exclusively as inhibitors toward hTK1. The same holds true for the newly synthesized complexes **11**, **12**, and **14**. Lineweaver–Burk plots allowed the attribution of a mixed type of inhibition and revealed K_{iu} values ranging from 4.4 to 334 μM (Table 1). The competitive components K_{ic} were measured to be more than 50 μM for all complexes (with the exception of **12**: $K_{ic} = 18$ μM). The site of uncompetitive interaction/inhibition of the metal complexes with hTK1 is difficult to predict at the moment. It is noteworthy that both complexes with a butyl-spacer (complexes **14** and **16**) but with different overall charge represent the extreme of the measured K_{iu}-spectrum. Therefore it appears that the overall charge has a significant impact on the potency of uncompetitive inhibition.

Table 1
Enzyme kinetic parameters of Re(CO)$_3$-aminothymidine complexes **11b**–**20b**

Complex	HSV-1 TK K_i (μM)	hTK1 K_i (μM)
11b	n.i.	112 ± 61[b]
12b	n.i.	54 ± 23[b]
13b	16.3 ± 4.6[a]	52 ± 35[b]
14b	n.i.	334 ± 22[b]
15b	n.i.	44 ± 15[b]
16b	n.i.	4.4 ± 1.5[b]
17b	n.i.	7.2 ± 3.7[b]
18b	n.i.	10.0 ± 4.9[b]
19b	n.i.	17.8 ± 6.5[b]
20b	n.i.	14.6 ± 1.3[b]

n.i. = no inhibition.
[a] Competitive inhibition.
[b] Mixed type of inhibition.

2.4. In vitro and in silico evaluation of complex *13*

From the fact, that complexes **11**, **12**, and **14**–**20** do not display inhibition of the HSV1-TK we concluded, that the tricarbonyl metal center can not be accommodated in the active site of the viral enzyme. This argument is likely since Martin et al. reported also the loss of activity of thymidine derivatives if the position 5′ was substituted with bulky phosphonates or sulphonates presumably due to steric hindrance of the lid domain of the enzyme [17,19]. We therefore reasoned to introduce a long spacers between the thymidine entity and the metal core to avoid any interaction of our metal center with the lid domain. We connected two 11-aminoundecanoic acids and formed the complex **13** allowing a maximal spatial separation of the complex moiety and thymidine entity of approximately 30 Å. To our delight, enzyme kinetic experiments revealed that compound **13b** inhibited HSV1-TK in a competitive manner. The K_i value was found to be in the low μM-range (16.3 ± 4.6 μM) (Fig. 2a). In addition, the complex **13b** is

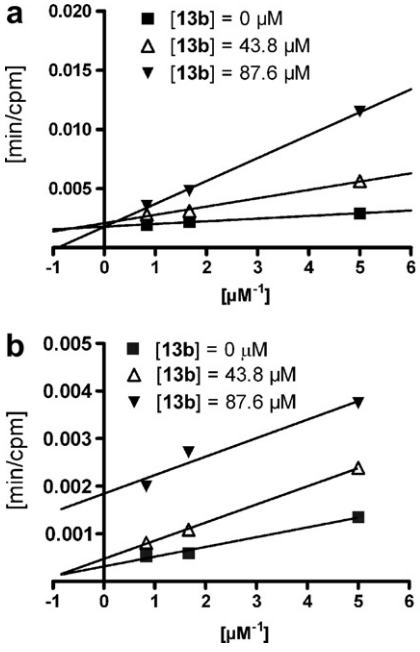


Fig. 2. (a) Lineweaver–Burk plot for inhibition of HSV1-TK by **13b**; (b) Lineweaver–Burk plot for inhibition of hTK1 by **13b**; cpm = counts per minute.

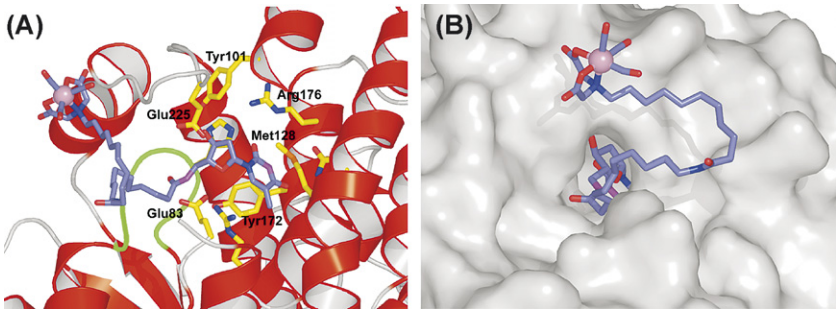


Fig. 3. (A) Predicted binding mode of complexes **13b** (metal center in pink; p-loop in green) in the active site of HSV-1 TK. (B) View along the thymidine-Re-tricarbonyl axis (protein surface presented in grey).

