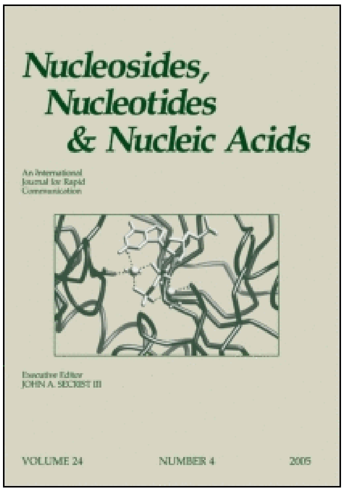


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Synthesis, Characterization, and In Vitro Assay of Folic Acid Conjugates of 3'-Azido-3'-Deoxythymidine (AZT): Toward Targeted AZT Based Anticancer Therapeutics

Anthony R. Vortherms[a]; Robert P. Doyle[a]; Dayuan Gao[b]; Olivia Debrah[b]; Patrick J. Sinko[b]
[a] Department of Chemistry, Syracuse University, Syracuse, New York, USA [b] Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey, USA

To cite this Article Vortherms, Anthony R. , Doyle, Robert P. , Gao, Dayuan , Debrah, Olivia and Sinko, Patrick J.(2008) 'Synthesis, Characterization, and In Vitro Assay of Folic Acid Conjugates of 3'-Azido-3'-Deoxythymidine (AZT): Toward Targeted AZT Based Anticancer Therapeutics', Nucleosides, Nucleotides and Nucleic Acids, 27: 2, 173 — 185

To link to this Article: DOI: 10.1080/15257770701795946

URL: http://dx.doi.org/10.1080/15257770701795946

Nucleosides, Nucleotides, and Nucleic Acids, 27:173–185, 2008
Copyright © Taylor & Francis Group, LLC
ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770701795946

Taylor & Francis
Taylor & Francis Group

SYNTHESIS, CHARACTERIZATION, AND IN VITRO ASSAY OF FOLIC ACID CONJUGATES OF 3′-AZIDO-3′-DEOXYTHYMIDINE (AZT): TOWARD TARGETED AZT BASED ANTICANCER THERAPEUTICS

Anthony R. Vortherms,[1] Robert P. Doyle,[1] Dayuan Gao,[2] Olivia Debrah,[2] and Patrick J. Sinko[2]

[1]*Department of Chemistry, Syracuse University, Syracuse, New York, USA*
[2]*Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey, USA*

□ *Conjugates of three components namely folic acid, poly(ethyleneglycol) and 3′-azido-3′-deoxythymidine (AZT) are presented. Folate-PEG units were coupled to AZT to facilitate delivery of the nucleoside into the cell. A convenient separation of the polydisperse PEGylated-folic acid regioisomers produced upon conjugation is described. This is to select for the active γ-regioisomer over the inactive α-regioisomer. In vitro cytotoxicity assays were conducted against an ovarian cell line (A2780/AD) that overexpresses the folate receptor (FR) and compared to a FR free control cell line. Compared to AZT a ∼20-fold greater potency against the resistant ovarian line was observed for the conjugates.*

Keywords Anticancer agent; 3′-azido-3′-deoxythymidine nucleoside; folic acid; isomer separation

INTRODUCTION

The synthesis of 3′-azido-3′-deoxythymidine (AZT) is credited to Jerome Horwitz and was first published in 1964.[1] Originally envisaged as an anticancer medication, AZT gained fame in 1987 as the first FDA approved anti-HIV drug.[2] The commonly accepted mechanism for anticancer and

Received 4 June 2007; accepted 23 August 2007.

The authors wish to thank Syracuse University, the iLEARN program, and Promega Corporation (Madison, WI) for funding. We also thank Professor Timothy J. Fairchild (Department of Exercise Science, Syracuse University) for assistance with statistical analysis and Karen L. Howard (State University of New York, ESF) and Nerissa A. Villegas (Syracuse University) for assistance obtaining MALDI-Tof mass spectra.

Address correspondence to Robert P. Doyle, Department of Chemistry, Syracuse University, Syracuse, NY 13208-4100. E-mail: rpdoyle@syr.edu

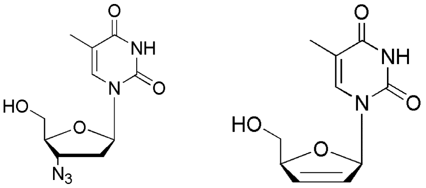


FIGURE 1 The structure of the nucleoside analogs 3′-azido-3′-deoxythymidine (AZT) and stavudine (d4T).

antiviral activity of AZT is the formation of ultrastructural changes and/or strand termination in growing DNA strands (especially mitochondrial DNA).[3] Other postulated mechanisms include thymidylate kinase inhibition (preventing di- and tri-phosphorylation of nucleosides),[4] succinate transport inhibition in muscle cells,[5] inhibition of telomerase activity,[6] and inhibition of chromosomal DNA polymerases.[7] There also is some evidence (and much debate[8b] of AZT being converted in vivo into an analog known as d4T, commercially known as stavudine (see Figure 1).[8] Of the eight Food and Drug Administration (FDA)-approved nucleoside analogs approved for human use, d4T shows some of the highest clinical toxicity.[3a] Conversion of some concentration of AZT into d4T also then has important implications for the drugs mode of action (and associated toxicity).

With renewed interest in AZT from its use in the treatment of HIV, researchers began reinvestigating AZT's toxicity to cancer cells. Such work demonstrated that AZT has typical micromolar range efficacy against such cells, for example, {(DU 145 (prostate) IC_{50} 24 μM), (MCF-7 (breast) IC_{50} 22 μM), (CX-1 (colon) IC_{50} 23 μM)}.[9] Even when AZT is given at IC_{10} or IC_{20}, the drug can be effective because cell division is sufficiently slowed. When this level of AZT dosage is cycled, growth inhibition can become pronounced.[10]

While many compounds have the bioactivity to be chemotherapeutics, they are often indiscriminate killers.[11] Given the multiple modes of action of AZT listed above, it comes as no surprise that there are numerous side effects to the use of AZT (and nucleoside drugs in general). The targeting of bone marrow by AZT as well as myopathy, lactic acidosis, and the development of anemia are just some of the problems associated with its use.[12] Developing a method to target AZT's potency against growing tumors may however make it possible to use AZT against specific tumor types. Given the multi-faceted modes of action of AZT it may be particularly suitable against resistant tumors. The targeting group used should also keep AZT away from problem areas such as bone marrow by providing the necessary tropism for the tumor. Since lower doses would be required to inhibit cancer growth and more would reach the desired areas, a patient can be spared the heavy dosing that would currently be required and still benefit

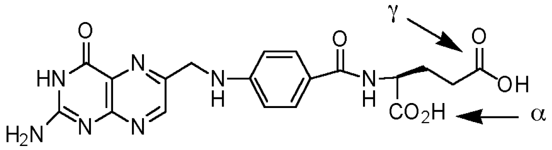


FIGURE 2 Folic acid with α-and γ-carboxylic acid groups indicated.

from AZT's activity against rapid cell division (i.e., improved dose-related toxicity).

Such a targeting system exists in the form of the active transport mechanisms in place for the absorption and cellular uptake of certain vitamin molecules.[13] Vitamins such as folic acid (see Figure 2) have the potential to act as drug delivery vectors if an active pharmaceutical can be tethered to them without disrupting their in vivo recognition.[14]

Folic acid (FA) has the potential to serve as a delivery agent, capable of specifically targeting folate-receptor (FR) positive tumors. FRs are membrane glyco-proteins overexpressed by a number of tumor cell types such as ovarian, breast, cervical, colorectal, renal, and nasopharyngeal cancers.[15] Cells overexpressing FR protein bind folate-drug conjugates tightly ($K_d \sim 10^{-9}$ M) and endocytose them inside, provided chemical modification of the folate upon conjugation does not disrupt recognition by the FR.[10] This can be achieved by modification of the γ-carboxylic acid moiety of folic acid, (unlike modification of the α-carboxylic acid group, which disrupts FR binding recognition).[16] Of great additional interest in terms of the use of folate to carry AZT is work by Antony et al. that demonstrated that FR overexpressing cells also overexpress thymidylate kinase, an enzyme, as mentioned earlier, required to convert nucleotides into their triphosphorylated nucleoside analogues necessary for incorporation into growing DNA.[17] The fact that FR overexpressing cells may also overexpress thymidylate kinase made the synthesis of a FR-targeted nucleoside system an intriguing idea since greater activity of the thymidylate kinase enzyme, combined with the greater selectivity and internalized transport facilitated by the overexpressed FR, could greatly improve the therapeutic index of AZT toward those cell lines.

We set out then to synthesize, purify, and assay new folate acid conjugates of AZT to compare their activity to unconjugated AZT. To achieve this we used long-chain poly(ethyleneglycol) (PEG) polymer linkers between the folic acid and AZT. PEGylation such as this has been shown to increase not just in vivo lifetimes (small molecular weight folates are rapidly cleared by the kidneys for example) but also can serve to prevent the cell from excreting the drug upon uptake.[18] Due to the problem of active γ-(versus inactive α-) isomer separation we also have developed a process of separating out the folate-PEG isomers produced upon conjugation necessary to test the activity of the final nucleoside conjugates. This separation has proved to be a tedious and problematic issue in the field with groups conducting multistep synthesis to overcome the problem or simply failing to separate out the isomers and using the regioisomer mix (obviously not ideal given the variable ratios of γ- to α-isomer that can be observed even from batch to batch and the effect this has on measuring accurate cytotoxicity and uptake).[19] The incorporation of such moderately polydisperse PEG units (approved for use by the FDA) makes this separation difficult, but given the desirability of having cheap and simple access to such γ-folate-PEG units suitable for further conjugation to the nucleoside or nucleotide of interest, such a separation would likely be of benefit to the field.

MATERIALS AND METHODS

Chemicals and Equipment

AZT was purchased from Toronto Research Chemicals, Toronto, Canada. FA, polyetheylene glycol (PEG), dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimde (NHS), N,N′-carbonyl-di-1,2,4-triazole (CDT), and sodium phosphate were purchased from Sigma as the highest purity available. Chromatography grade DMSO (Sigma, St. Louis, MO, USA) was dried through a column of molecular sieves (4 Å, Sigma) under dry dinitrogen. Water was distilled and deionized to 18.6 MΩ using a Barnstead Diamond RO Reverse Osmosis machine coupled to a Barnstead Nano Diamond ultrapurification machine (Barnstead, Dubuque, IA, USA). An Agilent (Santa Clara, CA, USA) 1100 HPLC with manual injection and automated fraction collector was fitted with a Zorbax C_{18} analytical column (4.6 (i.d) × 30 mm) to follow reactions and a semi-prep (10 (i.d) × 200 mm) column for purification. Ion-exchange chromatography (IEC) was conducted on a Low-pressure liquid chromatography (LPLC) machine (Akta Prime, GE, Piscataway, NJ, USA). Nuclear Magnetic Resonance (NMR) experiments were carried out on a Bruker (Billerica, MA, USA) 300 MHz machine. Solvent suppression and exponential transformation typically gave optimum results for NMR spectra containing PEG. Electronic absorption spectra were obtained using a Varian Cary 50 Bio UV-Vis spectrometer in 1 ml quartz cuvettes (Sigma) in a temperature chamber at 37°C regulated using a Peltier device. Electrospray mass spectrometry was performed on a Shimadzu LCMS-2010A system at a cone voltage of 5 kV. Infrared spectra were recorded on a Nicolet Magna-IR 850 Series II spectrophotometer as KBr pellets. Mass spectrometry was performed on an Applied Biosystems Voyager-DE MALDI-TOF mass spectrometer at SUNY ESF (Syracuse, NY, USA). Conditions were first elucidated using the commercial PEG molecule. In brief, samples were prepared by dissolving 10 mg of sample in chromatography grade acetonitrile (1 mL) and then mixed with a 10 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution in acetonitrile at 10:1, 5:1 and 1:1 matrix:analyte ratio. Elemental analysis was performed by QTI Inc.

(Whitehouse, NJ, USA). Due to the variable mass range of the commercial PEG compound component (Mr range 1900–2200 as observed by MALDI-TOF) however, elemental analyses were too varied to be useful. RPMI media (folate free) and Ham's media were purchased from the American type culture collection (ATCC). PeniStrep antibiotic cocktail and cell stripper were purchased from Sigma Aldrich. A Thermo Multiscan EX 96-well plate reader was used at a wavelength of 570 nm. Experiments were carried out in triplicate. The adriamycin resistant ovarian cell line A2780/AD is maintained at Rutgers University in the laboratory of Dr. Tamara Minko and in the lab of Dr. Robert Doyle (Donated by the Fox Chase Cancer Centre, Philadelphia, PA, USA). Chinese hamster ovary (CHO) cells were purchased from the American Type culture collection (Manassas, VA, USA).

Synthesis of α-,γ-FA-PEG-NH_2 1. Folic acid (0.392 g, 0.8 mmol), dicyclohexylcarbodiimide, (0.196 g, 0.9 mmol) and N-hydroxysuccinimde (0.112 g, 0.9 mmol) were dissolved in 40 ml of DMSO and stirred overnight in a dark room (15 W red light). The white precipitate (reaction by-product of dicyclohexyl urea) formed was removed by vacuum filtration. Bis-Amine PEG 2000 (200 mg, 0.1 mmol) was dissolved in 2 ml of DMSO and added to the now activated folic acid (10 ml, 0.1 mmol) and stirred for 6 hours under red light. **1** was purified by HPLC with a retention time of 12.56 minutes, yielding a yellow solid when dried in vacuo. Yield was 66% over the two steps based on PEG. ^{1}H NMR (D_2O): • 8.79 (s, 1H), 7.67 (d, 2H), 6.82 (d, 2H), 4.66 (s, 2H), 4.45 (m, 1H) 3.5-3.8 (PEG) 2.43 (m, 2H). Uv-Vis (H_2O): λ_{max} 280 nm (354 nm(sh)). MALDI-TOF MS *m/z* 2349 $[M + H]^+$.

Synthesis of γ-FA-PEG-AZT 2. AZT (10 mg, 0.04 mmol) was added to a solution of CDT in DMSO and stirred for 4 hours at room temperature. Compound **1** (91 mg, 0.04 mmol) was dissolved in 200 ml of DMSO and added to the solution. The reaction was then stirred overnight at room temperature under nitrogen. The solvent was removed in vacuo and the remaining yellow solid was redissolved in water. Compound **2** was isolated by HPLC with a retention time of 14.3 minutes. Yield is 40% based on **1**. ^{1}H NMR (DMSO): • 8.62 (s, 1H), 7.82–8.02 (b, 2H) 7.64 (m, 3H), 7.05 (b, 2H), 6.93 (b, 1H), 6.62 (d, 2H), 5.99 (t, 1) 4.48 (m, 2H), 4.47 (m, 1H) 3.5-3.8 (PEG), 2.92 (m, 2H) 1.33 (s, 3H). Uv-Vis (H_2O) λ_{max} 282 nm (352 nm (sh)) MALDI-TOF MS *m/z* centered at 2718 $[M + H]^+$.

Synthesis of 3-azido-3-deoxythymidine-5-hemisuccinate (HS-AZT) 3.[23] Succinic anhydride (10 mg, 0.1 mmol), AZT (11mg, 0.04 mmol), and 4-dimethlyamino pyridine (DMAP) were combined in 5 mL of DMSO and stirred for 3 hours. The solution was concentrated in vacuo and purified on an Akta Prime low pressure liquid chromatography machine with a 1 ml DEAE anion exchange column as follows: Buffer A: Water, Buffer B: 0.5 M NaCl. Conditions: 100% A for 5 column volumes at 1.0 ml/min then 50% A/50% B (1 ml/min) to elute the major band at a conductivity of ~2 mS/cm. The collected fractions were dried in vacuo. Yield was 67% based on AZT. ^{1}H NMR (D_2O): • 7,57 (s, 1H), 6.24 (t, 1H), 4.48 (q, 1H), 4.42 (d, 2H), 4.23 (q, 1H), 2.66 (t, 2H), 2.60 (t, 2H), 2.51 (m, 3H), 1.93 (s, 3H). ESMS (0.1% HCO_2H) 390 $[M + Na]^+$.

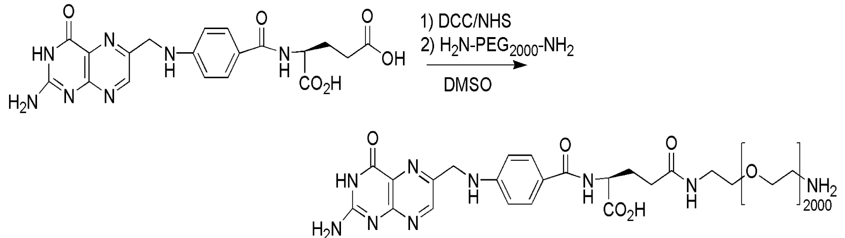
Synthesis of γ-FA-PEG-HS-AZT 4. **3** (0.04 mmol) was activated with DCC (10 mg, 0.048 mmol) and NHS (8 mg, 0.07 mmol) in DMSO over 4 hours. **1** (90 mg, 0.037 mmol) was dissolved in 2 ml of DMSO and then added drop wise to activated **3**. The resulting solution was stirred for 6 hours at room temperature. The yellow solution was then dried in vacuo and redissolved into water. This crude product was purified by HPLC with a retention time of 14.5 minutes and an overall yield of 40% (based on **1**). ^1H NMR (DMSO): • 8.55 (s, 1H), 7.91 (t, 1H), 7.65 (b, 2H), 7.5 (m, 3H), 6.82 (d, 2H), 6.74 (b, 1H), 6.21 (t, 1H), 4.42 (m, 2H), 4.24 (m, 1H) 3.5–3.8(PEG) 3.31 (s, 2H), 2.92 (m, 2H) 2.14 (m, 1H), 2.23 (s, 3H). Uv-Vis (H_2O): λ_{max} 278 nm (344 nm (sh)). MALDI-TOF MS: centered at 2775 m/z $[M+H]^+$. IR (KBR): ν 2112 (s) (azide), 1654 (br) (carbonyl group of hemisuccinate), 1102 (br) (PEG) cm^{-1}. The 5′-OH group of AZT at 3465 cm^{-1} is no longer observed.

HPLC purification. Compounds **1**, **2,** and **4** were initially purified by reverse phase gradient HPLC on a semi-prep C_{18} column (9.4 × 250 mm) with 5 mM sodium phosphate buffer (pH 7.0) run up to 10% acetonitrile over 5 minutes then up to 50% acetonitrile over an additional 12 minutes.

Ion-exchange chromatography. Separation of both isomer products (and removal of additional impurities) is described in the results and discussion section. Briefly, it was performed as follows: **1** was dried in vacuo and then redissolved in water to give a ∼ [50 mg ml^{-1}] concentration. Using a 1 ml ANX column (GE), samples ranging from 100–500 μL were loaded and the column washed with 5 column volumes of water at a flow rate of 0.2 ml/min. After the first peak was eluted the column was washed with 10% 0.1 M ammonium acetate for 5 column volumes, then increased to 30% ammonium acetate for an additional 5 column volumes. The column was then washed with 7 column volumes of 0.5 M NaCl. This procedure translates to semi-prep scale 20 mL ANX columns purifying over 275 mg per run.

Only the γ-isomer of **1** *was used in subsequent reactions.*

In vitro cytotoxicity (MTT) assays. The proliferation of logarithmic phase cultures of A2780/AD cells was assessed by calorimetric assay (MTT).[20] In brief, cells were plated onto 96-well microtiter plates at 10,000 cells per well. After a 24 hour incubation time to facilitate adherence, the folate free RPMI media was removed and replaced with 100 or 200 μl of fresh media containing different concentrations of AZT and compounds **2** and **4**. The AZT and conjugates **2** and **4** were dissolved in ethanol/water (3/1), filtered with a 0.22 μM filter, and diluted as appropriate with media. The cells were then incubated for 3 days at 37°C at 95% humidity and 5% CO_2. Formazan converted from MTT was dissolved with DMSO and measured at 570 nm with a plate reader. The percentage of cell viability was determined relative to untreated cells.



1

SCHEME 1 DCC/NHS coupling to give 1. Shown is the folate γ-isomer.

RESULTS AND DISCUSSION

Synthesis of Folate-PEG-NH_2 1. The synthesis of 1 is shown in Scheme 1. Coupling was achieved using the classic peptide bond forming DCC coupling reaction following the method of Park et al.[14c] No evidence of the rearrangement product (from the spontaneous rearrangement of the *O*-acylisourea to an inactive *N*-acylurea) was observed.

Separation of α- and γ-isomers of 1. RP-HPLC facilitated isolation of folate containing products but failed to give separation of both the α- and γ-isomer products (see Figure 3).

There was always sufficient overlap under all conditions tried to prevent our obtaining the desired γ-isomer by HPLC. This is a common problem in that field, further exacerbated by the use of polydisperse PEG units. The use of PEG, in moderate polydisperse form, is FDA approved and the use of these types of PEG units has been of increasing interest in nucleoside and nucleotide conjugates. The ability to separate out the active (γ) folate isomer of PEG conjugates in a straight forward and facile manner would be

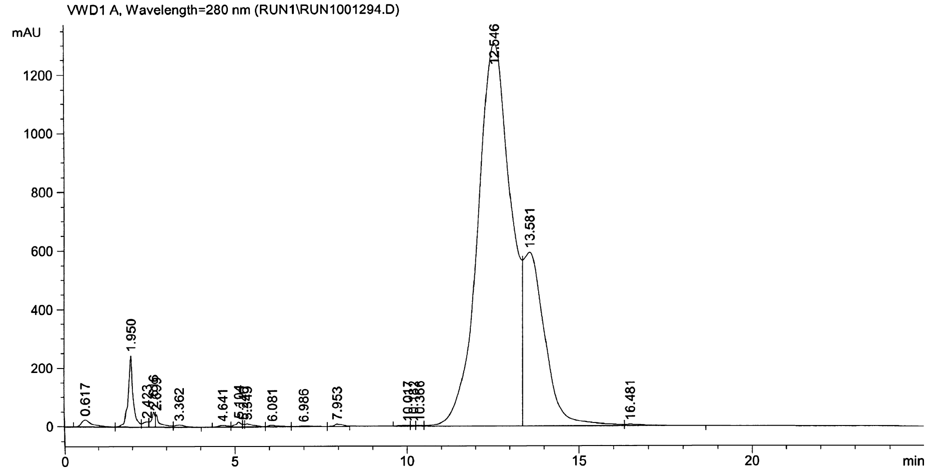


FIGURE 3 HPLC trace of 1 showing overlap of both folate isomer products (12.6 minutes = γ-isomer; 13.5 minutes α-isomer).

of great benefit therefore, and we decided to investigate this separation. We began with ion-exchange chromatography (IEC) using a low-pressure liquid chromatography device (Akta Prime, GE [21]) originally designed for peptide and protein purification inspired by the folate purification methods of Low et al.[18] Both weak (DEAE and ANX) and strong (Q-XL, Q-FF) anion-exchange columns were tested for isomer separation at pH 10 with different flow-rates and sample volumes. Before conducting anion-exchange, samples were desalted using 10 ml PD-10 G-25 sepharose columns. Typically, 2.5 mL of sample was loaded and the column washed with water with the yellow band containing **1** collected and used for IEC/LPLC. Analytically, 100–500 μL of sample was injected onto a 1 mL column with buffer A: water. Samples were eluted with a step-wise gradient of buffer B: 100 mM ammonium acetate (pH 10). [Gradient: A for 5 column volumes, then 10% B for 5 column volumes, followed by 30% B for 5 column volumes then 100% C buffer (0.5 M NaCl in water) for 7 column volumes]. Separation of both isomers from unreacted starting materials and bis-folate side-products was successful at flow-rates of 0.5 ml/min but yielded broad overlapping peaks containing both isomers for all resins tried. When the flow rate was reduced to 0.2 ml/min good separation was achieved using the ANX column (Figure 4). Interestingly, use of the DEAE resin, successful in the separation of other non-PEGylated folate conjugates, did not facilitate sufficient separation here. This is consistent with the known selectivity of ANX over similar resins such as DEAE, a result of the greater porosity of the ANX resin. This has made it more suitable to the separation of higher molecular weight compounds such as proteins for instance, or in this case, PEGylated systems.

The two isomers were identified by ^{1}H NMR, MALDI-TOF mass spectrometry and their characteristic electronic absorption spectra. Isomer

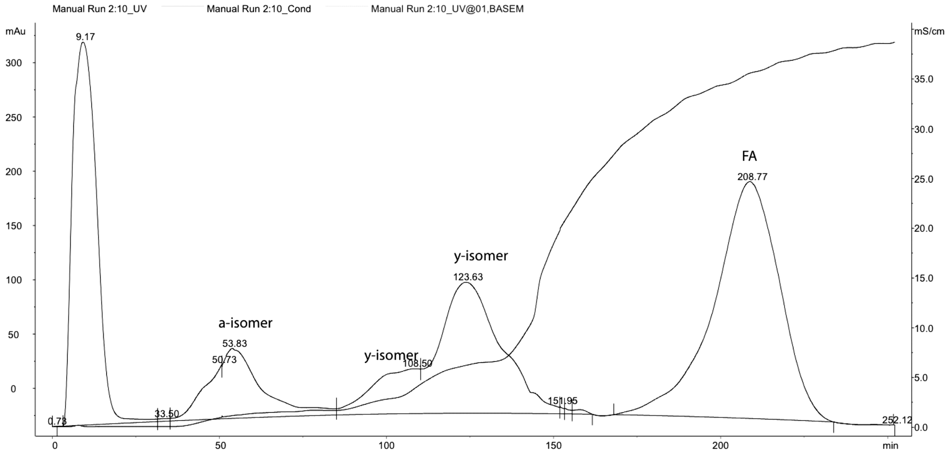
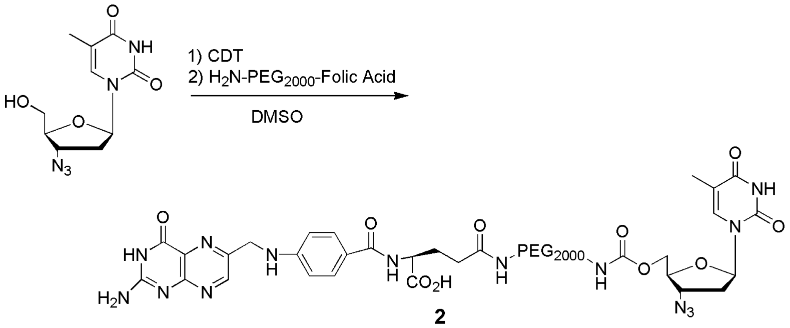


FIGURE 4 LPLC traces showing folate α- and γ-isomer separation using an ANX anion exchange column. FA is folic acid. Peak at ~50 minutes also contains trace gamma isomer.



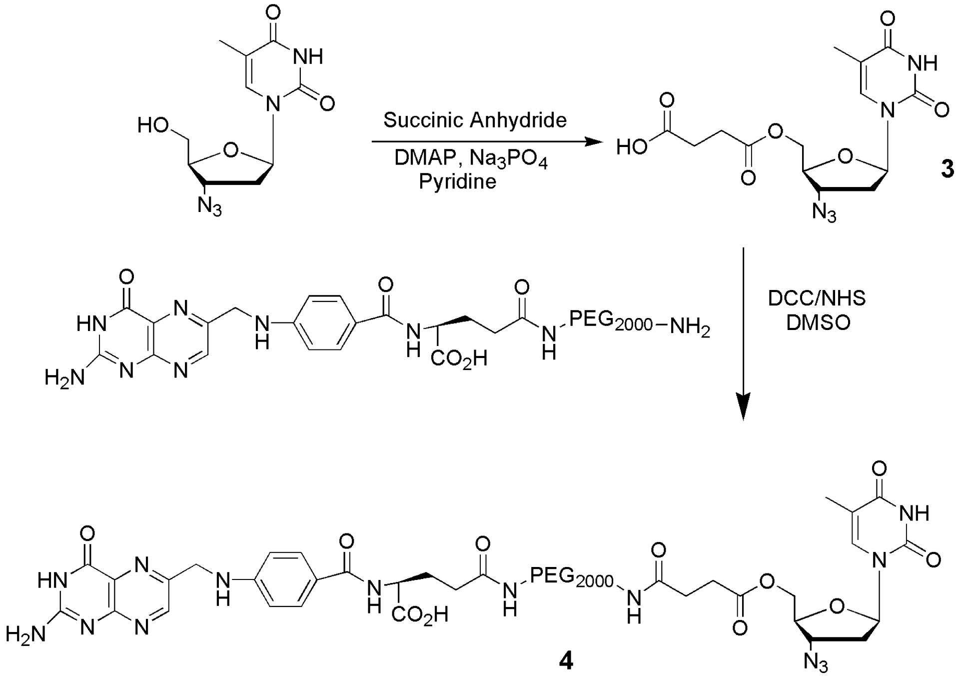
SCHEME 2 Synthesis of carbamate linked Folate-PEG-AZT **2**, shown as the γ-isomer.

assignment can be made using HPLC retention times and/or NMR as described by Sessler et al.[19b] The α-isomer elutes first on the LPLC, at lower conductivity (~0.5 mS/cm; C_{18} HPLC retention time (Tr) of 13.4 minutes) with the γ-isomer eluting at higher conductivity (~2 mS/cm; C_{18} HPLC retention time of 12.6 minutes) consistent with the pKa values of the uncoordinated carboxyl group on the folate conjugates (pKa for free α-~2.5 and for free γ-carboxyl ~4.5).[22] In all runs the peak that eluted at a conductivity of ~15 ms/cm was also collected and analytical C_{18} HPLC was performed and compared with the retention time of commercially obtained folic acid as an internal control (HPLC retention time of Tr = 5.2 minutes assigned as unreacted folic acid). This also was confirmed by NMR (see supplemental materials). This isomer separation approach has proven successful in our hands to date for folate-PEG conjugates containing PEG of molecular weights from 2,000 to 10,000 and allowed the purification of over 275 mg (5 mL sample volume [55 mg/mL]) of γ-folate-PEG in one run on a 20 mL ANX column. This makes it a practical and versatile route to isolating significant quantities of the desired PEGylated folate isomer quickly (typical run time is ~2 hours) and easily.

Synthesis of γ-folate-PEG-AZT conjugate 2. The synthesis of **2** is shown in Scheme 2. **1** was activated with CDT. Reaction proceeds in dry DMSO to produce the stable carbamate linker between the terminal PEG amine unit and the 5′-hydroxyl group of AZT. The reaction was carried out in non-aqueous solutions of DMSO and pyridine.

The structure of **2** was determined by ^1H NMR, MALDI-TOF MS and infrared spectroscopy. Mass spectrometry was conducted in CHCA matrix and gave indication of slight decomposition consistent with a folate-PEG unit under the conditions used (see supplementary material). We are currently attempting to synthesize a non-acidic ionic liquid matrix for use when such decomposition is observed.

Synthesis of γ-Folate-PEG-HS-AZT conjugate 4. The succinylated ester derivative of AZT was synthesized based on the method of Tadayoni et al. using DMAP coupling agent in phosphate buffer[23]. Purification was achieved



SCHEME 3 Synthesis of **4** shown as the γ-isomer. Coupling of succinate-AZT **3** to **1** was achieved in DMSO using DCC/NHS.

by ion-exchange chromatography using a DEAE sepharose column. Using succinic anhydride is a convenient route to allow the incorporation of a ‘spacer group’ (in this case a 4-carbon linker) between AZT and the PEG group. In addition to proving a carboxylic acid group for subsequent facile DCC/NHS coupling, the ester linkage between the SA and AZT may cleave under acidic conditions, such as occurs during receptor mediated endocytotic uptake in vivo. Thus we envisaged this providing a potential pH ‘trigger’ that may come in to play upon exposure to FR endosomal uptake pH of 5.5 or simply to prolonged exposure to cellular agents known to degrade ester bonds. The synthesis of HS-AZT **3** and subsequent coupling to **1** is shown in Scheme 3. Conjugate **4** was subsequently purified by RP-HPLC. Yield was 40% based on **1**.

Purified **1** and **3** were coupled using the DCC/NHS coupling system. Characterization of **4** was by mass spectrometry, ^{1}H NMR and infrared spectroscopy. The IR shows a shift in the AZT azide peak from 2084 cm^{-1} for uncoupled AZT to 2112 cm^{-1}, the carboxylate group at 1638 cm^{-1} (shifted from 1654 cm^{-1}) and the PEG peak at 1102 cm^{-1}.

In vitro biological activity. Table 1 shows the efficacy of conjugates **2** and **4** compared to free AZT and a **1** control (see also supplemental materials). Using an exponential fit, the IC_{50} values were calculated to be 410 μM for **2**

TABLE 1 IC_{50} values for **1**, **2,** and **4** versus AZT against A2780/AD ovarian cells and Chinese hamster ovary (CHO) cells

	A2780/AD	CHO
AZT	8 mM	8 mM
1	–	–
2	410 uM	–
4	380 uM	–

and 380 μM observed for **4**. This is in contrast to AZT with an IC_{50} of ~8 mM (~20 fold higher IC_{50}) under the same conditions. No toxicity was observed in either cell line for **1** up to concentrations of [10 mM].

In contrast, cytotoxicity assays of AZT and the conjugates against folate-receptor free (as confirmed by RT-PCR) Chinese hamster ovary (CHO) cells revealed a very different result. While AZT had an IC_{50} value of ~[8 mM], no IC_{50} value was obtained for either conjugate **1** or **2** over 72 hours at concentrations up to 10 mM. This control was conducted to demonstrate the involvement of FR-mediated cellular uptake.

CONCLUSIONS

Obviously the effectiveness and toxicity of any drug is the result of numerous factors such as stability, activation, transport and uptake. In the particular case of AZT, kinetics of incorporation work by Johnson et al.[3a] has clearly shown that the specific constant of incorporation of nucleoside analogs is correlated with their cellular toxicity. The results observed for conjugates **2** and **4** described herein may be rationalized then by increased uptake, in this case through FR mediated endocytosis. In addition, given efflux of nucleoside also plays a role in overcoming toxicity it stands that once inside the cell the conjugate maintains AZT's presence in the cell since such a large molecular weight molecule will not be readily effluxed. A role played by any overexpression of thymidylate kinase is not immediately evident here but given the success noted herein, and given ready access now to significant quantities of active folate-PEG isomer, further work in a variety of FR overexpressing cells, with a variety of additional nucleoside analogs, is currently underway.

Supplemental materials. Original MALDI-TOF, ^{1}H NMR, and electrospray mass spectra and typical cytotoxicity plots are provided. A comparative bar graph of cytotoxicity values is also included.

REFERENCES

1. Horwitz, J.P.; Chua, J.; Boel, M. Nucleosides. V. Monomethanesulfonates of 1-(2-deoxy-b -D-threo-pentofuranosyl) thymine. *J. Org. Chem.* **1964**, 29, 2076–2078.
2. Furman, P.A.; Fyfe, J.A.; St. Clair, M.H.; Weinhold, K.; