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Gadolinium texaphyrin–methotrexate conjugates. Towards improved cancer chemotherapeutic agents†

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Received (Pittsburgh, PA, USA) 14th March 2005, Accepted 12th July 2005
First published as an Advance Article on the web 4th August 2005

Conjugates between methotrexate (MTX, Matrex®, *N*-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-glutamic acid), an antifolate cancer chemotherapeutic to which resistance is often observed, and motexafin gadolinium (MGd), an experimental agent demonstrating selective tumor localization, are described. These systems were prepared in order to test whether linking these two species would produce agents with enhanced activity relative to MTX alone. Both ester- and amide-linked conjugates were synthesized starting from MGd and MTX. The ester conjugate showed greater *in vitro* anti-proliferative activity against the A549 lung carcinoma cell line at short incubation times than did MTX alone. Neither the amide conjugate, nor MGd, showed any observable activity under these *in vitro* conditions. These results are rationalized in terms of enhanced cellular uptake of both the ester and amide conjugates that is coupled with an effective rate of release (*e.g.*, inherent or enzyme-mediated hydrolysis) in the case of the ester-linked conjugate, but not the corresponding amide system.

Introduction

A defining characteristic of many malignant cancers is the rapid, unregulated growth of cell populations. These cells also typically possess genetic instability, predisposing them to develop resistance to chemotherapeutic agents.[1–3] During cancer chemotherapy, after single agent treatment, a resistant subpopulation of cells is often seen to survive. This resistance often poses a significant limitation to effective clinical treatment. The co-administration of a combination of at least two drugs, often multiple chemotherapeutic agents, administered simultaneously or sequentially to inhibit tumor growth and circumvent the development of a resistant tumor, is thus common.[1] Unfortunately, actively proliferating tissues such as bone marrow and intestinal mucosa, also tend to accumulate chemotherapeutic agents and be affected adversely by their presence.[4] Therefore, in an effort to reduce non-tumor cell toxicity, chemotherapeutic agents better able to localize to tumors, either intrinsically or *via* attachment to tumor-directing carriers, are being developed.[5–7] The latter species, often referred to as conjugates, show particular promise and have been shown to increase efficacy or decrease toxicity to non-tumor tissues in several cases.[8,9] In this work we report the synthesis of conjugates of motexafin gadolinium (MGd, **1**; Scheme 1), an experimental drug demonstrating significant tumor targeting, with methotrexate (Matrex®, MTX **2**; Scheme 1), a DNA synthesis inhibitor. The resulting conjugates, containing either ester or amide linkers, were tested *in vitro* for anti-proliferative activity using A549 lung cancer cells. Under conditions involving limited drug exposure times, the ester conjugate exhibited greater activity than the corresponding amide conjugate or methotrexate alone.‡

Methotrexate is an extensively studied member of the aminopterin antifolate class of chemotherapeutic agents. MTX inhibits the synthesis of DNA precursor thymidylate by binding strongly to dihydrofolate reductase (DHFR) and, to a lesser extent, thymidylate synthase (TS). This inhibition leads to reduced DNA synthesis.[4,10] Although effective as a cytotoxic agent, MTX has unfavorable biodistribution properties. The compound is rapidly cleared from the blood by the kidneys, $t_{1/2} = 3$ hours, and is taken up by only one of the two folate receptors, and then only at a low level (*ca.* 5% uptake).[4] Tumor cell lines have been shown to develop resistance to MTX rapidly *via* several mechanisms including reduced drug uptake.[11] Some of these effects can be mitigated by the use of high dose levels. Unfortunately, in some tumors, resistance to MTX cannot be overcome even at the highest doses.[1] Additionally, even at normal doses there is accompanying bone marrow and intestinal mucosa toxicity.