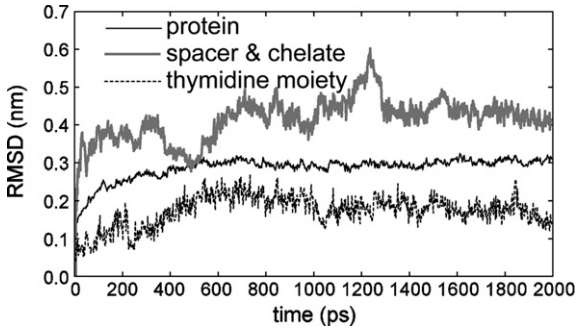


Fig. 4. Root-mean-square-deviation (RMSD in nm) of HSV-1 TK (thin black line) and complex **13b** (bold gray line: spacer and complex entities; dashed line: thymidine part) during 2 ns simulation.

also a mixed inhibitor of hTK 1 as observed for the complexes **11b**, **12b**, and **14b**–**20b** (Fig. 2b).

Parallel to the synthesis of complex **13** *in silico* docking experiments based on the crystal structure of the HSV1-TK were performed [26]. These calculations evidenced hydrogen bonds of Gln125 with O-4 and N-3 of thymine, as well as the hydrogen bonds between the 3′-OH group of the sugar moiety and Tyr101 and Glu225 similar to those found for natural substrates [24]. Formation of the typical sandwich-like complex with Tyr172 and Met128 was also observed (Fig. 3A). From Fig. 3B one can clearly see, that the chosen spacer entity enables hosting the metal core at the surface of the enzyme. Molecular dynamic calculations also predicted the formation of a stable system for the enzyme-thymidine complex. The RMSDs of different components reach a stable plateau around 0.3 nm after 600 ps. Relatively large temporary fluctuations were only observed for the spacer entity, which can be explained by its flexibility (Fig. 4).

3. Conclusions

A representative series of 5′-functionalized thymidine derivatives labeled with the organometallic technetium- and rhenium-tricarbonyl core have been synthesized and characterized. The enzyme kinetic experiments revealed mixed inhibition of human cytosolic thymidine kinase 1 for all complexes independent on the spacer length. On the other hand, the inhibition of the herpes simplex virus thymidine kinase 1 was dependent on the spacer length separating the thymidine moiety and the metal core. Competitive inhibition was found only for the complex offering the possibility to host the metal core at the surface of the protein avoiding interference with the enzyme's lid domain. On the other hand, one has to critically acknowledge that the relatively high K_i-values of complex **13** with respect to HSV1-TK and the mixed type of inhibition with respect to hTK1 presumably preclude any application in tumor imaging at the moment. Strategies to improve the affinity are the preparation of 5-ethyl-2′-deoxyuridine derivatives and/or derivatives, which are fluorinated at the ribose ring.

4. Experimental

4.1. General

Solvents and chemicals for synthesis were purchased from Aldrich Chemical or Fluka (Buchs, Switzerland). The organometallic precursor $(NEt_4)_2[ReBr_3(CO)_3]$ were synthesized according to the literature [3]. Complexes **15**–**20** prepared according to the literature [20]. $Na[^{99m}TcO_4]$ was eluted from a $^{99}Mo/^{99m}Tc$ generator (Mallinckrodt-Tyco, Petten, The Netherlands) using 0.9% saline. The radioactive precursor $[^{99m}Tc(H_2O)_3(CO)_3]^+$ was prepared with the IsoLink™ kit (Mallinckrodt-Tyco, Petten, The Netherlands) [2]. HPLC analyses of the rhenium and Technetium-99m complexes were performed on a Merck-Hitachi L-7000-system equipped with an L-7400 tunable absorption detector and a Berthold LB 506 B radiometric detector using a Macherey-Nagel C-18 reversed phase column (10 μm, 150 × 4.1 mm). HPLC solvents: Aqueous 0.05 M triethylammonium phosphate (TEAP) buffer, pH 2.25 (solvent A), methanol (solvent B). The HPLC system started with 100% of A from 0 to 3 min. The eluent switched at 3 min to 75% A and 25% B and at 9 min to 66% A and 34% B followed by a linear gradient 66% A/34% B to 100% B from 9 to 20 min. The gradient remained at 100% B for 2 min before switching back to 100% A. The flow rate was 1 ml/min. Nuclear magnetic resonance spectra were recorded on a 300 MHz Varian Gemini 2000

