Targeting Gallium to Cancer Cells through the Folate Receptor

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Abstract: The development of gallium(III) compounds as anti-cancer agents for both treatment and diagnosis is a rapidly developing field of research. Problems remain in exploring the full potential of gallium(III) as a safe and successful therapeutic agent or as an imaging agent. One of the major issues is that gallium(III) compounds have little tropism for cancer cells. We have combined the targeting properties of folic acid (FA) with long chain liquid polymer poly(ethylene glycol) (PEG) 'spacers'. This FA-PEG unit has been coupled to the gallium coordination complex of 1,4,7,10-tetraazacyclo-dodecane-N,N′,N″,N‴-tetraacetic acid (DOTA) through amide linkages for delivery into target cells overexpressing the folate receptor (FR). *In vitro* cytotoxicity assays were conducted against a multi-drug resistant ovarian cell line (A2780/AD) that overexpresses the FR and contrasted against a FR free Chinese hamster ovary (CHO) cell line. Results are rationalized taking into account stability studies conducted in RPMI 1640 media and HEPES buffer at pH 7.4.

Keywords: folate receptor, gallium, DOTA, targeting, cytotoxicity

Introduction

The anti-cancer properties of gallium(III) have been extensively investigated since 1971 (Hart et al. 1971). Gallium has numerous ways to induce cell death, including DNA binding and modification (Hedley et al. 1988), enzyme inhibition (especially ribonucleotide reductase) (Chitambar et al. 1988), and ion transport disruption (such as calcium efflux from mitochondria), a known trigger of cellular apoptosis (Collery et al. 1996).

In general, the poor pharmacokinetic properties of gallium salts investigated have prevented their widespread use in chemotherapy. Efforts to develop gallium complexes to improve its profile, by addressing the problems of hydrolysis, poor absorption, poor solubility, rapid renal excretion and little tropism for cancer cells are currently underway (Keppler and Jakupec, 2004; Desoize, 2004). Complexes such as those produced by the groups of Keppler (Keppler et al. 2006), Sharma (Sharma et al. 2007), Low and Green (Low and Green et al. 1996) have been successful in increasing plasma concentrations of gallium, providing better antiproliferative effects or improved imaging of cancer cells (when using gallium radioactive isotopes (^{67}Ga γ, ^{68}Ga β^+)) (Greenwood and Earnshaw, 2005). Problems still remain however especially in regards to renal retention times and cancer cell targeting.

Folic acid (FA) (see Fig. 1) is a vitamin potentially capable of delivering agents specifically to folic acid-receptor (FR) positive tumors (Lee and Sudimack, 2000). FRs are membrane glyco-proteins (Anderson et al. 1990) overexpressed by a number of tumor cell types such as ovarian, breast, cervical, colorectal, renal and nasopharyngeal cancers (Antony, 1996). Cells overexpressing the FR bind FA-drug conjugates tightly ($K_d \sim 0.42 \times 10^{-9}$ M) (Shen et al. 1995) and endocytose them inside (Anderson et al. 1988), provided that chemical modification of the FA upon conjugation does not disrupt recognition by the FR (Liu et al. 2005). 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 (Anderson et al. 1988).

We are primarily focused on ovarian tumors since they have been shown to greatly overexpress the FR (see Table 1). Current treatments for ovarian cancer have a number of serious side effects associated with their use including kidney damage, hearing loss and even secondary cancers (Sun et al. 2002). In addition, over 75% of patients are diagnosed when the disease has already progressed to stage III or IV, with only a 10%–20% 5 year survival rates, respectively (Ries, 1993). New ways to diagnose and/or treat this illness are therefore urgently needed.

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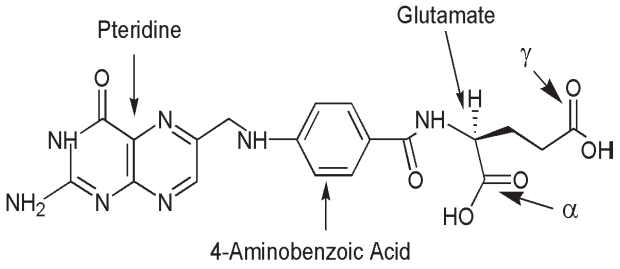


Figure 1. FA with its three major structural components including the α- and γ-carboxylic acid group of the glutamate moiety indicated.

We set out to synthesize, purify and evaluate *in vitro* a new FA bioconjugate of gallium and compare its activity to the unconjugated gallium analog. We began by initially complexing gallium (III) to the 1,4,7,10-tetraazacyclo-dodecane-N,N′,N″,N‴-tetraacetic acid (DOTA) ligand (Doyle et al. 2006). We have previously described the synthesis and solid state structure of this system (see Fig. 2). Preceding literature reports have demonstrated good kinetic and thermodynamic stability provided by the DOTA ligand in its coordination chemistry and we wished to exploit this coupled with folate receptor (FR) targeting conjugates. In addition to coupling the gallium-DOTA complex to folic acid we wished to include poly(ethylene glycol) (PEG) polymer linkers between the FA and gallium complex since 'PEGylated' FA conjugates have been shown to have greater affinity for the FR than free FA (Low and Lee, 1994). Pathways that can break down or efflux certain FA conjugates are inhibited by the polymer-FA conjugate and renal retention times of certain pharmaceuticals have been improved by conjugation to PEG units (Anderson et al. 2005). Hence, the use of FA-PEG conjugates yields synergistic traits that are of particular interest.

Table 1. Comparison of FR overexpression investigated in different cancer tissues via immunohistochemistry (IHC) and reverse transcriptase—polymerase chain techniques (RT-PCR) (Low and Leamon, 2005).

Tissue	IHC (%)	RT-PCR (%)
Ovarian	93	100
Endometrial	91	100
Breast	21	80
Lung	33	33
Colorectal	22	20
Kidney	50	100

Conjugation of PEG to FA through the glutamate moiety produces two regioisomer products at the α- and γ-carboxylic acid functional groups that need to be separated. FA modified at the α-carboxylic acid loses its affinity for the FR, making it unsuitable as a targeting agent (Yan and Ratnam, 1995). This separation is difficult when using polydisperse PEG units. Such PEG units are typically all that are commercially available but are approved for use by the FDA (Qui and Bae, 2006). A facile route to separation was previously reported by us and this route was used here to allow access to pure γ-FA-PEG-NH_2 for subsequent conjugation to the gallium-(HDOTA) complex (Doyle et al. 2008).

In vitro cytotoxicity assays were conducted against adriamycin resistant ovarian cancer cell line (A2780/AD), which overexpresses the FR, and contrasted against a non-FR expressing Chinese hamster ovary (CHO) control line.

Experimental

Chemicals

The following reagents were purchased and used without further purification: Folic acid (FA) (98%, Sigma), N,N'-Dicyclohexylcarbodiimide (DCC, ≥99%, Fluka), polyethylene glycol bis(amine) (PEG, MW: 2000) (Fluka), N-Hydroxysuccinimide (NHS, ≥97%, Fluka), and 1,4,7,10-tetraazacyclo-dodecane-N,N',N'',N'''-tetraacetic acid (DOTA, 98%, Strem Chemicals), N-Hydroxysulfosuccinimide sodium salt (sulfo-NHS, 98.5%, Fluka), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 98%, Alfa Aesar) and trifluoroacetic acid (99%, Aldrich). $GaCl_3$ (99.9%) was purchased from Alfa Aesar and dissolved in 100 mM ammonium acetate (pH 4.8) to make a stock concentration of 1.325 M. Dimethylsulfoxide (DMSO) (min. 99.9%, Sigma) was dried by running the solvent through a column of 4 Å molecular sieves (Mallincrodt) dried previously overnight at 120 °C. Solvents used for HPLC and growth media are filtered with 0.45 μm filter (Fisher). Pyridine (99.9%) was obtained from Fisher. Triethylamine (99.5%) was purchased from Sigma Aldrich. 3′-azido-3′-deoxythymidine (AZT; used as internal control in cytotoxicity assays) was purchased from Sigma Aldrich. All other reagents and buffers used were of reagent grade or higher. Ultra pure water (18.6 MΩ) was used through out the investigation. All syntheses except for **1** were performed in a

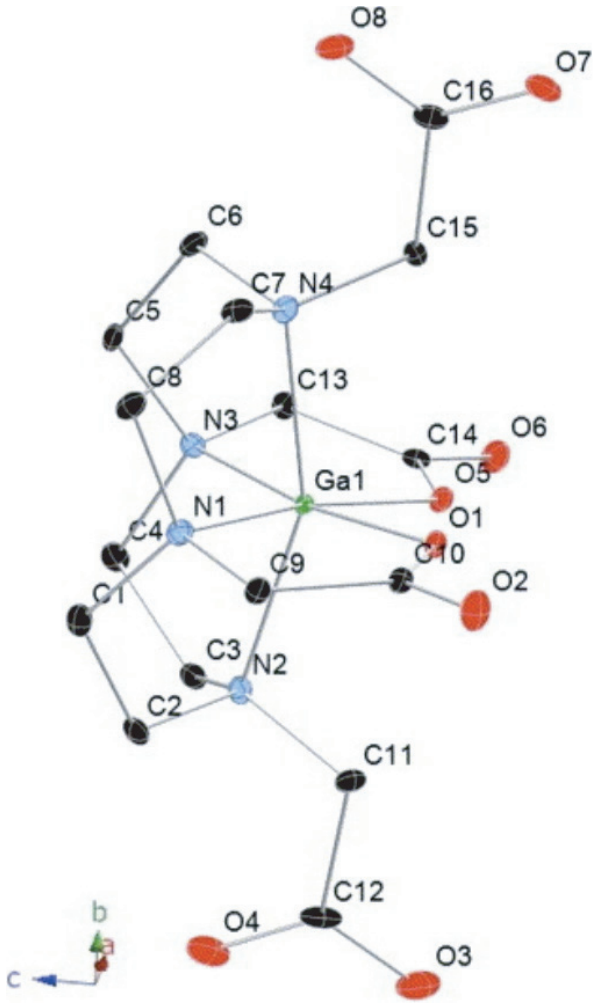


Figure 2. Crystal structure of Ga(HDOTA) [20].

dark-room under a 15 W red light. All reactions were conducted under nitrogen gas at ambient conditions unless otherwise stated with sample transfer conducted by cannula (24 inch, 16 gauge).

Physical measurements and instrumentation

An Agilent 1100 reverse phase high pressure liquid chromatography (HPLC) with manual injection and automated fraction collector was fitted with a Zorbax C_{18} analytical column (42 × 10 mm) for analytical trace analysis with a flow rate of 0.7 ml/min. Purification was achieved using a C_{18} (9.4 × 250 mm) semi-preparative column using a flow rate of 2 ml/min. Detection was by ultra violet monitoring at 280 nm. The linear gradient used was: (1) 90% 5 mM Na_2HPO_4 (pH 7.0) and 10% acetonitrile over 10 minutes; (2) 40% 5 mM Na_2HPO_4 and 60% acetonitrile over 20 minutes. Ion exchange chromatography (IEC) was conducted on an Akta Prime Plus with Primeview 5.0 software. The ANX (1 ml) and the PD10 Sephadex G-25M desalting (10 ml) columns were purchased from GE Healthsciences. ^{1}H nuclear magnetic resonance (^{1}H NMR) was performed on Bruker Avance DPX 500 MHz. A Shimadzu LCMS-2010 A mass spectrometer and An Applied Biosystems Voyager-DE linear Matrix Assisted Laser Desorption Ionization—Time of Flight mass spectrometer (MALDI-TOF) were used for mass spectrometry analysis. Infrared (IR) analyses were performed as KBr pellets on a Nicolet Magna-IR 850 series II spectrophotometer. A Perkin Elmer ELAN 6100 was used to conduct inductively coupled plasma analysis (ICP). Centrifugation was performed using a Sorvall Legend RT centrifuge typically as 10 minute runs at 4000 rpm at 4 ºC. Optical densities were measured with a Thermo Multiskan EX 96-well plate reader equipped with Ascent Software version 2.6 with 450 nm filter.

Chemical synthesis

Synthesis of GaHDOTA (1)

1 was synthesized as reported previously by Doyle et al. (Doyle et al. 2006).

Synthesis of γ-FA-PEG-NH_2 (γ-2)

FA (0.0441 g, 0.100 mmol) was dissolved in 3 ml of dry DMSO. To this solution, 0.0127 g of NHS (0.110 mmol) was added. The mixture was stirred for 5 minutes after which 0.023 g (0.110 mmol) of DCC was added. The solution was then stirred overnight. The activated FA was filtered through a 0.45 μM filter to remove the dicyclohexylurea side product. The FA-NHS solution was then added dropwise to PEG_{2000} (0.200 g, 0.100 mmol) previously dissolved in 3 ml DMSO. 100 μL of pyridine was then added and the reaction stirred overnight. Approximately 25 ml of chilled isopropanol (–78 °C) was added forming a light yellow precipitate. The precipitate was obtained via centrifugation. The γ- and α-isomers of **2** were separated via IEC using the following method [Doyle et al. 2008). **2** was redissolved in water to give a [20 mg/ml] concentration. This solution was desalted using a 10 ml sephadex PD10 desalting column eluting the product in water. 500 μL of this solution was injected into a 1 ml ANX weak anion exchange column. The flow rate was set at 0.1 ml/min. The column was then washed with

5 column volumes of water. After the first peak was eluted, the column was then washed following a gradient (solvent A, water; solvent B, 100 mM ammonium acetate, pH 10) of 10% B for 17 column volumes, 50% B for 17 column volumes, 80% B for 15 column volumes. A column volume of 5 ml of 0.5 M NaCl was used to flush the column. ^{1}H NMR (D_2O): δ 8.62 (s, 1H), δ 7.83 (t, 2H), δ 6.63 (d, 2H), δ 3.50–3.80 (m, PEG). Yield: 60% based on PEG.