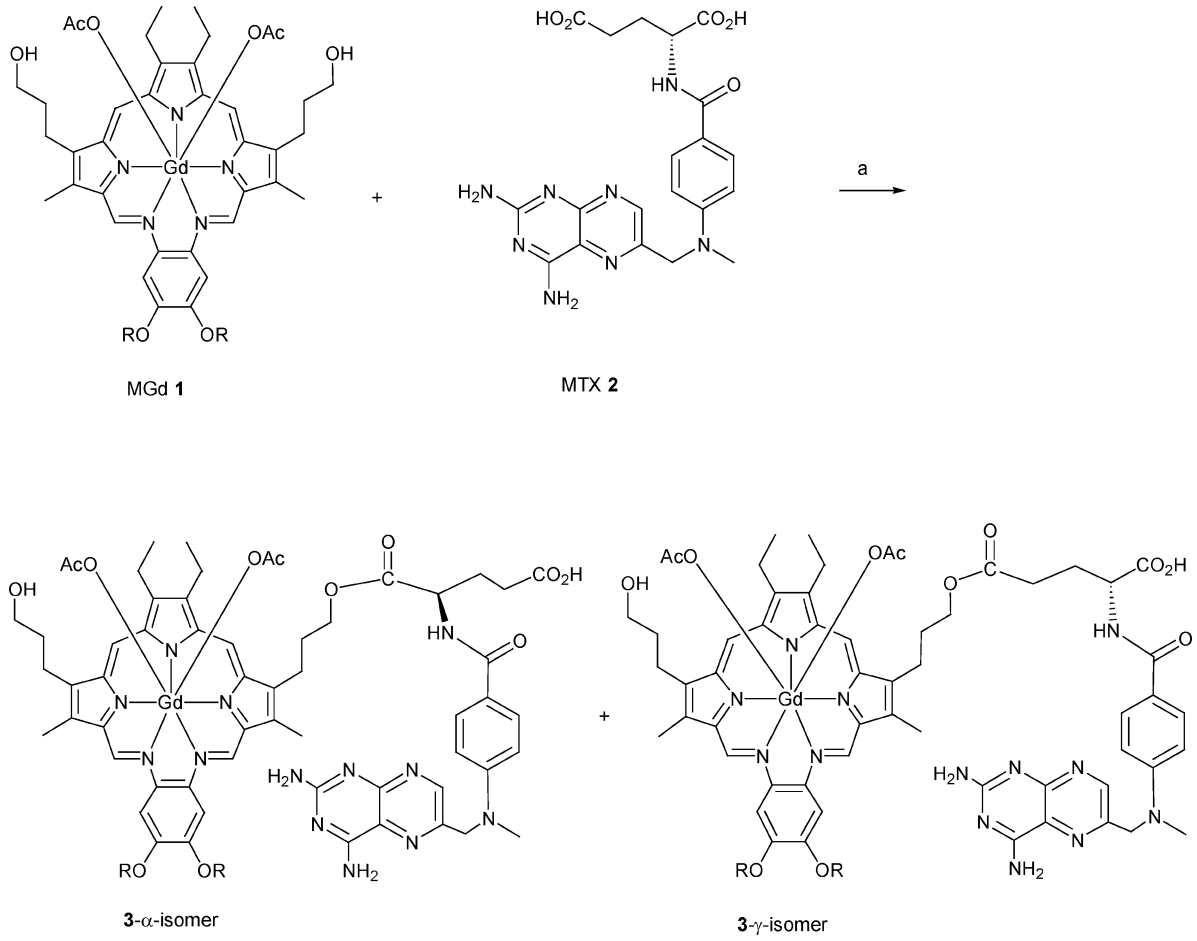
One approach to improving efficacy has been to synthesize MTX conjugates. To date, MTX has been conjugated to a number of carriers using the glutamic acid moiety, either α- or γ-carboxylate, as a point of attachment. Carriers studied to date include serum albumin, monoclonal antibodies, synthetic amphiphilic or lipophilic moieties such as polyethylene glycol or lipids, as well as smaller lipophilic molecules.[8,12–15] Currently, the most therapeutically advanced of these conjugate involves MTX linked to human serum albumin (HSA), which has completed Phase I and II clinical trials in Europe for renal cell carcinoma.[12,16] Studies of these and other conjugates have ascribed uptake to non-specific, fluid-phase endocytosis mechanisms, unrelated to the specific receptors for MTX.[6] *In vivo* tumor localization of the conjugates is often correlated with higher molecular weight and more lipophilic molecules, which extends circulation times and improves uptake of the conjugate.[6,15,16]

Modeling of the binding of MTX to DHFR suggests that the glutamyl moiety remains outside the binding pocket, and thus may not be fully necessary for binding.[17] Nonetheless, cleavage

† Electronic supplementary information (ESI) available: Supporting data. See http://dx.doi.org/10.1039/b503664j

‡ **MGd** The IUPAC name is bis(acetato-*O*)[9,10-diethyl-20,21-bis[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]-4,15-dimethyl-8,11-imino-6,3:13,16-dinitrilo-1,18-benzodiazacycloeicosine-5,14-dipropanolato-N^{1}, N^{18}, N^{23}, N^{24}, N^{25}]gadolinium hydrate. **MTX** The IUPAC name of this drug, also known by its tradename Matrex®, is *N*-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-glutamic acid.

DOI: 10.1039/b503664j



Scheme 1 Synthesis of ester conjugate **3** Reagents and conditions: (a) DEAD, Ph_3P; 28% yield; ratio **3**-α-isomer : **3**-γ-isomer = 38 : 62 (HPLC); R = $(CH_2CH_2O)_3CH_3$.

of the targeting agent from the glutamyl moiety appears to be required for activity in the case of MTX conjugates derived from large biomolecules, such as antibodies. In the case of smaller conjugates this requirement for hydrolysis may be relaxed. However, even with these systems, a free α-carboxyl group appears required for highest activity.[18] Independent of these considerations, low molecular weight conjugates are likely to be preferred in terms of cost, uniformity, ease of synthesis, stability, and characterization. These advantages of smaller conjugates notwithstanding, the recognized desirability of having what is essentially a "pro-drug," that cleaves under physiological conditions, imparts a significant design constraint that is not always easy to accommodate *a priori*.[5,7,19,20] A recognition of these factors led us to consider using texaphyrins as MTX carriers. Here, by using both amide- and ester-based coupling strategies it was thought that we might be able to test further the validity of the underlying cleavable linkage postulate.