spectrometer. The ^{1}H and ^{13}C chemical shifts are reported relative to residual solvent protons as a reference. IR spectra were recorded on a Perkin–Elmer FT-IR 16PC using KBr pellets. Mass and elemental analyses were performed at the ETH, Zurich.

4.2. In vitro assays

HSV1-TK and hTK1 were expressed as bacterial fusion proteins. The kinetic constants of both enzymes were determined by measurement of initial velocities. The reaction 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 [^{3}H]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 (MultiScreen®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 (Packard 1900TR). 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 calculations of K_i-values.

4.3. Molecular modeling

The initial conformation of HSV1 TK was taken from the structure published by Wild et al. [26]. Compound **13** was manually docked into its active by alignment of its thymidine part onto the thymidine molecule present in the crystal structure. The force field parameters of the compound were obtained using PRODRG tool [21] and on the basis of the crystal structure of an isostructural rhenium complex. Interaction of the metal atom with its ligands was treated with harmonic potential. Partial charges were calculated *ab initio* for thymidine and the organometallic part separately using the method of Kollman and Singh [23] on the basis of a wave function calculated on HF/MINI level. Energy minimizations and molecular dynamics simulations were performed in Gromacs 3.2.0 [15] force field. The solvated protein was simulated in NVT ensemble at 300 K during 2 ns.

4.4. Synthesis of ligand 7

5′-Amino-5′-deoxythymidine (**1**) (179 mg, 0.74 mmol) was suspended in MeOH (15 ml). Triethylamine (187 mg, 1.85 mmol) was added first. Then methyl bromoacetate (250 mg, 1.52 mmol) was added dropwise over a period of 2 h. The suspension was refluxed overnight. The solvent was removed and the product was purified by chromatography on silica gel (EtOAc/MeOH: 1/9). The hydrolysis of the ester groups was performed in equimolar amounts of 1 M NaOH. Yield: 75 mg (52%). ^{1}H NMR (CD_3OD): δ 7.62 (s, 1H) 6.32 (t, $J = 6.7$, 1H), 4.44 (m, 1H), 4.27 (m, 1H), 3.72 (s, 4H), 3.56 (m, 2H), 2.46 (m, 2H), 2.01 (s, 3H). ^{13}C NMR (CD_3OD): δ 173.4, 166.3, 152.2, 138.1, 111.8, 86.4, 85.9, 73.0, 56.9, 56.4, 52.0, 40.0, 12.4. MS (ESI): *m/z* (%) 378 (100) $[M + Na]^-$. Elemental analysis: Calc. for $C_{14}H_{17}N_3Na_2O_8 \cdot 0.7$ NaCl: C, 38.03; H, 3.88; N, 9.50. Found: C, 38.11; H, 3.67; N, 9.42%.

4.5. Synthesis of compound 2

Boc-glycine (200 mg, 1.14 mmol) was solved in dry DMF (15 ml). DCC (166 mg, 1.29 mmol) and NHS (148 mg, 1.29 mmol) were added. The solution was stirred at 50 °C for 90 min. 5′-Amino-5′-deoxythymidine (**1**) (286 mg, 1.11 mmol) was added in portions and the reaction mixture was stirred at RT for 3 h. The DMF was removed in vacuum and the product was purified by chromatography on silica gel (EtOAc/MeOH: 9/1). Deprotection of the amino group was performed in 20% boron trifluoride-ethyletherate in CH_2Cl_2 (10 ml). It was monitored by HPLC. The solution was neutralized with $NaHCO_3$ and dried in vacuo. Yield: 300 mg (91%). ^{1}H NMR (CD_3OD): δ 7.62 (s, 1H), 6.31 (t, $J = 6.5$ Hz, 1H), 4.43 (m, 1H), 4.03 (m, 1H), 3.85 (s, 2H), 3.66 (d, $J = 5.5$, 2H), 2.43 (m, 2H), 2.07 (s, 3H),1.52 (m, 9H); HPLC: R_t: 17.0 min. Yield: 301.2 mg (100%). ^{13}C NMR (D_2O): δ 171.3, 162.3, 153.0, 143.3, 109.4, 85.8, 80.3, 78.1, 58.4, 57.1, 46.3, 41.3, 14.3. MS (ESI): *m/z* (%) 298 (100) $[M^+]$. Elemental analysis: Calc. for $C_{12}H_{18}N_4O_5$: C, 48.3; H, 6.1; N, 18.8. Found: C, 48.2; H, 5.9; N, 18.6%.

4.6. Synthesis of ligand 8

Compound **2** (211 mg, 0.71 mmol) dissolved in dry methanol (20 ml). Triethylamine (147 mg, 1.45 mmol) was added. Methyl bromoacetate (250 mg, 1.52 mmol) was added drop wise over a period of 2 h. The suspension was refluxed overnight. The solvent was removed and the product was purified by chromatography on silica gel (EtOAc/MeOH.9:1). Hydrolysis of the ester groups was performed in equimolar amounts of 1 M NaOH. Yield: 278 mg (67%). ^{1}H NMR (D_2O): δ 7.72 (s, 1H), 6.50 (t, $J = 6.8$ Hz, 1H), 4.67 (m, 1H), 4.31 (m, 1H), 3.83 (m, 5H), 3.74 (s, 2H), 3.55 (s, 4H), 2.62 (m, 2H), 2.21 (s, 3H). ^{13}C NMR (D_2O): δ 179.3, 174.4, 162.9, 152.1, 137.7, 118.6, 114.7, 112.0, 110.9, 85.3, 84.1, 71.5, 37.9. MS (ESI): *m/z* (%) 436.1 (100) $[MNa]^-$. HRMS Calc. for $C_{16}H_{21}N_4O_9$ 413.1309. Found: 413.1311.

4.7. Synthesis of compound **3**

11-Aminoundecanoic acid (200 mg, 0.99 mmol) and NEt_3 (0.413 ml, 2.97 mmol) are dissolved in 50 ml MeOH. This mixture is stirred at RT for 30 min. Afterwards methyl bromoacetate (0.191 ml, 2.08 mmol) is added drop wise. After addition the mixture is stirred at refux for 12 h. The MeOH was removed under vacuum and the residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH: 9.5/0.5) to give the 11-[bis(carboxymethyl)amino]undecanoic acid **3**. Yield: 165 mg (48%). ^{1}H NMR (CD_3OD) δ 3.69 (s, 6H), 3.52 (s, 4H), 2.66 (t, $J = 7.5$ Hz, 2H), 2.27 (t, $J = 7.2$ Hz, 2H), 1.60 (m, 2H), 1.45 (m, 2H), 1.31 (s, 12H); ^{13}C NMR (CD_3OD) δ 177.8, 173.2, 55.6, 55.5, 52.0, 35.0, 30.6, 30.5, 30.4, 30.2, 28.6, 28.2, 26.1; MS *m/z* 346.4 $[M + H]^+$. Elemental analysis: Calc. for $C_{17}H_{31}NO_6$: C, 59.1; H, 9.1; N, 4.1. Found: C, 58.8; H, 8.8; N, 4.1%.

4.8. Synthesis of compound **4**

11-[Bis(carboxymethyl)amino]undecanoic acid (158 mg, 0.45 mmol) was dissolved in DMF (15 ml). DCC (103 mg, 0.5 mmol) and NHS (57.5 mg, 0.5 mmol) were added. The reaction was stirred at 55 °C for 90 min. The temperature was allowed to come back to RT. 11-Aminoundecanoic acid (90 mg, 0.45 mmol) was added and the solution was stirred at room temperature for 5 h. The reaction was filtered. The filtrate was evaporated and the residue was purified by chromatography on silica gel (CH_2Cl_2/EtOAc: 1/1) to give **4**. Yield: 214 mg (88%). ^{1}H NMR (CD_3OD) δ 7.95 (m, 1H), 3.69 (s, 6H), 3.53 (s, 4H), 3.16 (m, 2H), 2.66 (m, 2H), 2.27 (t, $J = 7.5$ Hz, 2H), 2.14 (t, $J = 7.5$ Hz, 2H), 1.84 (m, 1.5H), 1.72 (m, 1.5H), 1.56 (m, 3H), 1.47 (m, 3H), 1.25 (s, 30H), 1.15 (m, 2H); ^{13}C NMR (CD_3OD) δ 177.7, 176.2, 173.2, 55.6, 55.6, 52.0, 40.3, 37.1, 35.0, 30.6, 30.6, 30.6, 30.5, 30.4, 30.4, 30.2, 28.6, 28.2, 27.9, 27.1, 26.8, 26.1; MS *m/z* 527.3 $[M]^-$. Elemental analysis: Calc. for $C_{28}H_{52}N_2O_7$: C, 63.6; H, 9.9; N, 5.3. Found: C, 63.5; H, 9.9; N, 5.3%.