*Only the isolated γ-isomer (**γ-2**) was used for subsequent coupling.*

Synthesis of γ-FA-PEG-H_3DOTA (γ-3)

The sulfo-succinamide ester of DOTA was prepared by activating 0.121 g (0.300 mmol) of the ligand with 26.5 μL (0.150 mmol) EDC and 0.0260 g (0.120 mmol) sulfo-NHS in 2 ml water. **γ-2** (0.0726 g, 0.03 mmol) was dissolved in 2 ml water and cooled to 4 °C. To the DOTA solution, **γ-2** was added dropwise and the pH was adjusted to 8.5. The reaction was left to stand overnight. **γ-3** was obtained via HPLC with a retention time, of T_r = 20.4 minutes. ^{1}H NMR (D_2O): δ 8.76 (s, 1H), δ 7.62 (d, 2H), δ 6.73 (d, 2H), δ 4.61 (s, 2H), δ 4.48 (m, 2H), δ 3.90 – 3.29 (m, PEG), δ 2.87 (d, 2H). Yield: 64.4% based on **γ-2**.

Synthesis of γ-FA-PEG-Ga(HDOTA) (γ-4)

1 (3.42 mg, 0.00726 mmol) was dissolved in 1 ml of 20:80 water:DMSO solution. A volume of 1 ml containing dissolved NHS (0.800 mg, 0.00695 mmol) and DCC (1.5 mg, 0.00727 mmol) was added to the solution of **1**. This mixture was stirred for 30 minutes. **γ-2** (17.6 mg, 0.00726 mmol) was subsequently dissolved in 1 ml of DMSO. A volume of 100 μl of triethylamine was added to this solution and was also stirred for 30 minutes. The solution of **γ-2** was then added dropwise to the solution of **1**. The mixture was left to react overnight. The resulting solution was filtered with a 0.45 μm filter and the crude product precipitated with 25 ml of chilled isopropanol (–78 °C). A yellow solid was isolated by centrifugation and redissolved with 1 ml water and purified by HPLC. ^{1}H NMR (D_2O): δ 8.64 (s, 1H), δ 7.68 (d, 2H), δ 6.85 (d, 2H), δ 4.61 (s, 2H), δ 3.83 – 3.20 (m, PEG), δ 2.32 (d, 12H), δ 1.18 – 1.13 (t, 11H). MALDI-TOF: 2715.00 m/z ($M+H^+$) calculated 2876.25 for **γ-4**. Yield: 82.9% based on **γ-2**.

Cell lines and culture conditions

Adriamycin resistant ovarian cancer cell line (A2780/AD) and Chinese hamster ovary (CHO) cell line were cultured as adherent monolayers in RPMI 1640 (Invitrogen) growth media containing L-glutamine and FA supplemented with 10,000 units penicillin and 10 mg/ml streptomycin (Sigma), 10% (v/v) fetal bovine serum (Sigma). CHO cells were obtained from the ATCC. The A2780/AD cell line used for testing was provided by the Fox Chase Cancer Centre, Philadelphia. Cells were incubated and grown in a VWR mammalian incubator at 5% CO_2 and 95% humidity. The presence of the FR in the A2780/AD line (and indeed absence in CHO cells) was followed by RT-PCR and confocal microscopy (Doyle et al, unpublished results). All preparations for cell culture and assays were conducted in a sterile environment under a Labconco Purifier I Laminar flow hood. Cells were cultured in Millipore 250 mL culture bottles with vented lids.

Drug cytotoxicity

The proliferation of the exponential phase cultures of A2780/AD and CHO cells was assessed by colorimetric assay. WSK-8 (Dojindo) was performed according to manufacturer's instructions.

Adherent cell cultures were harvested by stripping of culture flasks by a non-enzymatic cell stripper (Mediatech) after a 30 minute incubation period. The cells were then collected. The cell densities were adjusted using FA-free RPMI 1640 media to 3.0×10^4 cells/ml to guarantee exponential growth for the period of drug exposure. To each well, aliquots of 100 μL were inoculated. After a 24 hour incubation time to facilitate adherence, the FA free RPMI media was removed and replaced with 200 μl of fresh media containing different concentrations of **1**, **γ-2**, **γ-3**, **γ-4**, DOTA and a control of AZT. The cells were then incubated for 72 hours. Optical densities were measured at 450 nm using a plate reader. The percentage of cell viability was determined relative to untreated control microcultures.

Stability studies

2 mM solutions of **γ-4** were prepared from 25 mM HEPES (pH 7.4) and RPMI 1640 FA-free media. These solutions were incubated over 72 hours at 37 °C. Solutions made from the media were filtered by centrifugation using a centrifugal filter (Pall Life Sciences, MW: 1000 g/mol) at 4,000 rpm over

15 minutes. Analytical C_{18} reverse phase HPLC analysis was conducted at 0, 1, 24, 48 and 72 hours. At 72 hours, fractions were analyzed for the presence of gallium via inductively coupled plasma (ICP).

Results and discussion

Chemical synthesis

The synthesis of **1** (Scheme 1) was prepared by direct addition of stoichiometric equivalents of gallium to DOTA under acidic (pH 4.8) conditions. Crystals were grown after concentrating the solution to its saturation point. Five volume equivalents of acetone was then added and the suspension filtered. The clear, colorless solution was then placed at 4 °C. Colorless needle-shaped crystals formed after 24 hours.

FA was activated by a reaction with DCC/NHS to couple it to PEG. **α/γ-2** was subsequently separated via IEC using a weak anion exchange column as shown in Scheme 2. The first and second peak eluted both isomers. Upon increasing the conductivity to 3 mS/cm, a third peak eluted to give **γ-2**. Analytical HPLC runs of fractions collected from IEC confirmed the identity of the isomers. Previous work separating both isomers established the **α-2** eluting at a later time than the γ-isomer with reverse phase HPLC (Doyle et al. 2008). The identities of the peaks from fractions collected from the IEC were confirmed with **γ-2** eluting at $T_r = 23.64$ minutes and **α-2** subsequently eluting at $T_r = 26.34$ minutes.

γ-3 was made with sulfo-NHS/EDC water soluble cross linkers to give the metal-free compound. EDC formed an *O*-acylisourea intermediate with DOTA. This converts to the sulfo-hydroxysuccinimide DOTA ester in the presence of the amine reactive sulfo-NHS. Amidation proceeds upon addition of the amine group of **γ-2** (see Scheme 3).

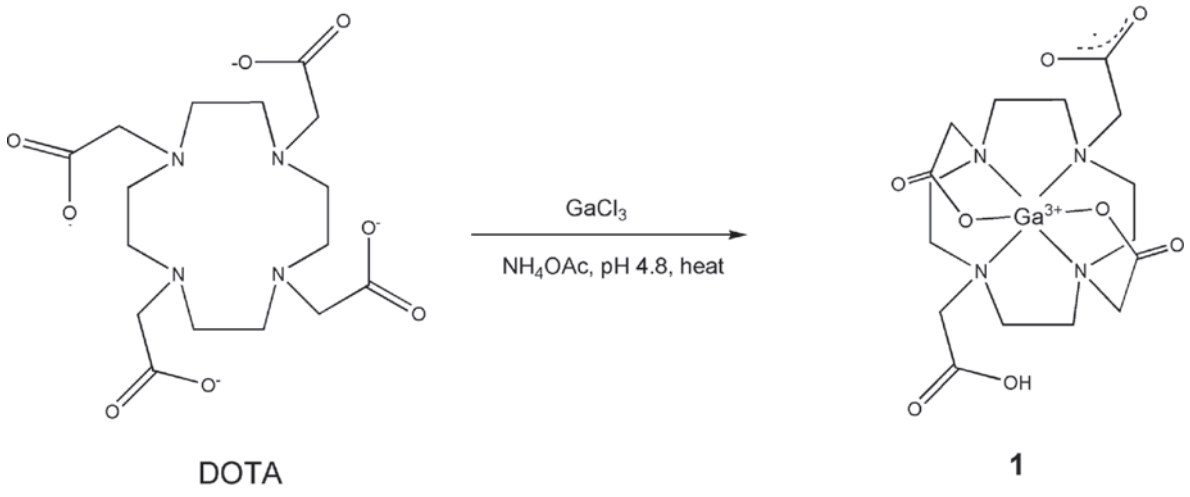
Coupling of **γ-2** and **1** proceeded using DCC/NHS coupling agents (see Scheme 4). TEA was added to **γ-2** improving the nucleophilicity of the amine end of the conjugate. Since **1** is only soluble in water, it was dissolved in a minimal amount of water followed by addition of DMSO. The water to DMSO ratio was 20:80.

Mass spectra

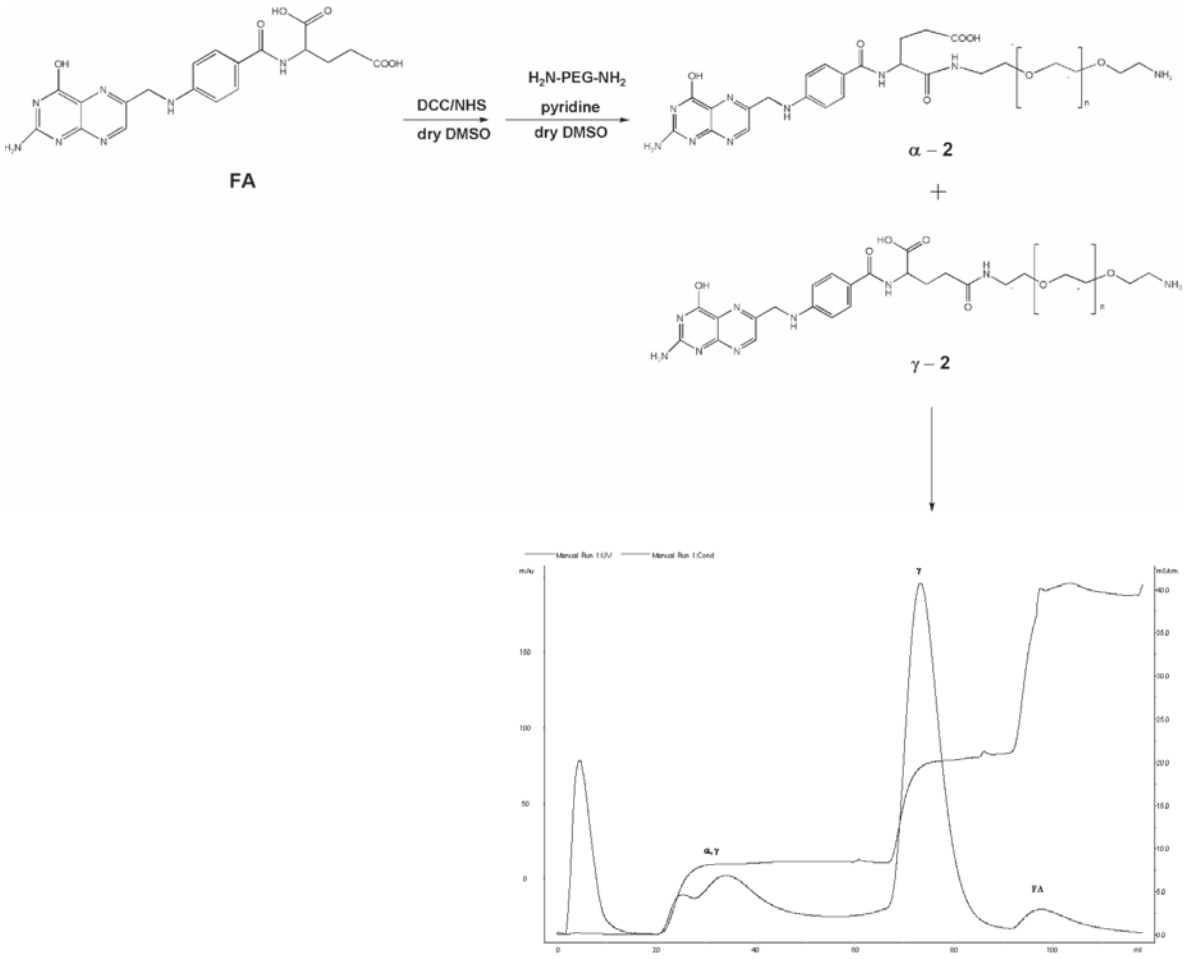
A MALDI-TOF mass spectrum of the commercial PEG displayed a mass range of 1900–2100 m/z due to the polydispersity of the polymer. The observed mass of **γ-2** is centered at 2422 m/z in agreement with the calculated theoretical mass of ~ 2400 m/z for the polydisperse PEG containing system. The calculated theoretical mass of **γ-4** is ~ 2900 m/z. A central range at 2846 m/z was observed from MALDI-TOF mass spectrometry analysis. The 44 m/z spacing is indicative of one unit of ethylene glycol (MW: 44 g/mol). Figure 3a–b displays the mass spectra obtained for **γ-2** and **γ-4** respectively.

In vitro biological activity

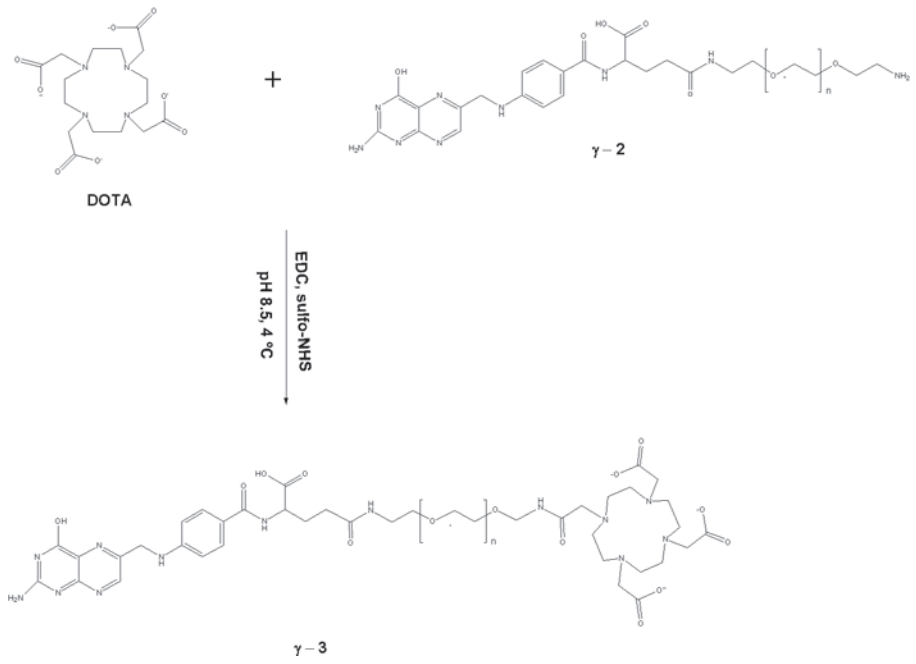
IC_{50} concentrations were calculated using an exponential fit. Table 2 shows the potency of **1**, **γ-2**, **γ-3**,



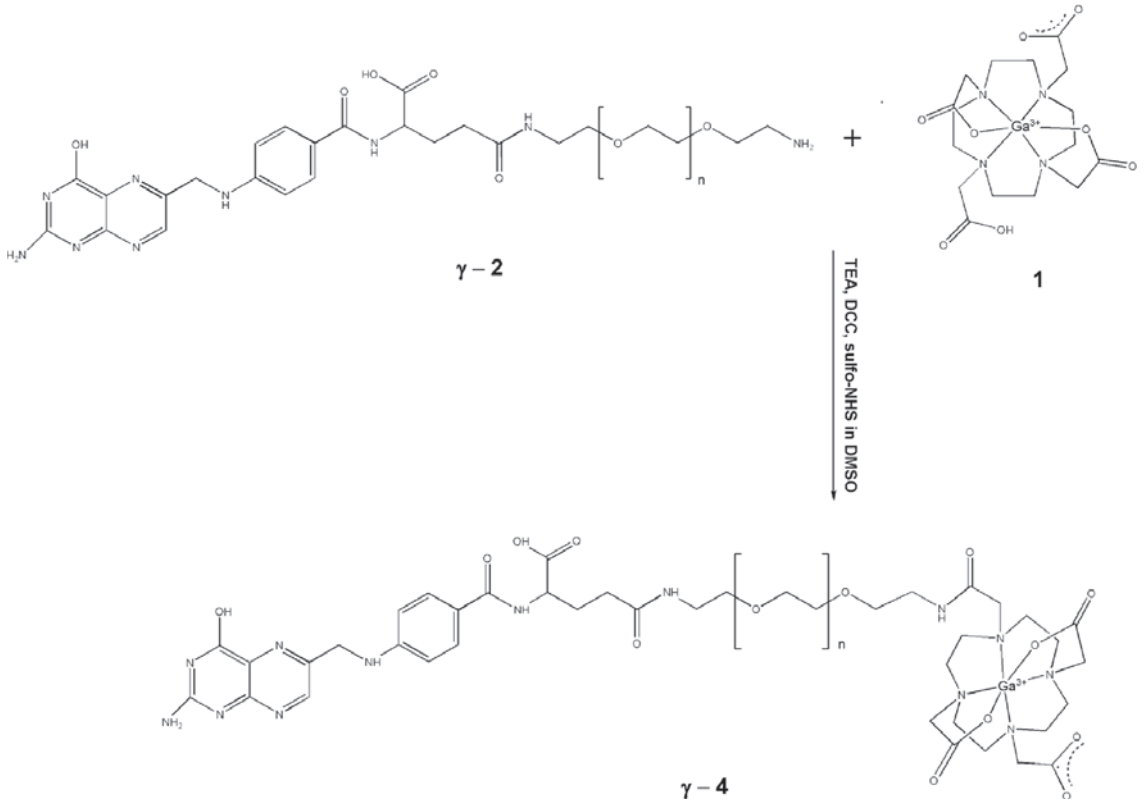
Scheme 1. Synthesis of **1** involving chelation of Ga(III) to DOTA in ammonium acetate buffer (pH 4.8).



Scheme 2. Synthesis and separation, via IEC of the regioisomers of the α- and γ-isomers of **2**.



Scheme 3. Synthesis of γ-**3** using EDC/sulfo-NHS as coupling agents.



Scheme 4. Synthesis of **γ-4** illustrates the coupling of **γ-2** and **1** using DCC and NHS as coupling agents in dry DMSO.

γ-4 and DOTA against both the A2780/AD and CHO cell lines. AZT was used as an internal control (data not shown). In all cases toxicity was greater in the A2780/AD line over the CHO line. **1**, **γ-3**, **γ-4** and DOTA displayed between [0.18 and 1.85 mM] activity against A2780/AD cells and between [0.8 and 2.93 mM] in CHO cells. **γ-2** provided no IC_{50} concentration over 72 hours at concentrations up to [100 mM]. The fact that the FA-PEG moiety is not toxic indicates that the activity of the completed conjugates stems from the gallium metal or DOTA ligand itself.

Interestingly gallium compounds were noted as less toxic than free DOTA containing controls. This was the case in both cell lines. The IC_{50} concentrations for both compounds containing gallium (namely **1** and **γ-4**) indicate that gallium is in fact reducing the toxicity of the DOTA moiety, presumably by chelation, hence preventing the scavenging by DOTA of other essential metals.

Toxicity of the FA-PEG containing DOTA compound (**γ-3)** against CHO cells can then also be explained as metal scavenging outside the cell, with uptake not necessary (and not possible in CHO without the FR). DOTA and **γ-3** displayed IC_{50} concentrations of 800 μM and 1.35 mM against CHO and 580 μM and 180 μM against A2780/AD cell lines respectively. The presence of the FR receptor in A2780/AD and more rapid division in A2780/AD over CHO helps explain the greater toxicity.

Toxicity of **γ-4** in CHO cells was noted as 2.93 mM and 1.85 mM in A2780/AD cells. The reduced toxicity of the DOTA compounds previously complexed with gallium is consistent with the observed toxicity of free DOTA and supports the idea that the toxicity lies with metal scavenging by DOTA and supercedes any gallium related toxicity. The toxicity in CHO cells can only be explained then if gallium is leaching from the DOTA macrocyle, since this would produce a gallium salt or complex and would leave DOTA now uncomplexed and free to chelate other metals.

Gallium decomplexation from a ligand such as DOTA that renders such thermodynamic and kinetic stability is possible even with a reported stability constant of log K ~ 21.33 (Clark and Martell, 1991) (compared to say to open-chain multidentate ligands like ethylenediamine (log K ~ 17.2) (Harris and Martell, 1976)). A key

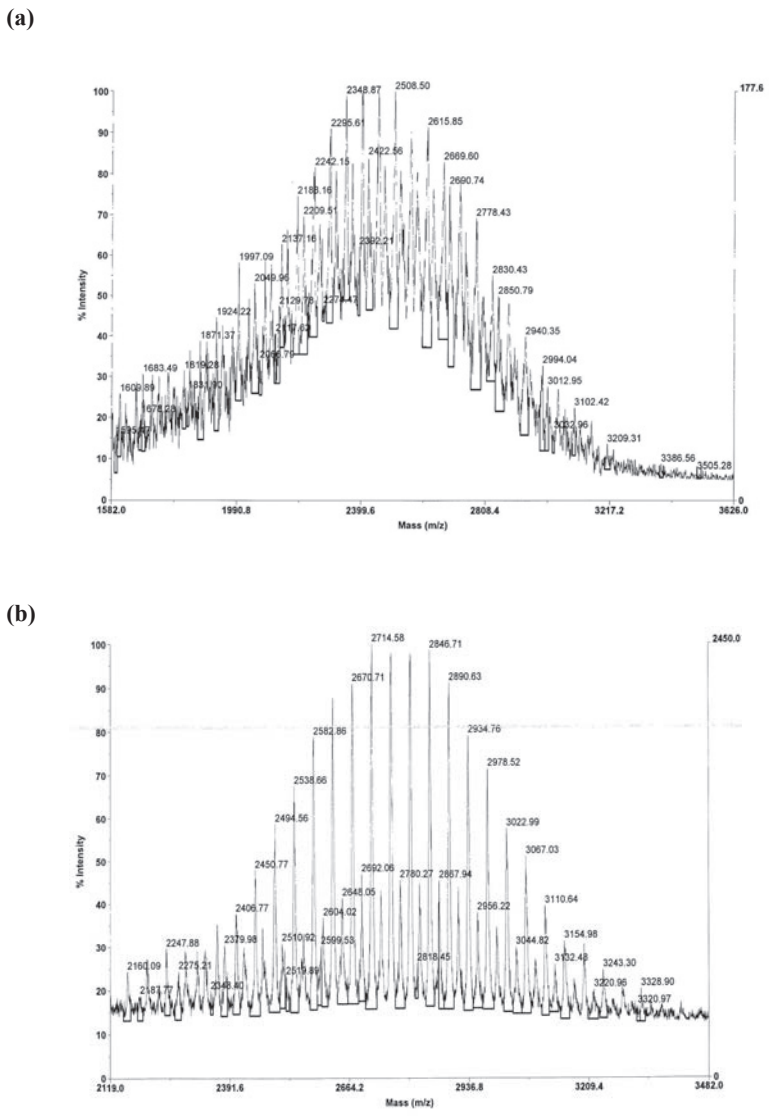


Figure 3. MALDI-TOF mass spectrometry analysis of **a**) **γ-2** and **b**) **γ-4** showing a central peak at *ca.* 2400 m/z and 2846 m/z respectively. ICP also confirmed the presence of gallium in **γ-4**.

factor in this release is an increase in DOTA's electron density due to inductive effects contributed by the ethylene bridges (Hancock and Martell, 1995).

A plausible explanation for the possibility of gallium release concerns the formation of the FA conjugate. The stability of **γ-4** may be affected by the conjugation of one of the pendant carboxylate arms of DOTA. This phenomenon has been observed by several investigations that involve modification of the DOTA side arms. Sherry et al. in their work involving gadolinium-DOTA conjugated to a propylamide group via one carboxylate arm has reported a stability constant that is considerably lower (10^5 fold) than the DOTA complex owing to the decrease in basicity of the amine macrocycles (Sherry et al. 1989). A recent investigation reported that substitution with a *p*-NO_2-benzyl group at either one of the DOTA arms resulted in a reduction in the cooperative binding of the ligand and a lower thermodynamic stability constant compared to unmodified metal-DOTA complex (Sherry et al. 2004). Of course thermodynamic stability does not necessarily translate into in vivo stability with kinetic inertness often being of greater importance. This may also have a role to play in gallum's release.

Structural studies by Csajbok et al. via ^{1}H NMR reveal the occurrence of ring inversion and fluxionality in DOTA with an increase in temperature

Table 2. IC_{50} concentrations for **1**, **γ-2**, **γ-3**, **γ-4** and DOTA against A2780/AD ovarian cells and Chinese hamster ovary (CHO) cells. (–) indicates no IC_{50} was recorded. AZT was used as a control and returned an IC_{50} concentration of ~ 6–8 mM consistent with literature values (Doyle et al. 2008).

Compound	IC_{50} (mM) (72hrs)	
	CHO	A2780/AD
1	1.61	0.77
γ-2	(–)	(–)
γ-3	1.35	0.18
γ-4	2.93	1.85
DOTA	0.80	0.58

(Csajbok et al. 2004). Similarly, proton exchange can occur between ring amine groups and the carboxylate pendant arms, which may trigger decomplexation (Goddard et al. 2001). With the ligand's dynamic exchange process occurring in solution decomplexation can occur. A whole series of gallium compounds have been screened for *in vitro* cytotoxicity. A series of gallium compounds with significant toxicity are shown for comparision in Table 3.

To prove that gallium has indeed been "freed" from its macrocyclic cage, stability studies in HEPES buffer and RPMI 1640 media over 72 hours coupled with HPLC and ICP techniques were conducted. New peaks were observed between 48 and 72 hours indicative of gallium release (see supplemental material). These peaks were analyzed via ICP and gallium was noted. In addition, a slight precipitate could be removed by filtration (0.22 μM filters) and ICP confirmed the presence of gallium in the collected solid fraction. Attempts to identify the new species were unsuccessful by electrospray mass spectrometry and ^{1}H NMR and attempts to obtain crystals for X-ray structural analysis also proved unsuccessful. It is likely that both soluble and insoluble gallium salts (such as gallium hydroxides) are forming and/or gallium is complexing with compounds found in the RPMI media. The comparable observed cytotoxicities on both cell lines can then be ascribed to gallium leaching from DOTA over 72 hours.

Table 3. Ga compounds of various ligands (L) tested on cell lines showing significant antiproliferative activity. IC_{50} concentrations were obtained at 72 hours unless otherwise noted. PIH is Pyridoxal Isonicotinoyl Hydrazone.

Ligand (L)	IC_{50}	Cell line	References
2-acetylpyridine ^{4}N-dimethylthiosemicarbazone	1.33 +/– 0.43 nM – 96 hr 2.10 +/– 0.90 nM – 96 hr 0.18 +/– 0.02 nM – 96 hr	***Ovarian***: 41M ***Mammary***: SK-BR3 ***Colon***: SW480	26
Kenpaullone	<1 μM – 48 hr	***Lung***: CCRF-CEM; K-562; MLT-4	27
	<5 μM – 48 hr	***Colon***: HCT-116; HCT-15; HT29; SW-620	
	<1 μM – 48 hr	***Melanoma***: SK-MEL-28; SK-MEL-5	
	<10 μM – 48 hr	***Ovarian***: OVCAR-3	
	<10 μM -48 hr	***Breast***: MCF7	
PIH	50 μM	***Lung***: CCRF-CEM	28
Chloride	175 μM – 48 hr 16 μM – 96 hr	***Leukemia***: L1210	29
Transferrin	1.1 +/- 0.2 μM	***Leukemia***: HL60	30
Nitrate	120 μM	***Lung***: CCRF-CEM	2
	80 μM	***S-phase arrest Lung***	
1	1.61 mM	***Ovarian***: CHO	This work
	0.77 mM	***Ovarian***: A2789/AD	
γ-3	1.35 mM	***Ovarian***: CHO	This work
	180 μM	***Ovarian***: A2789/AD	
γ-4	2.93 mM	***Ovarian***: CHO	This work
	1.85 mM	***Ovarian***: A2789/AD	

Conclusion

We have successfully synthesized, characterized, and investigated the *in vitro* cytotoxicity studies of DOTA based gallium complexes and conducted controls to track the source of the toxicity. These results demonstrate that while a ligand of extraordinary kinetic and thermodynamic stability gallium can 'leach' from DOTA over a 72-hour period. What is also clear is that DOTA itself has between [500–800 μM] toxicity, an interesting note in and of itself. Toxicity in both lines could be explained by the uptake, by diffusion, of free DOTA or gallium-DOTA, or the presence of uncomplexed DOTA and/or the release of gallium from the conjugate as applicable in the FA-PEG containing systems. Clearly the fact that free DOTA has greater toxicity than the gallium complexed forms described herein, make them unsuitable as anticancer agents themselves. There is however a significant difference on FR containing cells over non-FR containing cells in terms of selectivity, *as well as sufficient stability*, to suggest that coupling the γ-emitting ^{67}Ga isotope or the β-emitting ^{68}Ga isotope to the FA-PEG conjugate unit may provide a suitable route to targeting radioisotopes of gallium to cell lines for use as diagnostic agents. This work is currently being investigated in the group.

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Supporting Material

HPLC stability traces and IC_{50} graphs showing exponential plots.

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Targeting Gallium to Cancer Cells through the Folate Receptor

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Supplementary Data

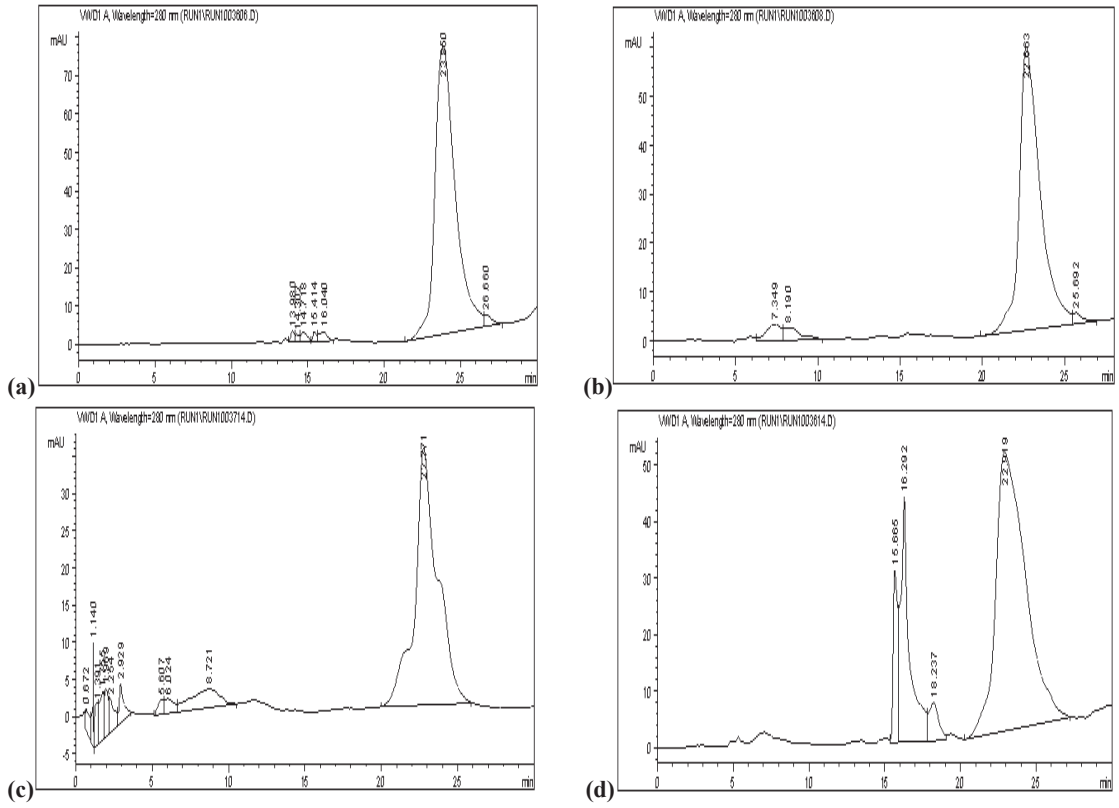

Figure S1. HPLC traces of **γ-4** displaying peaks after incubation at 37 °C in 25 mM HEPES buffer (pH 7.4) at **a**) 0 hr **b**) 24 hrs **c**) 48 hrs **d**) 72 hrs. ICP confirmed presence of gallium at the new peaks growing after 72 hrs at a retention time of Tr = 15.6–16.3 min.

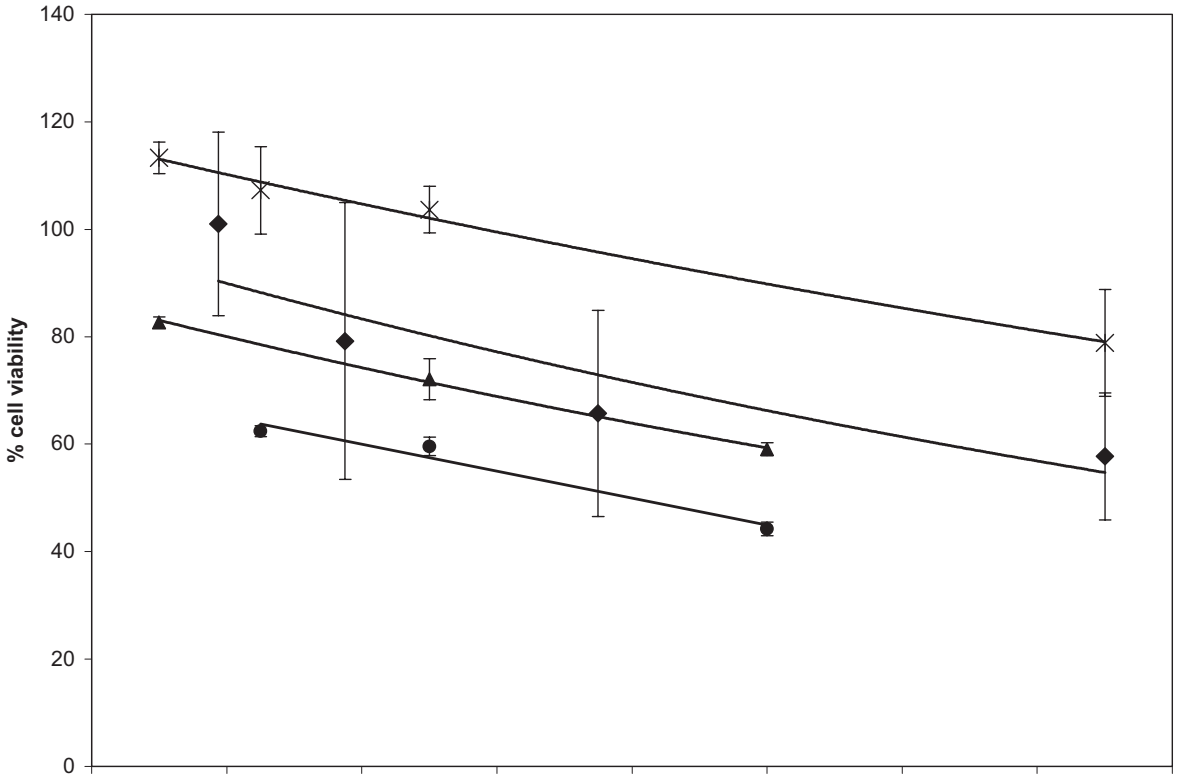

Figure S2. Cytotoxic effects of **1** (♦), **γ-3** (▲), **γ-4** (×) and DOTA (●) against CHO cancer cells. Error bars represent the standard deviation of the mean of three experiments (where n = 3 for each experiment) calculated for each concentration. Lines are exponential fits with R^2 values of 0.8333, 0.9982, 0.9942 and 0.9652 for **1**, **γ-3**, **γ-4** and DOTA respectively.

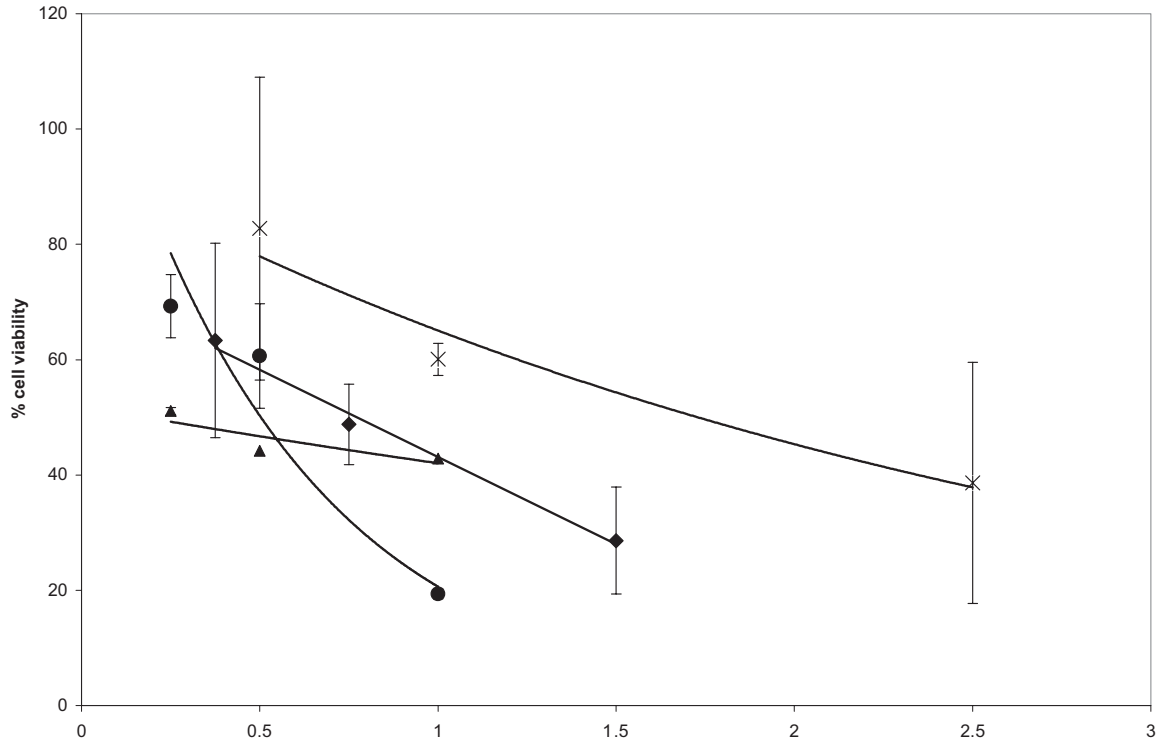

Figure S3. Cytotoxic effects of **1** (♦), **γ-3** (▲), **γ-4** (×) and DOTA (●) against A2780/AD cells. Error bars represent the standard deviation of the mean of three experiments (where n = 3 for each experiment) calculated for each concentration. Lines are exponential fits with R^2 values of 0.9905, 0.7274, 0.9444, and 0.9645 for **1**, **γ-3**, **γ-4** and DOTA.