Texaphyrins are large planar porphyrin-like macrocycles that coordinate trivalent lanthanide metal cations, giving molecules with improved therapeutic properties.[21–23] One clinical derivative, motexafin gadolinium (Xcytrin®, MGd, **1**), a water solubilized gadolinium complex, is taken up into, and retained in tumors with a high degree of selectivity, as judged from a substantial body of preclinical work, as well as data from initial clinical trials.[24,25] MGd, which is MRI-detectable, has been extensively studied in combination with X-ray radiation therapy (XRT), and, more recently as a direct chemotherapeutic agent either alone or in conjunction with other anticancer agents.[26–29] Currently MGd is undergoing clinical trials for a range of cancer indications.[30] The most advanced of these trials involves the use of MGd and radiation therapy for metastatic brain cancer arising from non-small-cell lung cancer.[31] Based on *in vitro* studies, MGd is believed to be taken up by endocytosis, and to localize in several cytoplasmic compartments.[32,33] To the extent that this localization is retained in larger systems containing the texaphyrin core, the use of MGd could provide MTX conjugates with increased activity. This approach has the further advantage that it should permit the construction of conjugates containing both cleavable and non-cleavable linkages, thus allowing the importance of methotrexate release to be more fully assessed. A further advantage of such conjugates is that they might allow for tumor-localized imaging and therapeutic activity at a targeted tumor site. Such localized imaging and therapeutic activity was recently demonstrated in a chlorophyll-a aminobenzyl–Gd-DTPA conjugate derivatized with PEG-methyl ether.[34] In this conjugate the Gd-DTPA functionalization allowed *in vivo* MRI imaging of tumors, while the chlorophyll moiety allowed PDT therapeutic treatment.

Results and discussion

Synthesis of MGd-MTX conjugates

Ester conjugate. The synthesis of the ester conjugate **3** is summarized in Scheme 1. As indicated, the Mitsonobu reaction was used to esterify directly MGd **1** with MTX **2** to give **3**. To proceed well, the esterification requires more than 3 equiv. of diethyl azodicarboxylate (DEAD) and Ph_3P. When fewer than 3 equiv. of DEAD and Ph_3P were added, only a trace of the desired product **3** was found according to HPLC analysis. The fact that MTX **2**, as purchased commercially, is a dihydrate may account for this finding. We also found that the yields were reduced when more than 7 equiv. of DEAD and Ph_3P were used. This proved true even when 2–3 equiv. of **2** were also added. The best conditions were those where 4.5–5 equiv. of DEAD were used and Ph_3P was added; this gave 40% conversion to **3** (by

HPLC). The overall, isolated yield was 28% (based on **1**). The loss of product during purification is thought to reflect the fact that hydrolysis of the ester bond takes place to give back the initial building blocks, namely MGd **1** and MTX **2**.

The HPLC trace revealed two closely spaced peaks, ascribed to the **3**-γ- (t_r. = 6.3 min) and **3**-α-isomers (t_r = 6.7 min), at an integrated ratio of 2 : 1. The **3**-γ- and **3**-α-isomers were individually purified to a purity of ≥90% on a small scale and characterized by UV/Vis (280–800 nm) spectroscopy, ESI, FAB-HRMS, and HPLC. Unfortunately no readily interpretable ^{1}H-NMR spectrum could be obtained, due to the presence of the paramagnetic gadolinium metal center.

The UV/Vis spectra are shown in Fig. 1. Conjugate **3** has a Soret-like band with a shoulder at 474 nm and a Q-like band at 742 nm. These two bands are characteristic of the MGd core present in conjugate **3**. Likewise, the peak seen at 305 nm is typical of MTX **2**, providing important support for the proposal that this subunit is present in **3**. Consistent with this conclusion is the finding that the λ_{max} for this latter peak is slightly red-shifted relative to MTX **2** (298 nm). The ESI-MS spectra of the **3**-γ- and **3**-α-isomeric conjugates showed a molecular fragment corresponding to $[M - 2(OAc)]^+$, rather than the $[M - OAc]^+$ peak, which is normally seen for most gadolinium texaphyrin complexes. This difference is ascribed to the presence of the carboxylate group on the MTX portion of these conjugates. This group is expected to coordinate tightly to the gadolinium center in the gas phase, thus competing effectively with the normal axial ligand, AcO^-.

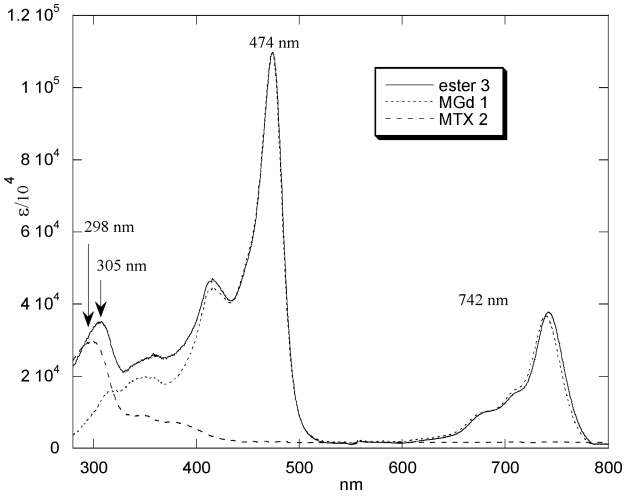
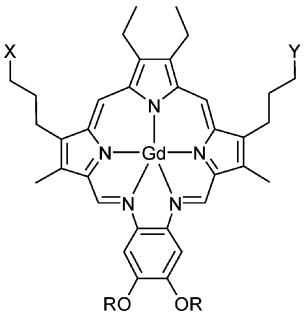


Fig. 1 UV/Vis Spectra of MGd **1**, MTX **2**, and ester **3**.

The HPLC peak assignment of the **3**-α-isomer ester conjugate was confirmed by derivatization and hydrolysis. Thus, the **3**-α-isomer free γ-carboxylic acid was coupled with propylamine, giving the γ-propylamide-MTX-α-MGd ester. Hydrolysis of the α-ester linkage gave γ-propylamide-MTX. This same γ-propylamide-MTX was made by an independent route involving the direct EDC mediated coupling of MTX **2** to propylamine. The α- and γ-amide MTX isomers prepared in this way were separated by chromatography. The structures were then assigned based an analysis of the ^{1}H NMR shift patterns for the amide NH proton and comparisons of these shifts, and the HPLC retention times to those of previously reported MTX-amides.[10] The HPLC retention times, of the γ-amide prepared by direct synthesis from MTX and from the 3-α isomer of the ester as described above proved identical. On this basis, it is proposed that the **3**-γ-isomer is the product that elutes first, and that it is the **3**-α-isomer that elutes second from the reverse-phase column. This is in agreement with previous reports that the γ-esters of MTX (α-free) are more polar than the corresponding α-esters.[35,36]

Amide conjugate. The amide conjugate **9** was synthesized in accord with the strategy summarized in Schemes 2 and 3. First the ammonium acetate texaphyrin **7** intermediate was prepared in 42% yield using a Mitsonobu reaction (Scheme 2).[37] Thus, MGd **1** was reacted with dimethoxytrityl (DMT) chloride in the presence of base to give **4**, the mono-DMT protected product. DMT *O*-protected texaphyrin **4** was reacted with phthalimide in the presence of diethyl azodicarboxylate (DEAD) and Ph_3P to give a phthalimide texaphyrin derivative **5**. Removal of the phthalimide in aq. 40% methylamine ($MeNH_2$) then gave the amine texaphyrin **6**. Finally, treatment of **6** with an acidic buffer (1% HOAc in 0.1 M NH_4OAc, pH = 4.3) gave the ammonium acetate texaphyrin **7**. In order to avoid oxidation, the buffer was added without delay after most of $MeNH_2$ had been removed under argon. Neither the phthalimide **5** nor the amine **6** was purified prior to being carried on to the next step.



R = -$(CH_2CH_2O)_3CH_3$

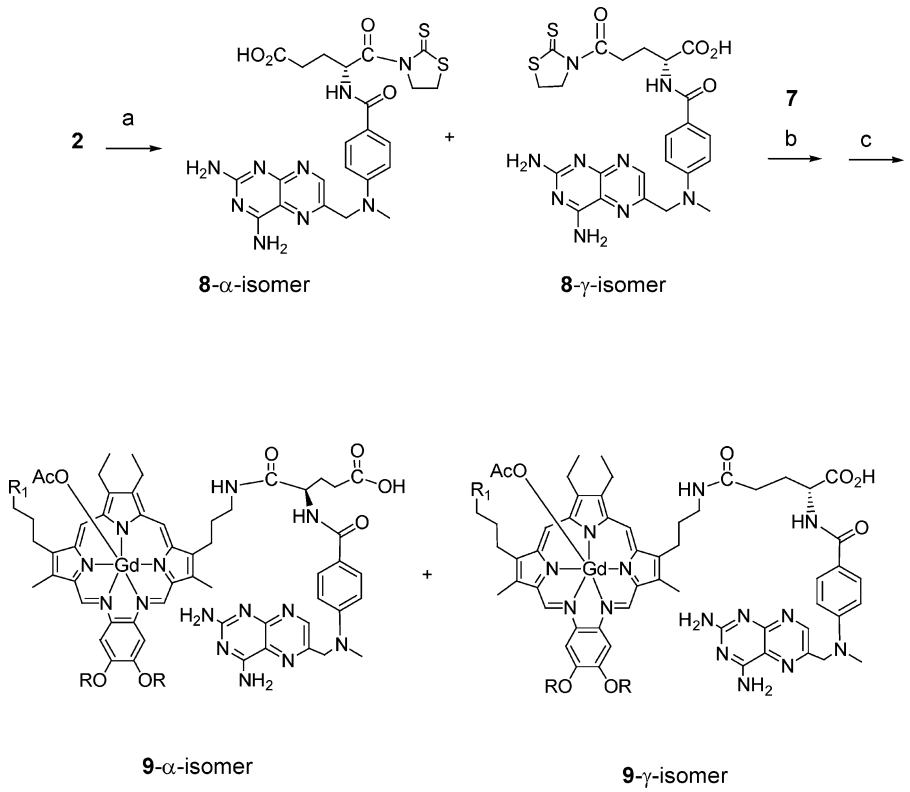
a **1**, X = Y = OH
b **4**, X = ODMT, Y = OH
c **5**, X = ODMT, Y = N-phthalimide
d **6**, X = ODMT, Y = NH_2
7, X = ODMT, Y = NH_3^+ AcO^-

Scheme 2 Synthesis of **7** (a) DMT-Cl, diisopropyl ethylamine; (b) DEAD, Ph_3P, phthalimide; (c) 40% $MeNH_2$, MeOH, RT, 2 h; (d) AcOH in NH_4OAc pH = 4.3; 42% yield (first three steps).

Separately (Scheme 3), the carboxylic groups present in MTX **2** were activated[38] by treatment with a slight excess of 3-thiazolidine-2-thione (TTH, 1.1 equiv.) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) to give a 1 : 2 mixture of **8** as a mixture of isomers eluting with retention times of 7.7 and 7.9 minutes, respectively, on reverse phase HPLC (pH 4.3, MeCN–NH_4OAc gradient). In analogy to the ester linked products described above, these were assigned as being the γ– and α-isomers, respectively. Unfortunately, this compound (as a mixture of isomers) proved very hard to purify. In fact, a small amount of MTX **2** was always found to be present after chromatographic purification, as judged from TLC analysis. Therefore, the mixture of the **8**-α- and **8**-γ-isomers was used in the next reaction step. Here, the activated carboxylic group of the MTX moiety in these products was coupled directly with compound **6** (produced *in situ* from **7**) to give, following deprotection, the target amide conjugate **9**, as shown in Scheme 3. The product (**9**) was isolated as a mixture of the two isomers, in 29% yield for the combined coupling and deprotection steps. The total yield based on **1** was 4.4%.

In vitro biological activity

The relative proliferation of A549 lung cancer cells treated with 60 μM MGd **1**, MTX **2**, a mixture of MGd **1** and MTX **2**, amide conjugate **9** or ester conjugate **3** is compared in Fig. 2. Cells were incubated in the presence of these agents for 4, 8 or 24 hours. The medium was replaced, and the cells were analyzed by colorimetric assay (MTT) 72 hours later. No effect was seen after 4 hours of treatment with any agent (data not shown). After 8 hours of treatment, only ester conjugate **3** displayed activity,



Scheme 3 Synthesis of amide conjugate **9** Reagents and conditions: (a) TTH, EDC, DMAP, DMF, 0 °C, 74%; (b) DIEA, DMF, 0 °C for 5 h and rt overnight; (c) HOAc–DCM (1 : 4 v/v), 2–3 h, 29% (two steps); R = $(CH_2CH_2O)_3CH_3$.

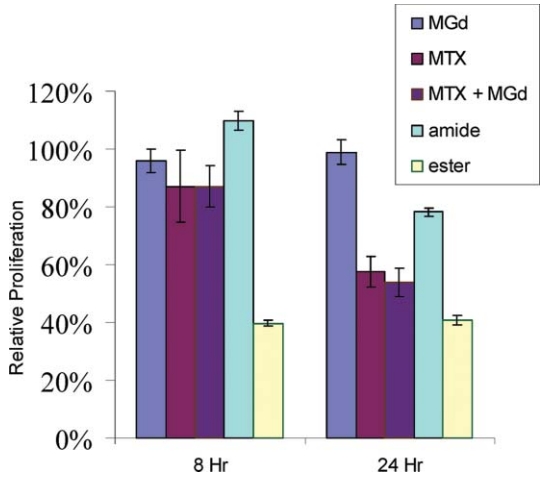


Fig. 2 Cell proliferation after 8 and 24 hour incubation with MGd, MTX, MGd and MTX, ester conjugate **3**, and amide conjugate **9**.

inhibiting cell proliferation to *ca.* 40% that of untreated control. When an incubation period of 24 hours was used, the activity of the ester conjugate was found to be unchanged, relative to what was observed at the 8 h timepoint. The amide conjugate **9** was seen to display modest activity, *ca.* 80% of control at 24 h, *vs.* no observable effect at 8 h. Finally, the effect of MTX, either alone or analyzed in the presence of MGd, was found to be substantially enhanced after 24 hours incubation relative to what was seen at 8 h.