4.9. Synthesis of ligand **9**

Compound **4** (78 mg, 0.14 mmol) was solved in DMF (10 ml). DCC (30 mg, 0.14 mmol) and NHS (17 mg, 0.14 mmol) were added. The solution was stirred at 55 °C for 90 min. The reaction was monitored by TLC (silica gel; CH_2Cl_2/EtOAc: 1/1). 5′-Amino-5′-deoxythymidine (35 mg, 0.14 mmol) was added in portions and the reaction mixture was stirred at RT for 3 h. The DMF was removed in vacuo and the product was purified by chromatography on silica gel (EtOAc/MeOH: 4/1). The hydrolysis of the ester groups was performed in equimolar amount of 1 M NaOH at 50 °C overnight. Yield: 92 mg (78%). ^{1}H NMR (CD_3OD): δ 7.52 (s, 1H), 6.20 (t, $J = 6.7$ Hz, 1H), 4.25 (m, 1H), 4.10 (s, 4H), 3.91 (m, 1H), 3.48 (m, 2H), 3.15 (t, $J = 7.0$ Hz, 2H), 3.2 (t, $J = 3.1$Hz, 2H), 2.19 (m, 6H), 1.91 (s, 3H), 1.73 (m, 2H), 1.60 (m, 4H), 1.46 (m, 2H), 1.21-1.34 (m, 24H),. ^{13}C NMR (D_2O): δ 177.6, 170.7, 166.5, 151.7, 137.5, 111.6, 85.5, 85.4, 71.6, 57.1, 56.3, 40.8, 38.1, 35.9, 32.7, 29.2, 28.6–28.1, 25.5, 23.8, 11.7. MS (ESI): *m/z* (%) 768 (100) $[M + Na]^-$. Elemental analysis: Calc. for $C_{38}H_{31}N_5O_{10} \cdot 0.8\ H_2O$: C 55.6, H 8.5; N 8.5. Found: C, 55.4; H, 8.7; N, 8.5%.

4.10. Synthesis of compound **5**

N-Boc-4-aminovaleric acid (493 mg, 2.27 mmol) was solved in 20 ml DMF. DCC (529 mg, 2.56 mmol) and NHS (295 mg, 2.56 mmol) were added. The solution was stirred at 55 °C for 2 h. 5′-Amino-5′-deoxythymidine (543 mg, 2.25 mmol) was added portion wise and the reaction was monitored by TLC (EtOAc/MeOH). The solvent was removed under vacuum and the product was purified by chromatography on silica gel (EtOAc/MeOH: 9/1). The collected Boc-protected product was dissolved in 1 M HCl (5 ml). This solution was stirred at RT for 2 h and the conversion was monitored with TLC (silica gel; EtOAc/MeOH: 9/1). After evaporation of the solvent the compound was isolated as the corresponding hydrochloride. Yield: 130 mg (75%); ^{1}H NMR (CD_3OD): δ 7.52 (s, 1H), 6.23 (t, $J = 6.8$ Hz, 1H), 4.63 (m, 1H), 4.21 (m, 2H), 3.97 (m, 1H), 3.46 (m, 2H), 3.05 (m, 2H), 2.25 (m, 2H), 1.91 (s, 3H), 1.66 (m, 4H). ^{13}C NMR (CD_3OD): δ 172.4, 163.1, 155.0, 147.2, 111.8, 85.8, 80.3, 77.1, 58.4, 57.1, 42.4, 39.3, 35.1, 33.615.9. MS (ESI): *m/z* (%) 340 (100) $[M]^+$. Elemental analysis: Calc. for $C_{15}H_{25}ClN_4O_5$: C, 47.8; H, 6.7; N, 14.9. Found: C, 47.7; H, 6.5; N, 14.7%.

4.11. Synthesis of compound **6**

Compound **5** (342 mg, 0.91 mmol) and triethylamine (93 mg, 0.91 mmol) were solved in dry MeOH (15 ml) and 2-pyridine carbaldehyde (96 mg, 0.90 mol) was added drop wise and stirred for 30 min at RT. Sodium triacetoxyborohydride (190 mg, 0.90 mmol) was added under a stream of nitrogen and the solution was stirred at RT for 12 h. The reaction was quenched with 10 ml of an acidic solution pH 2 and the solvents were removed under vacuum. The product was purified by chromatography on silica gel (CH_2Cl_2/MeOH: 9/1 + 1% NH_3). Yield: 303 mg (77%). MS (ESI): *m/z* (%) 432 (100) $[M^-]$. Compound **6** was used without further characterization for the next reaction step.

4.12. Synthesis of ligand **10**

Compound **6** (202 mg, 0.46 mmol) and triethylamine (71 mg, 0.7 mmol) were solved in MeOH (15 ml). Methyl bromoacetate (72 mg, 0.46 mmol) was added dropwise at RT. After addition the mixture was refluxed for 5 h. The solvent was removed and the product was purified by chromatography on silica gel (CH_2Cl_2/MeOH: 9/1 + 1% NH_3). Deprotection of the ester functionalities was achieved

during evaporation due to the presence of NH_3. Yield: 51 mg (22%). ^{1}H NMR (CD_3OD): δ 7.3–8.4 (m, 4H), 7.62 (s, 1H), 6.25 (t, $J = 6.7$ Hz, 1H), 4.25 (m, 2H), 3.91 (m, 3H), 3.52 (d, $J = 5{,}6$ Hz, 2H), 3.37 (s, 2H), 2.69 (m, 2H), 2.23 (m, 4H), 1.93 (s, 3H), 1.66 (m, 2H), 1.53 (m, 2H). ^{13}C NMR (CD_3OD): δ 176.7, 173.7, 166.8, 161.0, 152.7, 149.7, 139.1, 138.6, 125.3, 124.2, 112.1, 87.4, 86.9, 73.5, 61.1, 55.8, 55.3, 42.9, 40.5, 37.2, 28.3, 25.0, 12.8. MS (ESI): m/z (%) 512.3 (100) $[M + Na]^-$. Elemental analysis: Calc. for $C_{23}H_{30}N_5NaO_7$: C, 54.0; H, 5.9; N, 13.7. Found: C, 53.9; H, 5.7; N, 13.5%.

4.13. Synthesis of the complexes *11a–14a*

The Technetium-99m complexes were prepared according to the following general procedure: 900 μL of a solution of *fac*-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ (pH 7.4) and 100 μL of a 10^{-4} M solution of the corresponding ligands in PBS buffer (0.1 M NaCl/ 0.05 M sodium phosphate buffered, pH 7.4) were placed in a 10 ml glass vial under nitrogen. The vial was sealed and the reaction heated to 75 °C for 60 min and cooled on an ice bath. The reaction was checked by HPLC (γ-trace). The complexes were characterized by comparison with the corresponding Rhenium complexes (UV; 254 nm).

4.14. Synthesis of complexes *11b–14b*

All complexes were prepared according to the following general procedure. $(NEt_4)_2[ReBr_3(CO)_3]$ and 1.1 equivalent of the corresponding thymidine derivative were dissolved in 5 ml H_2O/MeOH (1:1). The reaction was stirred at 50° and monitored by HPLC. After completion the solvent were removed under reduced pressure. The residue was purified by chromatography on SepPak®-cartridge (Waters, C18-cartridges) using a H_2O/MeOH gradient starting with 100% H_2O and finishing with 50% H_2O. The fractions were analyzed via HPLC and those containing exclusively the desired rhenium complexes dried in vacuo. From HPLC analyses one could conclude that the rate of product formation was generally >90%. Isolated yields varied between 30 and 60%. Analytical data for complex **11b**: ^{1}H NMR (CD_3OD): δ 7.48 (s, 1H), 6.19 (m, 1H), 4.20 (m, 2H), 3.73 (m, 6H), 2.42 (m, 2H), 1.90 (s, 3H), 1.29 (m, 8H); ^{13}C NMR (CD_3OD): δ 198.8 (CO), 197.8 (CO), 183.4, 182.5, 166.4, 152.1, 138.9, 112.1, 87.4 83.5, 73.6, 72.2, 65.7, 63.9, 53.3, 39.1, 12.6, 7.7; Elemental analysis for Calc. $C_{17}H_{17}N_3O_{11}Re(C_8H_{20}N)_{0.7}Na_{1.2}Br_{0.9}$: C, 33.2; H, 3.8; N, 6.3. Found: C, 33.4; H, 4.0; N, 6.1%. HPLC: R_t: 16.8 min; MS (ESI): m/z (%) 625.9 (100), 623.9 (60) $[M^-]$. Analytical data for complex **12b**: ^{1}H NMR (CD_3OD): δ 7.53 (s, 1H), 6.21 (t, $J = 6.7$ Hz, 1H), 4.26 (m, 1H), 4.14 (s, 2H), 3.91 (m, 5H), 3.50 (m, 2H), 2.28 (m, 2H), 1.93 (s, 3H), 1.29 (m, 10H). ^{13}C NMR (CD_3OD): δ 195.7 (CO), 195.0 (CO), 180.2, 167.1, 135.7, 109.1, 84.3, 83.2, 70.2, 67.4, 62.3, 51.1, 50.4, 49.8, 39.5, 37.1, 4.9; Elemental analysis for Calc. $C_{19}H_{20}N_4O_{12}Re(C_8H_{20}N)_{0.7}Na_{0.3}$: C, 37.8; H, 4.4; N, 8.4. Found: C, 37.3; H, 5.0; N, 7.9%. HPLC: R_t: 15.9 min; MS (ESI): m/z (%) 682.9 (100), 680.9 (60) $[M^-]$. Analytical data for complex **13b**: ^{1}H NMR (CD_3OD): δ 7.52 (s, 1H), 6.21 (t, $J = 6.6$ Hz, 1H), 4.25 (m, 1H), 3.92 (m, 1H), 3.70 (d, $J = 16.0$ Hz, 2H), 3.53 (d, $J = 16.0$, 2H), 3.48 (m, 4H), 3.15 (m, 2H), 2.20 (m, 6H), 1.93 (s, 3H), 1.73 (m, 2H), 1.60 (m, 4H), 1.48 (m, 2H), 1.31 (m, 24H). ^{13}C NMR (CD_3OD): δ 198.9 (CO), 198.0 (CO), 183.0, 176.6, 152.3, 138.2, 111.8, 86.9, 86.5, 73.1, 70.8, 64.2, 53.2, 42,4, 40.1, 37.1, 30.5, 27.9, 27.0, 26.2, 12.4, 7.6; Elemental analysis for Calc. $C_{47}H_{79}N_6O_{13}Re$: C, 50.3; H, 7.1; N, 7.5. Found: C, 50.6; H, 7.5; N, 7.5%. HPLC: R_t: 19.9 min; MS (ESI): m/z (%) 992 (100), 990 (60) $[M^-]$. Analytical data for complex **14b**: ^{1}H NMR (CD_3OD): δ 8.82 (m, 1H), 8.08 (m, 1H), 7.70 (m, 2H), 7.53 (s, 1H), 6.21 (m, 1H), 4.67 (m, 1H), 4.53 (m, 1H), 4.26 (m, 2H), 3.90 (m, 2H), 3.53 (m, 2H), 2.34 (m, 2H), 2.26 (m, 2H), 1.91 (s, 3H), 1.72–1.88 (m, 4H). ^{13}C NMR (CD_3OD): δ 197.1 (CO), 196.3 (CO), 182.1, 174.6, 159.0, 152.7, 152.2, 140.8, 137.2, 125.9, 124.0, 110.6, 86.1, 85.5, 72.2, 69.7, 68.2, 60.8, 41.5, 38.9, 35.2, 24.3, 22.8, 11.1; Elemental analysis for Calc. $C_{26}H_{30}N_5O_{10}Re \cdot 1.2H_2O$: C, 40; H, 4.1; N, 8.9. Found: C, 40.2; H, 4.2; N, 8.57%. MS (ESI): m/z (%) 781.9 (100), 779.5 (60) $[M^-]$.

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