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Targeting the Folate Receptor (FR): Imaging and Cytotoxicity of Re^{I} Conjugates in FR-Overexpressing Cancer Cells

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*The synthesis, characterization, in vitro imaging, and cytotoxic properties of a new folate conjugate of rhenium(I) are reported. The conjugate [FA-PEG-BQAV-Re(CO)$_3$]$^+$ (γ-**4**) was screened against an adriamycin- and cisplatin-resistant human ovarian cancer cell line (A2780/AD) that overexpresses the folate receptor (FR). Compound γ-**4** was internalized by a folate-receptor-mediated endocytotic pathway, which results in internal accumulation of γ-**4**. This was contrasted with a FR-negative Chinese hamster ovary cell line in which no internalization of γ-**4** was observed. γ-**4** was found to be cytotoxic with IC_{50} values of 189 and 78 μM at 6 and 24 h, respectively, toward the FR-positive cell line. This is in contrast to the IC_{50} value of 502 μM at 6 h and 84 μM at 24 h for cisplatin in the same cell line, with a significantly greater toxicity at the earlier time point. The cytotoxicity of γ-**4** as explained by interactions that occur between the rhenium(I) complex moiety and DNA is described.*

Introduction

Specific targeting of cancer cells remains one of the major themes in medicinal chemistry. The goal is one of overcoming the problems that are associated with the loss of healthy tissue by using classic chemotherapy and has driven the search for, and identification of, unique markers of tumors such as prostate-specific membrane antigen or overexpressed folate receptor (FR). The ability to selectively target tumors would be predicted to assuage patients' treatment burden in that it provides a tropism for the drug and greatly improves its therapeutic index. The use of overexpressed FR in particular as a tumor marker has generated considerable interest.[1] Targeting the FR is attractive because in addition to being overexpressed in tumor lines, it is down-regulated (and inaccessible to blood circulation) in healthy adult cells. The FR facilitates the uptake of folic acid (FA) (see Figure 1), a vitamin (B_9) that is necessary for cell growth and proliferation.[1] The FR has a high binding affinity ($K_D \sim 0.42 \times 10^9$ M) for FA,[2] and once bound, FA is rapidly delivered into the cell through endocytosis.[3] Several tumors overexpress the FR, including ovarian (A2780), breast (MDA-MB-231), cervical (HeLa-IU$_1$), nasopharyngeal (KB), and colon (Caco-2).[4] Consequently the FR is ideal for delivery of antiproliferative compounds or imaging agents to locate such cancerous tissue in vivo, and a number of examples of such a use have been reported.

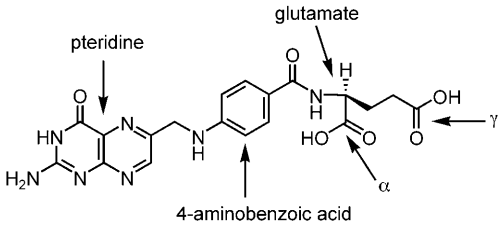

Figure 1. FA with its three major structural components including the α- and γ-carboxylic acid group of the glutamate moiety indicated.

Low et al. have investigated a ^{67}Ga–deferoxamine complex that is conjugated to FA,[5] while other groups have looked at FA-based conjugates of metal radionuclide agents such as: $^{66/68}$Ga,[6] ^{111}In,[7] and ^{64}Cu.[8] The FR has also been exploited for single-photon emission computed tomography (SPECT) by attaching a ^{99m}Tc–diethylenetriamine pentaacetic acid complex with a polyethylene glycol spacer to FA.[9] This resulted in localized imaging in lymphatic tumors that express FR, and low uptake in other tissues except for the kidneys.[9] Recently, a ^{99m}Tc/Re^{I} tricarbonyl core that is chelated by a picolylamine monoacetic acid with an aminohexane spacer and conjugated to FA has been reported.[10a] In this investigation, Schibli and co-workers showed effective cell binding and internalization of the folate derivatives of the metal fluorophore.[10a]

This widespread use of ^{99m}Tc in diagnostic medicine has also drawn attention to the potential of technetium's heavier congener, rhenium and in particular isotopes ^{186}Re and ^{188}Re. For example, Re^{I} has been complexed to boronic acid derivatives of bipyridine as an in vivo optical glucose detector to eliminate the need for blood sampling to determine blood glucose levels.[10b] This approach is based on fluorescence switching through photoinduced electron transfer, in which the absence of glucose quenches the fluorescence of the metal complex

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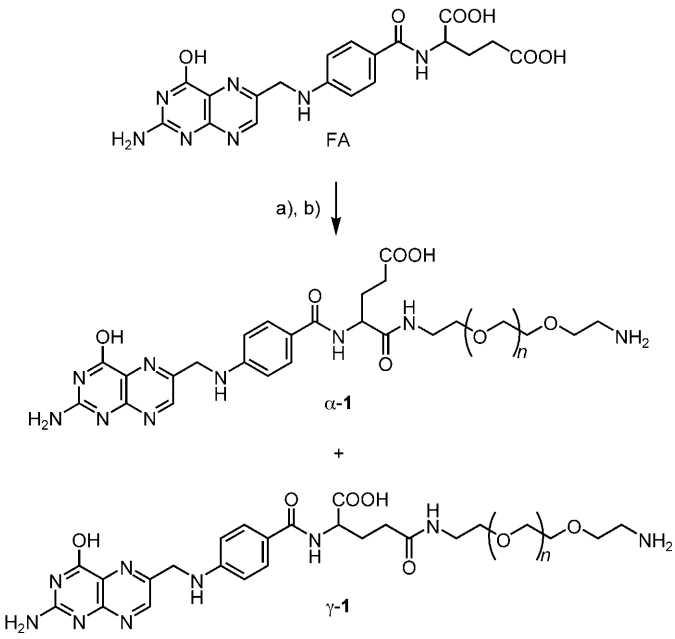
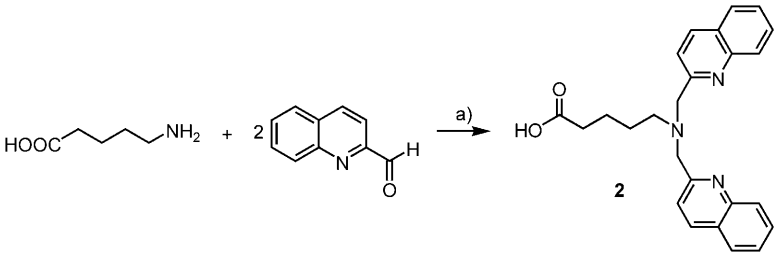
transducer and the presence of glucose increases luminescence intensity.[10b] ^{188}Re ($t_{1/2}$ = 17 h, ε_β = 2.12 MeV, ε_γ = 155 keV) has also been explored mainly for its radiotherapeutic properties. A recent study targeted metastatic melanomas by using a heptapeptide conjugate of the radionuclide, ^{188}Re-HYNIC-Asn-Pro-Asn-Trp-Gly-Pro-Arg (HYNIC = 6-hydrazinonicotinamide), which resulted in considerable tumor retardation.[11] Recently, $^{186/188}$Re systems that were complexed to 1-hydroxyethylidene-1,1-diphosphonate showed promise as a radiopharmaceutical for the palliative treatment of bone cancers, with patients reporting significant pain relief.[12] Furthermore, Spitzweg and colleagues have explored the therapeutic properties of ^{188}Re, which is transported by a sodium iodide symporter and results in tumor volume decrease in prostrate cancer.[13a] The first ^{188}Re radiofolate was prepared and its pharmacokinetic profile was investigated alongside with ^{99m}Tc.[13b] Biodistribution experiments of the ^{188}Re conjugate showed excellent tumor uptake with low tumor-to-kidney ratios upon administration of an antifolate.[13b]

Combining then the intriguing imaging and therapeutic properties of rhenium isotopes with the molecular target that is offered by the folate receptor could produce effective pharmaceuticals for the diagnosis and/or treatment of FR-positive cancers. Based on these considerations, we have set out to investigate the properties of a bisquinoline-based Re^{I} chelate complex bound to FA–polyethylene glycol conjugates. Herein we present the synthesis and characterization of a new Re^{I} conjugate, and demonstrate its selectivity for FR cell lines and its intriguing cytotoxic properties.



Scheme 1. Synthesis of γ-**1** via DCC/NHS coupling: a) DCC/NHS, DMSO (anhyd); b) H_2N-PEG-NH_2, pyridine, DMSO (anhyd).



Scheme 2. Preparation of **2** via reductive amination: a) $Na[BH(CH_2COO)_3]$.

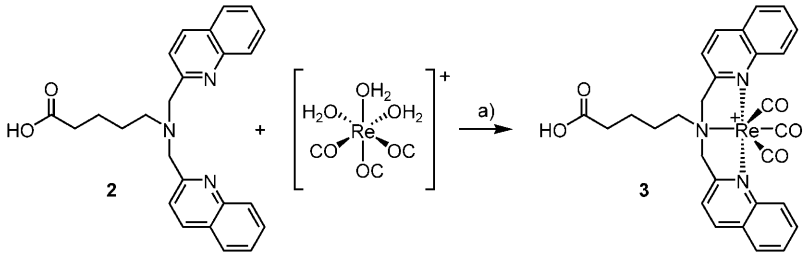
Results and Discussion

Synthesis and characterization

The synthesis of γ-**1** was performed by coupling of polyethylene glycol (PEG, $M_r \sim 2000$) to the glutamate moiety of FA. FA was activated by *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) coupling agents as shown in Scheme 1. The reaction yielded two FA-based regioisomers, designated α- and γ-**1**. These isomers were separated by ion-exchange chromatography and characterized as described previously.[14] Such separation is necessary to remove the inactive α-**1** system, which is not recognized by the FR.[15]

Compound **2** was prepared by reductive amination of two moles of quinoline-2-carboxaldehyde and the amino acid linker, 5-aminovaleric acid as previously described and shown in Scheme 2.[16] A modification in the literature purification step was made by precipitating **2** by using ethyl acetate/hexane (2:8) at room temperature. This eliminated the need for column chromatography. The expected mass (*m*/*z* 399) was verified by electrospray ionization mass spectrometry (ESI-MS, see Supporting Information), and the ^{1}H NMR spectrum was consistent with **2**.

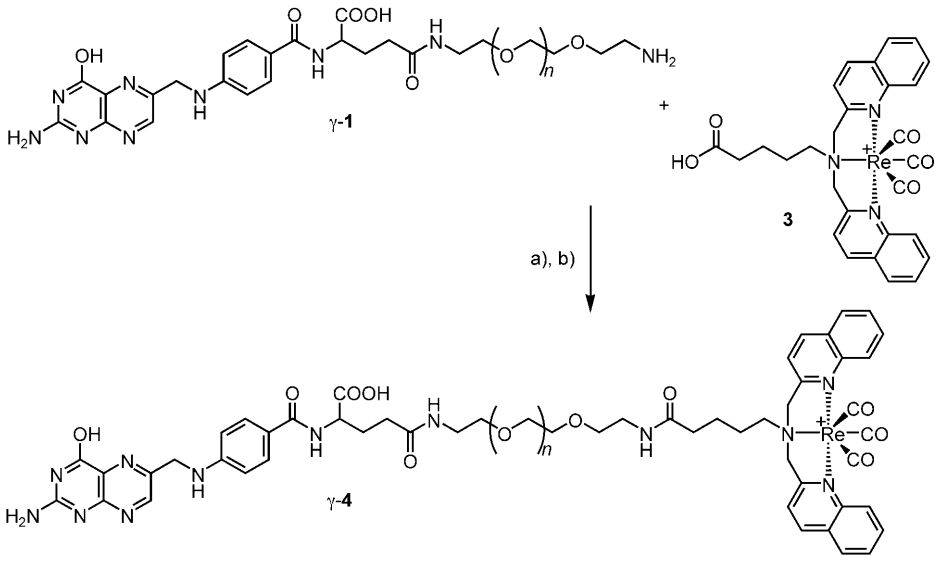
The complexation of $[Re(CO)_3(H_2O)_3]^+$ to **2** (see Scheme 3) was carried out in methanol by reflux at 70 °C for 16 h. Compound **3** was isolated by using reversed-phase C_{18} HPLC (RP-HPLC); it had a retention time of $t_R \sim 10.2$ min, in contrast to that of **2**, which eluted at ~6.0 min. Compound **3** was characterized by ^{1}H NMR spectroscopy and ESI-MS. Furthermore, the isotopic distribution pattern observed in ESI-MS was also con-



Scheme 3. Labeling of **2** with $[Re(CO)_3]^+$: a) MeOH, 70 °C.

sistent with rhenium incorporation (see Supporting Information). Electronic absorption spectroscopy provided molar extinction coefficients (ε) at 232 nm ($108\,625\ M^{-1}cm^{-1}$) and 314 nm ($23\,776\ M^{-1}cm^{-1}$), which are assigned to $\pi\rightarrow\pi^*$ transitions from the aromatic quinoline rings.

Compound γ-**1** was conjugated to **3** via a 1,1′-carbonyl-di-(1,2,4-triazole) (CDT) coupling reaction in dry DMSO as shown in Scheme 4; **3** was activated by CDT at 60 °C over 30 min. A yellow solution of γ-**1** was then added to this solution. An amber-colored solution was observed after 16 h. 1,2-Dichloroethane was added to this solution, and the solution was washed with water. The organic layer was dried in vacuo and redissolved in water/acetonitrile (1:9). Purification was carried out with RP-HPLC. Compound γ-**4** eluted at a retention time of $t_R \sim 13.4$ min.



Scheme 4. Conjugation of γ-**1** and **3** via CDT coupling: a) CDT, 60 °C, 30 min; b) TEA.

The identity of γ-**4** was confirmed by MALDI-TOF MS; the compound displayed a mass peak that was centered at *m/z* ~3007, which corresponds to the desired theoretical mass (see Supporting Information). The broad variance is the result of PEG polydispersity (peak separation of *m/z* 88 corresponds to two ethylene glycol units (*m/z* 44)). Further characterization by ^{1}H NMR spectroscopy was also consistent with the identity of γ-**4**.

In vitro cell imaging

Confocal microscopy experiments were performed on FR-overexpressing A2780/AD cells, with CHO cells as negative control (CHO cells do not express FR, as confirmed by RT-PCR). Compound γ-**4** was added to the cells at a concentration of 10 μM, and the solution was incubated for 45 min and 24 h. No time dependence was noted in this range, and an incubation period of 45 min was sufficient for uptake and internalization; this is consistent with FR's known ability to bind and internalize FA within ~3 min.[17] Uptake of γ-**4** was visualized by fluorescence in A2780/AD cells (see Figure 2). Scanning from the surface through the intracellular regions to determine internalization of the drug was performed by slicing through the cell in 1 μm increments (see Supporting Information). Fluorescence was uniformly distributed in and throughout the cell, which clearly indicates the successful uptake of γ-**4.** No internalization of γ-**4** in CHO was observed, although some nonspecific surface binding was noted. This is due to the presence of PEG and has been reported.[17] Performing an acidified saline wash decreases this occurrence.[18] Scanning from the surface through the cell in 1 μm increments was also performed on the CHO cells, and no fluorescence was observed. This is consistent with no internalization of γ-**4** in CHO cells; γ-**4** is bound only to the cell surface membrane (data not shown).

To determine whether the presence of FA inhibited the binding of γ-**4** to the FR, an excess of FA (~500 mM) was added to a solution of γ-**4** (10 μM) and incubated, for between 45 min and 24 h, with the FR overexpressing A2780/AD cells growing in FA-containing RPMI 1640 media. No uptake was observed as followed by confocal microscopy in contrast to the rapid and extensive internalization noted in A2780/AD cells that were grown in the absence of FA. This is consistent with the idea that binding of γ-**4** to the FR is completely blocked by the presence of free FA. Even after 24 hours of incubation with γ-**4** and excess FA, no internalization was observed.

In vitro cell cytotoxicity

To the best of our knowledge, there has been limited investigation of the antiproliferative properties of Re^{I} conjugates, and only limited reports of IC_{50} values for Re^{I} compounds.[19,20] We have conducted antiproliferative studies to determine the impact of the presence Re^{I} in terms of cytotoxicity. Compounds γ-**1**, **2**, **3**, and γ-**4** were tested against the FR-overexpressing A2780/AD and FR-free CHO cell lines for suitable comparison over a time period of 6 and 24 h (see Table 1). Results indicate that both the rhenium-free systems, namely γ-**1** and **2**, have low toxicity, marked by the high-micromolar IC_{50} values that were determined for **2** and by the fact that no IC_{50} value was noted for γ-**1** up to a concentration of 5 mM (see Table 1). The cytotoxicity on either of the two lines, FR-free or FR-overexpressing cells was also similar for both γ-**1** and **2**. In contrast, **3** and γ-**4** were shown to have significantly greater toxicity, especially in the earlier 6-hour time point, for FR-expressing cells

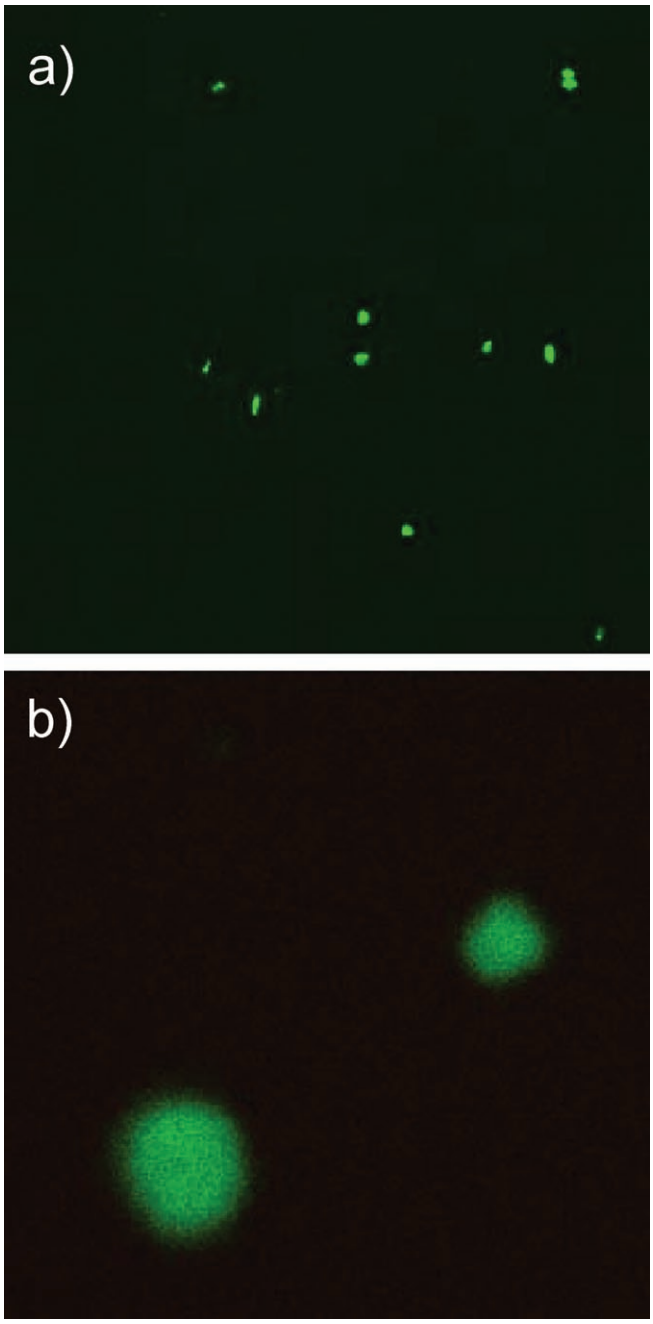


Figure 2. A2780/AD cells incubated with γ-**4**: a) 10× magnification showing fluorescently labeled cells; b) 63× magnification showing a closer look of the cellular pool.

Table 1. IC_{50} values in A2780/AD and CHO cells [μM].

Drug	A2780/AD		CHO	
	6 h	24 h	6 h	24 h
γ-**1**[a]	–	–	–	–
2	2837 ± 0.310	1765 ± 0.0400	2979 ± 0.210	1526 ± 0.0200
3	1684 ± 1.80	590 ± 5.88	2627 ± 2.80	746 ± 3.90
γ-**4**	189 ± 15.0	78.2 ± 0.210	204 ± 12.0	80.8 ± 13.0
cisplatin	502	84	12	4.8

[a] Not cytotoxic at concentrations up to 2 mM.

than FR-free cells (see Table 1). Clearly the presence of the FR and the presence of rhenium itself are playing a key role in toxicity. The greater toxicity of γ-**4** over **3** might be related to uptake and cellular retention. Compound γ-**4** undergoes facilitated transport through the FR, but **3** can only gain entry through passive diffusion. This facilitated uptake and the fact that FA–PEG-containing systems have been shown to have increased intracellular residency times[21] would explain this greater antiproliferative activity. This postulated increased residency might also play a role when comparison is made between the toxicity of γ-**4** and cisplatin in A2780/AD cells; γ-**4** is significantly more toxic than cisplatin in the multi-drug-resistant A2780/AD cell line at 6 h. This can be rationalized by the efficient uptake of γ-**4** via the FR endocytotic pathway, which evidently out-competes the rate of intracellular cisplatin accumulation.

IC_{50} values were also determined for non-FR-expressing CHO cells (see Table 1). As in the A2780/AD cells, the presence of rhenium has significantly increased the cytotoxicity over γ-**1** and **2**. Also of interest here is the observed toxicity, which is similar to that observed in the FR-expressing A2780/AD cells, for γ-**4** (189 and 204 μM at 6 h and 78.2 and 80.8 μM for A2780/AD and CHO respectively) in CHO cells. The toxicity that is associated with **3** can be explained by passive diffusion for cellular entry, but, as evidenced by confocal microscopy experiments, nonspecific cell surface binding was observed for γ-**4**, and no internalization was observed after 45 min and 24 h incubation. Toxicity then might be due to these surface interactions. Previous work describing the effects of metal ions on membrane surfaces has certainly shown toxicity can occur,[22–24] but the exact effects that are induced by γ-**4** on the membrane surface requires further investigation. Finally, unlike the resistance to cisplatin that is observed in A2780/AD cells, cisplatin had IC_{50} values in the low μM range (12 and 4.8 μM at 6 and 24 h respectively, compared with 502 and 84 μM for the same time points in A2780/AD cells).

Ligand challenge experiments

Competitive binding experiments were performed with a 100-fold excess of histidine (100 mM) or 5 mM glutathione on 1 mM **3** in phosphate-buffered saline (PBS) at pH 7.4 to gauge the stability of **3**. This is important given that the presence of rhenium clearly impacts the antiproliferative activity of the compounds and noting that rhenium release would aid to our understanding of mechanism. Three solutions were used: 1) **3** in PBS, 2) **3** in PBS with histidine and 3) **3** in PBS and glutathione. The solutions were incubated at 37 °C over a period of 48 h. Analytical C_{18} RP-HPLC runs of all three solutions yielded one major peak with a retention time of $t_R \sim 11.8$ min. No new peaks were observed over 48 h aside from a new peak in the glutathione run in which a peak at 3.77 min was observed (data not shown). This peak was collected and analyzed by ESI-MS and inductively coupled plasma analysis (ICP). Mass spectrometry indicated this new peak was simply oxidized glutathione and ICP confirmed that no rhenium was present in the sample (data not shown). These observations therefore sup-

port the stability of the bisquinoline ligand in binding rhenium under these conditions.

DNA gel mobility shift assays

pUC19 DNA (15 nM) was incubated with **3**, over 24 to 48 h, with metal-ligand-to-DNA ratios (r_b) ranging from 1.0 to 5.0. No scission of the DNA was observed as shown in Figure 3. No effect on DNA mobility was observed either based on control, with no change in the open circular (oc), or supercoiled (sc) DNA.

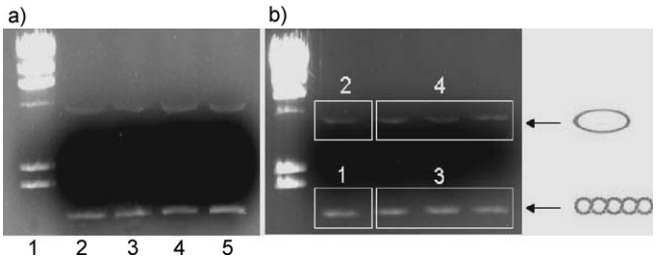


Figure 3. DNA mobility shift assays of incubation time points of a) 24 and b) 48 h, showing the two forms of DNA (oc and sc) present in the control and with the presence of **3**. Lane 1: [$\phi+\lambda$] DNA marker (72 bp–23 kbp marker); Lane 2: DNA control; Lane 3: $r_b=1.0$; Lane 4: $r_b=2.0$; Lane 5: $r_b=5.0$. For the gel at 48 h, DNA bands 1, 2, 3, and 4 were collected, purified and analyzed for rhenium(I) ICP. All bands were determined by ICP to have no detectable limits of rhenium.

Interestingly, Alberto et al. reported that an equilibrium exists between the oc and sc forms of pDNA after exposure to Re[I] prodrugs.[25] Rhenium was reported to bind to N7 in guanine with high kinetic stability as elucidated by its solid state structure.[26] It was still not conclusive then whether **3** was interacting with DNA. To bridge this gap, intercalation studies were conducted by using electronic absorption spectroscopy.

DNA Intercalation Study

Electronic absorption spectroscopy of **3** in the presence of 0.3 μg pUC19 DNA at 37 °C over 24 h was performed (see Figure 4). A slight hypochromicity that was associated with a 0.021 decrease in absorption can be attributed to intercalation interactions of **3** with the DNA base pairs. Studies showing alkyl-linked diquinolines have reported selective binding to A+T-rich regions of the DNA.[27] The binding affinity was shown to increase as the alkyl chain length increased.[27] Luzopeptin, which contains two quinoline moieties that are linked by a cyclic depsipeptide was shown to bifunctionally intercalate and induce DNA cross-linking.[28] In addition, Crooke et al. reported that the half molecule of luzopeptin (i.e., quinoline with a pentapeptide chain) can effectively interact with DNA but does not intercalate.[28]

In another study of quinoline-derivatized echinomycin (i.e., a naturally occurring quinoxaline antibiotic), 2QN was reported to behave as a bisintercalator with preference to poly(dA–dT) rich sites.[29] In addition, **3** is positively charged, which enhances the interaction with the negatively charged helix. Work by Schneider et al. has shown that destacking of nucleobases by small aromatic systems (e.g., quinoline) is promoted by a positive charge in the ligand system.[30] In addition to intercalation is the possibility of minor-groove binding. Minor-groove binders have structural motifs that are i) crescent-shaped ii) positively charged and iii) moderately rotational to fit in the minor groove of the nucleic acid.[31] The crescent form corresponds well with the helicity of the minor groove.[32] These previous reports can be correlated to the mechanistic activity of **3**. A closer look at the solid state structure of **3** shows a crescent-shaped structure with the bond angle of 77.72° between the rhenium(I) atom and the nitrogen atoms on the quinoline rings.[33] The structure of **3** is then consistent with the criteria for minor-groove binding to DNA with the quinoline rings preferring the A+T sites of the helix, and with the positive charge contributed by the metal ion. The toxicity of **3** might then be attributed to interactions between DNA and **3**. To investigate whether either this interaction or intercalation led to topoisomerase inhibition, we also performed a topoisomerase inhibition assays.

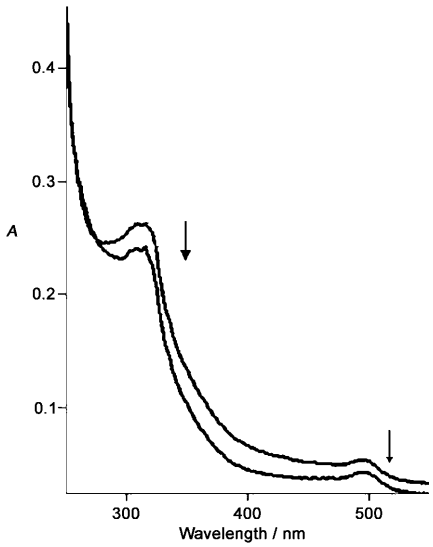


Figure 4. UV/Vis absorption spectrum of 3 with pUC19 DNA at 37 °C over 24 h showing a decrease in absorption indicative of DNA intercalation.

Topoisomerase I Inhibition Assay

Many anticancer therapeutics interfere with the activity of topoisomerase I and II (Top I and II). Topoisomerases are isomerase enzymes that act on the topology of DNA, and are critical for the unwinding of DNA that is necessary for replication. Cancers of the kidney,[34] colon,[34] prostrate,[34] ovary,[35] and esophagus[36] have been determined to have elevated topoisomerase levels, which is a necessary consequence of rapid and unchecked proliferation. Top I assays with **3** on plasmid ϕX174, which contains both oc and sc DNA indicated that the ability of Top I to unwind sc into oc DNA was not inhibited by concentrations of **3** of up to 5 mM (see Figure 5). Clearly, no inhibition of the Top I activity was observed. No re-supercoiling of

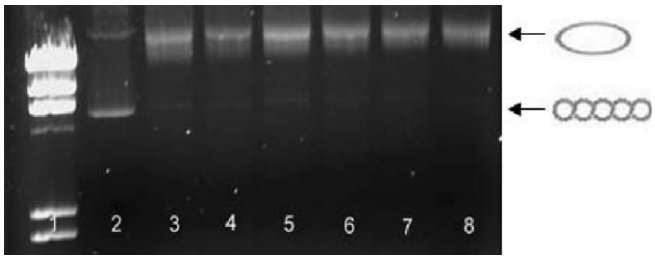


Figure 5. Top I inhibition assay with ϕX174 RF I. Lane 1: λDNA marker (564 bp–23.13 kbp); Lane 2: DNA control; Lane 3: DNA with Top I; Lanes 4–8 contain DNA, Top I, and various concentrations of **3** (1, 10, 100, 1000, 5000 μM).

the DNA was subsequently observed upon removal of the topoisomerase and the addition of **3**; this indicates that **3** was not intercalating, or even weakly intercalating (also suggested by Figure 4) at concentrations of less than 5 mM.[37] A DNA-based mechanism of cytotoxic action seems unlikely then for this system and as with many metal-based drugs, the exact mode of action requires further investigation.

Conclusions

We have successfully synthesized a FA–PEG-derivatized Re^{I} conjugate and tested its in vitro imaging and cytotoxic properties. Addition of the FA–PEG moiety to the Re–BQAV complex affords significant internalization of the complex in FR-expressing cells compared with no internalization in non-FR-expressing cells; this is consistent with a receptor-mediated endocytotic uptake mechanism. This selectivity of uptake between FR-devoid and FR-overexpressing cell lines suggests that γ-**4** might be a suitable candidate for diagnostic imaging of FR-overexpressing carcinoma in vivo by replacing Re with ^{99m}Tc. The significant cytotoxicity suggests that it might actually be possible to use this system with $^{186/188}$Re as a targeted therapeutic in cell lines where drug-resistance is evident. This work then could lead to a novel diagnostic radioimaging or therapeutic agent that targets FR-overexpressing carcinoma in vivo and work to investigate this is underway.

Experimental Section

Materials: All reactions were performed under an inert argon or N_2 atmosphere by using Schlenk techniques. Drying of dimethylsulfoxide (DMSO, 99%, Sigma) was performed by charging through a column of molecular sieves (4 Å, Mallincrodt) dried overnight at 120 °C. The following reagents were purchased and used without further purification: folic acid (98%, Sigma), *N*,*N*′-dicyclohexylcarbodiimide (DCC, ≥99%, Fluka), polyoxyethylene bis(amine) (PEG, M_r ~ 2000) (Fluka), *N*-hydroxysuccinimide (NHS, ≥97%, Fluka), 1,1′-carbonyldi-(1,2,4-triazole) (≥90%, CDT, Fluka), quinoline-2-carboxaldehyde (98%, Alfa Aesar), 5-aminovaleric acid (97%, Sigma), sodium triacetoxyborohydride (95%, Sigma), 1,2-dichloroethane (DCE, ≥99%, Sigma), dichloromethane (≥99.5%, Sigma), methanol (MeOH, ≥99.8%, Sigma), acetonitrile (MeCN, ≥ 99.8%, Sigma), Na_2SO_4 (≥99.9%, anhydrous, Sigma) Et_3N (99.5%, Sigma), and trifluoroacetic acid (TFA, 99%, Aldrich). $[Re(CO)_3(H_2O)_3]^+$ was prepared by following a method that was described previously.[38]

For in vitro cell studies, all manipulations were performed in a Labconco Purifier I laminar flow hood that had been disinfected with 70% ethanol and irradiated with UV light. CHO cells were obtained from the American Type Culture Collection (ATCC). The A2780/AD cell line that was used for testing was provided by the Fox Chase Cancer Centre, Philadelphia (USA) and was generated in 1984. Fetal bovine serum (FBS) was purchased from Hyclone. Penicillin–streptomycin solution with 10000 units penicillin and 10 mg mL^{-1} streptomycin in 0.9% NaCl was obtained from Sigma. Gibco RPMI 1640 1× growth media that contained L-glutamine and phenol red without folic acid was supplied by Invitrogen. Cellgro Cellstripper, a nonenzymatic cell dissociation solution, was obtained from Mediatech. F-12K Ham's media was purchased from ATCC. Growth media were filtered with 0.45 μm filter (Fisher). 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt solution (WSK-8) was purchased from Dojindo. Cells were incubated and grown in an incubator that was purchased from VWR. Optical densities of the cell cultures were measured at 450 nm with Thermo Multiskan EX that was equipped with Ascent software version 2.6. Confocal microscopy experiments were conducted with a Zeiss LSM 4 Pascal confocal microscope and Image Analysis equipped with argon and HeNe laser.

The following materials were used for DNA experiments: Topoisomerase I (Top I) and bovine serum albumin (BSA) were purchased from New England Biolabs. Agarose and Tris(boric acid)–EDTA (10×) were obtained from Merck. Ethidium bromide and gel-loading dye (6×) were purchased from Promega and Amresco, respectively. ϕX174 DNA was purchased from Invitrogen. pUC19 was transformed from chemically competent *E. coli* and isolated and purified by using the Wizard Plus SV Miniprep DNA purification system from Promega. Gels were viewed by using a Kodak Gel Logic 100 imaging system UV transilluminator.

All other reagents and buffers that were used were of reagent grade or higher. Ultrapure water (18.2 MΩ) was used through out the investigation.

An Agilent 1100 reversed-phase HPLC instrument with manual injection and automated fraction collector was fitted with a Zorbax C_{18} analytical column (42×10 mm) for analytical runs. The flow rate was 0.7 mL min^{-1}. Purification was made with a C_{18} semipreparative column (9.4×250 mm) at a flow rate of 2 mL min^{-1}. Detection was carried out by UV monitoring at 254 nm. The gradient used was 55% 0.1% TFA in H_2O and 45% 0.1% TFA in MeCN to 40% 0.1% TFA in H_2O and 60% 0.1% TFA in MeCN over 15 min. Ion-exchange chromatography was conducted on an Akta Prime Plus instrument by using Primeview 5.0 software. The ANX (1 mL) and the PD10 Sephadex G-25M desalting (10 mL) columns were purchased from GE Health Sciences. ^{1}H NMR spectroscopy was performed with Bruker Avance DPX 500 MHz and Bruker Avance DPX 300 instruments. A Shimadzu LCMS-2010 A mass spectrometer and a Bruker Autoflex matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer were used for ESI and MALDI-TOF MS analysis, respectively. A PerkinElmer ELAN 6100 instrument was used to conduct inductively coupled plasma analysis (ICP). A Varian Cary 50 Bio UV/Vis spectrometer was used to record the electronic absorption spectra of the compounds and was also used for DNA intercalation studies. Centrifugation was performed for 10 min at 4000 rpm at 4 °C with a Sorvall Legend RT centrifuge. All reactions

except for **2** and **3** were performed in a darkroom under a 15 W red light.

Synthesis of FA-PEG-NH_2 1: The synthesis of **1** was described previously.[39] The isomers, α- and γ- of **1** were purified by using a procedure that was established in our research group.[14]

Synthesis of {[bis(quinolin-2-ylmethyl)amino]-5-valeric acid} (BQAV) (2): Compound **2** was prepared by using a previously established procedure.[16] 5-aminovaleric acid (0.633 g, 5.4 mmol) was dissolved in DCE (20 mL). A solution of quinoline-2-carboxaldehyde (1.79 g, 11.34 mmol) in DCE (10 mL) was added dropwise to this solution. The mixture was stirred for 2 h at room temperature and subsequently cooled down to 0 °C. Sodium triacetoxyborohydride (2.63 g, 12.41 mmol) was slowly added, and the mixture was stirred under N_2 for 2 h. The solution was then extracted with CH_2Cl_2 (3× 50 mL) and water (3×50 mL). The organic layer was washed with 3.4 M NaCl (50 mL) and dried with Na_2SO_4. The organic layer was then dried in vacuo. The dark-red oily substance that was obtained was redissolved in a solution of EtOAc/hexane (1:1) and the red precipitate that was formed was then collected by centrifugation at 4000 rpm over 10 min; yield: 215 mg (34.0%). ^{1}H NMR (300 MHz, MeOD, 25 °C): δ = 8.28 (d, $^3J_{H,H}$ = 8.7 Hz, 2H), 7.97 (d, $^3J_{H,H}$ = 8.1 Hz, 2H), 7.86 (d, $^3J_{H,H}$ = 2.4 Hz, 2H), 7.77–7.69 (m, 4H), 7.55 (t, $^3J_{H,H}$ = 7.2 Hz, 2H), 4.10 (s, 4H), 2.76 (t, $^3J_{H,H}$ = 6.6 Hz, 2H), 2.19 (t, $^3J_{H,H}$ = 6.6 Hz, 2H), 1.66 ppm (br m, 4H); UV/Vis (MeCN/H_2O, 9:1): λ_{max} (ε) = 230 nm (95760 $M^{-1}cm^{-1}$); ESI-MS (10 eV, 0.1% TFA in MeCN): *m/z* calcd for $C_{25}H_{25}N_3O_2$: 399.1; found: 400.0 [M+H^+].

Synthesis of $[Re(CO)_3$-BQAV$]^+$ 3: $[Re(CO)_3(H_2O)_3]^+$ (24.3 mg, 0.075 mmol) was dissolved in MeOH (20 mL). A solution of **2** (30 mg, 0.075 mmol) in MeOH (5 mL) was added dropwise to the flask. This mixture was heated to reflux at 70 °C under an argon atmosphere overnight. The solution was then dried in vacuo and redissolved in CH_2Cl_2 (10 mL). H_2O (3×10 mL) was then added to the solution, and an extraction was performed. Na_2SO_4 (~25 g) was added to remove the residual aqueous solvent. The mixture was then dried in vacuo and redissolved in MeOH. Purification and isolation of **3** were performed by using C_{18} RP-HPLC with t_R = 10.2 min; yield: 12.1 mg (40.4%). ^{1}H NMR (300 MHz, MeOD, 25 °C): δ = 8.43 (d, $^3J_{H,H}$ = 8.4 Hz, 2H), 8.02 (d, $^3J_{H,H}$ = 8.4 Hz, 2H), 7.98 (d, $^3J_{H,H}$ = 8.4 Hz, 2H), 7.82 (t, $^3J_{H,H}$ = 8.1 Hz, 2H), 7.64 (t, $^3J_{H,H}$ = 7.2 Hz, 2H), 7.53 (d, $^3J_{H,H}$ = 8.7 Hz, 2H), 4.97–5.25 (m, 6H), 3.67 (d, $^3J_{H,H}$ = 1.8 Hz, 1H), 3.46 (br s, 5H), 2.48 (t, $^3J_{H,H}$ = 7.2 Hz, 2H), 2.06 (br s, 2H), 1.77 ppm (m, 2H); UV/Vis (MeCN/H_2O, 9:1): λ_{max} (ε) = 232 nm (108625 $M^{-1}cm^{-1}$), 314 nm (3776 $M^{-1}cm^{-1}$); ESI-MS (10 eV, H_2O/MeCN, 2:3): m/z calcd for $C_{28}H_{25}N_3O_5Re$: 669.2; found: 669.8 [M^+].

Synthesis of [FA-PEG-$Re(CO)_3$-BQAV]$^+$ γ-4: Compound **3** (10.2 mg, 0.0152 mmol) and 1,1′-carbonyl-di-(1,2,4-triazole) (7.48 mg, 0.456 mmol) were dissolved in dry DMSO (1 mL) in a 50 mL Schlenk tube. The mixture was stirred and heated at 60 °C for 30 min. A solution of γ-**1** (24.6 mg, 0.0102 mmol) in DMSO (1 mL) and Et_3N (75 μL) was added dropwise to **3**. The mixture was stirred overnight at room temperature under N_2. The mixture was then dried overnight in vacuo. A yellow solid was collected and was subsequently purified by using C_{18} RP-HPLC (t_R = 13.4 min); yield: 5.71 mg (23.2%). ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 8.67 (d, $^3J_{H,H}$ = 9.5 Hz, 2H), 8.39 (d, $^3J_{H,H}$ = 9.3 Hz, 2H), 8.11 (d, $^3J_{H,H}$ = 7.8 Hz, 2H), 7.96 (t, $^3J_{H,H}$ = 7.5 Hz, 4H), 7.72 (m, 4H), 6.61 (s, 2H), 5.14 (m, 5H), 3.8 (m, 2H), 3.38 (m; PEG), 2.75 (s, 1H), 2.28 (s, 1H), 2.10 (s, 1H), 1.91 (m, 3H), 1.63 (m, 3H), 1.22 ppm (s, 4H); MALDI-TOF MS: *m/z* calcd for $C_{137}H_{228}N_{12}O_{53}Re$: 3074; found: 3000 [$M^+$]

Cell lines and culture conditions: An adriamycin-resistant ovarian cancer cell line (A2780/AD) and a Chinese hamster ovary (CHO) cell line were cultured as adherent monolayers in RPMI 1640 growth media that contained L-glutamine and folic acid and F-12K Ham's media respectively. These are supplemented with penicillin (10000 units) and 10 mg mL^{-1} streptomycin (Sigma), 10% (*v/v*) FBS (Sigma). Prior to testing, cells were then passed in RPMI 1640 FA-free growth media with FBS and penicillin–streptomycin solution. It is important to note that cell growth in FA-free media declined after several passages. Cells were incubated and grown in a VWR mammalian incubator at 5% CO_2 and 95% humidity. Cells were cultured in Millipore 250 mL culture bottles with vented lids.

Confocal microscopy experiments: CHO and A2780/AD ovarian cancer cells (150000 cells/dish) were plated separately on 35× 100 mm vented dishes. The cells were incubated at 37 °C overnight in FA-free RPMI 1640 media. To each plate, 10 μM γ-**4** (1 mL) was added and incubated for 45 min and 24 h. The drug was then removed, and the cells were washed with 50 mM phosphate buffered saline (PBS; 3×1 mL). The cells were then washed with acidified saline solution (3×1 mL; 3.4 mM NaCl, pH 3.0) and fixed with ice-cold MeOH.

For the competitive binding affinity assays with folic acid, A2780/AD ovarian cancer cells (150000 cells) were plated on 35×100 mm vented dishes. The cells were incubated at 37 °C in FA-containing RPMI 1640 media. The media was then removed and a 10 μM solution of **4**(1 mL) that contained 500 mM FA was added. The cells were incubated for 45 min and 24 h. The drug was removed and the cells were washed with of 50 mM PBS (3×1 mL),and then acidified saline solution (3×1 mL; 3.4 mM NaCl, pH 3.0) to reduce nonspecific cell surface interactions. The cells were then fixed with cold MeOH.

Drug cytotoxicity: The proliferation of the exponential phase cultures of A2780/AD and CHO cells was assessed by WSK-8 colorimetric assay. WSK-8 was performed according to manufacturer's instructions (Dojindo). Adherent cell cultures were harvested by stripping of culture flasks by using a nonenzymatic cell stripper after a 30 min incubation period. The cell densities were then adjusted to 5.0×10^4 cells mL^{-1} for exponential growth over the period of drug exposure. To each well, aliquots (100 μL) were inoculated, which resulted in 5000 cells per well. After a 24-hour incubation time to facilitate adherence, the FA-free RPMI media was removed and replaced with 200 μL of fresh media that contained different concentrations of **2**, **3**, and γ-**4**. The cells were then incubated for 6 and 24 h. Optical densities were measured by using a plate reader. The percentage of cell viability was determined relative to untreated control microcultures. The IC_{50} values were calculated based on an exponential fit by using OriginLabs 8 software with R^2 values ≥0.90 in all cases. All experimental points were measured in triplicate and each experiment was performed at least three times on separate 'batches' of compound on cells with no more than 14 passages.

Ligand challenge experiments: Three 1 mM solutions of γ-**4** were prepared in 50 mM PBS buffer (pH 7.4). One solution contained 100 mM histidine and another with 5 mM glutathione. These solutions were incubated over 48 h at 37 °C. C_{18} RP-HPLC analysis was conducted at 0, 24 and 48 h. Peaks collected were analyzed by ESI-MS and ICP.

DNA gel mobility shift assays: pUC19 DNA (15 nM) solutions were incubated with **3** at 37 °C with [**3**]/[pUC19] ratios of 0–5.0:1 over a period of 24 h. These solutions were then loaded into a 1% agarose gel prepared in 1× TBE buffer. Gels were then run at 70 V for 90 min with 1× TBE as running buffer. The gel was then stained

with ethidium bromide (10 μL to 100 mL TBE buffer) for 1 h and rinsed with H_2O for 20 min.

Topoisomerase I inhibition assays: ϕX174 RF I DNA was used for Top I inhibition assays. Each reaction mixture had a final volume of 25 μL that contained 0.5 μg of plasmid DNA, one unit of Top I and varying concentrations of **3** [1–5000 μM] in buffer that contained the following: 50 mM Tris–HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, 30 μg mL^{-1} BSA. Solutions were incubated at 37 °C for 30 min. The samples were then analyzed on a 1% agarose gel that was prepared in 1× TBE at 70 V for 90 min with 1X TBE as running buffer. The gel was then stained with ethidium bromide (10 μL to 100 mL TBE buffer) for 1 h, and rinsed with H_2O for 20 min.

DNA intercalation studies: This procedure followed the protocol of Strekas et al. with modifications.[40] To 12.5 mM **3** in DMSO/H_2O (1:9), 0.3 μg pUC19 DNA was added. The electron absorption was then scanned with a UV/Vis spectrometer from 270–550 nm at a maintained temperature of 37 °C. The absorption was monitored every 30 min over 24 h. The difference in initial and final absorption readings were calculated and analyzed for peak and/or absorption shifts.

Supporting Information: MS data and confocal microscopy depth scanning showing images taken from layers through a single A2780/AD cell are provided.

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