To understand better the difference in activity observed for the ester and amide conjugates, we measured the stability of conjugates **3** and **9** under the conditions of the experiment. These conjugates were extracted and analyzed by HPLC following incubation with A549 cells in the medium used for the study (RPMI 1640 supplemented with fetal bovine serum). The ester conjugate **3** and amide conjugate **9** remained substantially (>99%) uncleaved in culture medium over the 24 hour time course of incubation. The amide conjugate **9** associated with the cell pellet remained uncleaved (>99.7%) over this time interval, whereas the corresponding ester conjugate **3** was cleaved to the extent of 9.5 to 17.5%. Although a greater amount of ester conjugate **3** was taken up after 24 hours, the percentage of total conjugate cleaved at this time was lower (9.5%), possibly indicating that the capacity of cell associated esterase activity had been exceeded. These findings lead us to suggest that ester conjugate **3** is more active than amide conjugate **9** as a consequence of being more labile towards intracellular enzymatic hydrolysis.

We also suggest that the greater activity of ester conjugate **3** relative to MTX after an incubation time of 8 hours is due to intracellular release of MTX. It is clear from the data in Fig. 2 that extracellular MTX levels equivalent to those presumably produced by ester hydrolysis did not result in significant activity after an 8 h treatment. The currently accepted postulate is that uptake of MTX occurs *via* saturable, folate receptors, and is followed by metabolic activation of MTX to potentiate cellular retention, a process that generally takes place during a 12–24 h time period.[4] The experimental consequence is that good activity is seen for MTX, but only after long (*ca.* 24 h) incubation times. By contrast, more rapid cellular uptake of MGd, or its derivatives, is expected to occur through non-saturable, folate receptor-independent endocytosis, thus providing higher intracellular concentrations as the external concentrations are raised and at shorter incubation times.[32,39] On this basis we suggest that the greater activity of ester conjugate **3** relative to MTX after an incubation time of 8 hours is due to more rapid cellular uptake, enhanced retention and intracellular release of MTX from the conjugate. This latter release is not expected to take place to an appreciable extent in the case of the corresponding amide conjugate **9**.

Conclusion

Texaphyrin ester conjugate **3** and amide conjugates **9** were successfully synthesized. The ester conjugate **3** showed greater

anti-proliferative effect than MGd **1**, MTX **2**, a mixture of MGd and MTX, or the amide conjugate **9** in A549 lung cancer cells after 8 hours of treatment. Both conjugates were stable in the extracellular milieu. Current efforts are thus being devoted to improving the yield of **3** and to carrying out *in vivo* biological studies with conjugate **3** and related agents. It is anticipated that the current strategy of attaching known, active anticancer drugs to the tumor-targeting texaphyrin derivative, MGd, may provide anti-cancer agents with improved selectivity and bioavailability.

Experimental

All of reactions were monitored by HPLC using a reverse-phase column, and a mixture of MeCN and 0.1 M aqueous buffer (pH = 4.3, 0.1 M NH_4OAc and 1% HOAc in water) as the mobile phase. Purity was recorded in terms of the percent area of the peaks seen at different retention times (t_r). The 0.1 M NH_4OAc buffer described above (termed "buffer") was used in all procedures and reverse phase purifications, unless otherwise noted. Unfortunately, the paramagnetic nature of most products produced in this study, specifically the MTX–MGd conjugates, precluded characterization by ^1H NMR spectroscopy. All starting materials, including methotrexate **2** (MTX), were purchased and used without further purification unless other noted. Motexafin gadolinium (MGd, **1**) was prepared as described previously.[40] DMF was dried with molecular sieves. Liquid chromatographic (HPLC) analyses were performed on a Beckman Coulter instrument equipped with a reverse-phase Agilent Eclipse XDB-C18 column, System Gold 168 Detector and 32 Karat Software. UV/Vis spectra were taken on Beckman DU-640B Spectrophotometer. Sep-pak reverse-phase tC18 cartridge columns were purchased from Waters. Proton-NMR spectra were recorded at 300 M Hz.

Synthesis of ester conjugate 3

Diethyl azodicarboxylate (142 mm^3, 0.90 mmol) was slowly added dropwise to a solution of MGd **1** (230 mg, 0.2 mmol), Ph_3P (276 mg, 1.0 mmol) and MTX **2** (91 mg, 0.20 mmol) in dry DMF (12 cm^3) under argon at room temperature. The solution was stirred for 3–6 hours. HPLC was used to check the progress of the reaction. When conversion to the ester conjugate **3** appeared complete, the green solution was poured into the buffer. The tC18 column and eluents consisting of various concentrations of MeCN in buffer were used to effect separation. In the initial separation, a 25% MeCN-buffer was used to elute MGd **1**, while a 30–35% MeCN-buffer was used to elute a mixture of target conjugate **3** and a MGd-OAc ester by-product. The partially purified conjugate mixture was again subject to separation on the same solid phase using 30% MeCN-buffer as the eluent. Under these conditions, the ester conjugate **3** eluted first. Desalting with deionized water on a new tC18 column gave a green solid in 90 mg, a yield of 28%. This proved to be pure ester conjugate **3** as inferred from HPLC analysis. ESI-MS (1% AcOH): m/z 1465.6 $[M - H]^+$; FAB-HRMS $[M]^+$ calculated for $C_{68}H_{86}Gd[158]N_{13}O1_4$: 1466.5735; found: 1466.5746; UV/Vis in MeOH [λ_{max}, nm (log ε)]: 305 (4.61), 414 (sharp shoulder), 474 (5.04) and 742 (4.58).

Synthesis of amide conjugate 9

DMTO-MGd 4. To a dark green solution of MGd **1** (2.29 g, 2 mmol) in a mixture of dry dichloromethane (DCM, 300 cm^3) and dry tetrahydrofuran (THF, 30 cm^3), diisopropylethylamine (DIEA, 1.05 cm^3, 6 mmol) was added under argon at room temperature. Then, 4,4′-dimethoxytrityl chloride (DMT-Cl, 1.7 g, 5.0 mmol) was added to the solution in one portion. The solution was stirred for 8–12 h at room temperature. The reaction was monitored by HPLC. There were three major peaks: MGd **1**, DMTO-MGd **4** (mono-substituted) and DMTO-MGd-ODMT (disubstituted) in the HPLC spectrum. When the integrated area of the peak corresponding to DMTO-MGd **4** was maximal (around 39–43%), the reaction was quenched with 10 cm^3 of methanol. At this juncture, the solution could be stored in refrigerator overnight. A reverse-phase tC18 cartridge column was used for the separation, with a mixture of MeCN and 0.1 M buffer being used as the eluant. MGd **1** eluted first from the column with 25–30% MeCN-buffer, DMTO-MGd **4** with 50–55% MeCN-buffer, and the doubly protected species DMTO-MGd-ODMT with 65–70% MeCN. DMTO-MGd **4** was desalted with a new tC18 column using deionized water. A green solid, **3**, was obtained (1.05 g, 36% yield). UV/Vis in MeOH [nm]: $\lambda_{max} = 425$, 475 and 740. ESI-MS (1% AcOH): m/z 1391.6 $[M - 59\ (AcO^-)]^+$ (*i.e.*, without OAc).

DMTO-MGd-NH_2·HOAc 7. DMTO-MGd **4** (530 mg, 0.36 mmol), phthalimide (269 mg, 0.83 mmol) and triphenylphosphine (Ph_3P, 479 mg, 8.3 mmol) were dried for 1 hour using a vacuum pump and dissolved in DCM (50 cm^3, dried over CaH_2 and redistilled under argon). To the resulting green solution, diethyl azodicarboxylate (287 mm^3, 0.83 mmol) was added dropwise at 0 °C and stirred for 4–5 hours. An HPLC chromatogram was taken, and the reaction stopped when the area of the peak as ascribed to the coupling product from DMTO-MGd **4** and phthalimide was maximal. Solvent (DCM) was removed under reduced pressure. Methylamine (40% in water, 20 cm^3) and MeOH (20 cm^3) were added to the flask. The solution was stirred for 2–3 h under argon and then was bubbled with argon to remove the bulk of the methylamine (around 30 min). MeOH and any remaining methylamine was then evaporated off under reduced pressure. The residue was dissolved in MeCN (25 cm^3) and buffer (50 cm^3) was added. The solution was extracted with 3 × 50 cm^3 $CHCl_3$. The organic layers were combined and evaporated to dryness. The resulting crude product was loaded onto a reverse-phase tC18 cartridge column. A mixture of MeCN and buffer were used as the elutant. Finally, **7** was subject to further purification on a new tC18 column to remove the ammonium salt. A green solid was obtained in 42% yield. ESI-MS (1% AcOH): m/z 1391 $[M - 59\ (AcO^-)]^+$ (*i.e.* without HOAc).

MTX-TTH 8

Methotrexate-TTH 8. To a solution of MTX **2** (29.4 mg, 0.060 mmol) and 1,3-thiazolidine-2-thione, TTH (7.8 mg, 0.066 mmol, 1.1 equiv) in dry DMF (4 ml) cooled to 0 °C with an ice-bath, was added EDC (14.4 mg, 0.075 mmol, 1.25 equiv) and 4-(*N*,*N*′-dimethyl)aminopyridine, DMAP (0.020 g). The mixture was stirred at 0 °C for 5 h, then at room temperature overnight. The product was collected as the second yellow tinged fraction from a tC18 column. Finally, the crude yellow solid **8** was dried under vacuum overnight. The crude **8** was obtained in a yield of 74% and was not subject to further purification prior to the next coupling reaction. CI-HRMS: calculated for $C_{23}H_{25}N_9O_4S_2$: 555.1471; found: 555.1489. δ_H (DMSO-d_6): 8.75 (d, $J = 6.3$, 1H, α-isomer), 8.56 (s, 1H), 7.76 (d, $J = 6.3$, 1H, γ-isomer), 7.72 (d, $J = 9.0$, 2H), 7.43 (s, br, 2H), 6.81 (d, $J = 9.0$, 2H), 6.61 (s, br, 2H), 4.77 (s, 2H), 4.35–4.25 (m. 3H), 3.28 (br, 2H), 3.19 (s, 3H), 2.31–2.26 (m, 2H), 2.05–1.96 (m, 2H).

Amide conjugate 9 from the reaction of 7 + 8

To a solution of **7** (78 mg, 0.05 mmol) and DIEA (18 µl, 0.10 mmol) in DMF (6 ml), was added **8** (28 mg, 0.05 mmol) in one portion. The solution was stirred at 0 °C for 1 h and room temperature for 3 h. The mixture was loaded onto a tC18 column. The pure DMT *O*-protected amide conjugate was obtained in 33 mg, 37% yield. ESI-MS (1% HOAc): m/z 1766.5 $[M - 59\ (AcO^-)]^+$ The pure green solid (33 mg, 0.018 mmol) was dissolved in DCM (2 cm^3) under argon at room temperature. Acetic acid (0.5 cm^3) was added *via* syringe. The ensuing hydroxyl deprotection was monitored by

HPLC. When the peak corresponding to the starting material could no longer be observed (after *ca.* 3 h), the solvent, DCM, was removed quickly using a vacuum pump. The resulting liquid was immediately poured into an aqueous buffer solution (50 cm^3, 0.3 M aq. NH_4OAc) and the resulting green solution was quickly loaded onto a tC18 column. A mixture consisting of 25% MeCN-buffer was used to elute off MGd **1**, whereas an eluent consisting of 30–33% MeCN-buffer was used to obtain the desired product, namely the amide conjugate **9**. A pure green solid, conjugate **9** was obtained in 41% yield (11.4 mg) after removing the ammonium salt *via* passage through a new tC18 column with deionized water, eluting with methanol, and drying under reduced pressure. ESI-MS (1% HOAc): m/z 1464.5 $[M - H]^+$; FAB-HRMS $[M]^+$ calculated for $C_{68}H_{87}Gd[158]N_{14}O_{13}$: 1466.5735; found: 1466.5746; UV/Vis in MeOH [λ_{max}, nm (log ε)]: 306 (4.61), 415 (shoulder), 474 (5.04) and 743 (4.58). Note: The overall yield was 15% for the two step coupling and deprotection procedure. The total yield increased to 29%, when the crude DMTO-protected amide conjugate obtained after the first step was used directly for next coupling reaction without subjecting it to intermediate purification.

In vitro biological activity

The proliferation of exponential phase cultures of A549 cells was assessed by colourimetric assay.[41] In brief, A549 lung cancer cells were seeded on 96 well microtiter plates at 2000 cells per well and allowed to adhere overnight in RPMI 1640 medium supplemented with 20 mM HEPES, 2 mM L-glutamine (Invitrogen), 10% heat inactivated fetal bovine serum, and antibiotics (200 U cm^{-3} penicillin and 200 μg cm^{-3} streptomycin). Stock solutions of MGd-methotrexate ester **3**, MGd-methotrexate amide **9**, methotrexate **2**, and MGd **1** were formulated in 5% mannitol, then diluted in medium for secondary stocks of 180 μM. Stock solutions were serially diluted 1 : 3 on Rows B–F, whereupon plates were incubated at 37 °C under a 5% CO_2–95% air atmosphere. Medium was replaced with fresh medium after 4, 8, or 24 hours treatment. After a total of 72 hours, medium was exchanged for fresh medium (150 mm^3 per well) supplemented with the tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical, 0.5 mg cm^{-3}). The plates were incubated at 37 °C for approximately 2 hours, whereupon medium was removed and isopropyl alcohol (100 mm^3 per well) was added. Microplates were vortexed briefly and well absorbances at 560–650 nm were measured using a microplate reader (Molecular Devices, Sunnyvale, CA). The average absorption of wells in the absence of cells was subtracted from each well as background absorbance. Plate absorbances were normalized to wells containing untreated cells to allow plate-to-plate comparison. Data shown at each concentration of test article is the average of data from 10 wells. Error bars represent the associated standard deviation.

Analysis of texaphyrin conjugate stability in A549 cell pellets and the extracellular milieu

To assess conjugate stability, cell pellets and extracellular milieu from plateau phase cultures of A549 cells treated as described in Fig. 2 were sampled after 4 h, 8 h, and 24 h. Samples were stored at −20 °C for subsequent extraction and analysis using reversed-phase HPLC. In accord with standard procedures, cell pellets and extracellular medium were extracted by the addition of a 50 : 50 v/v solution of methanol–acetonitrile containing 0.16 M glacial acetic acid and zinc sulfate. Extraction efficiency was corrected using an internal standard. HPLC was performed using an Agilent HP1100 chromatography system with detection based on MGd absorbance at 470 nm. Values are the average of 3 measurements (see supporting information†).

Acknowledgements

This work was supported in part by the NIH (grant no. CA 68682 to J.L.S.)

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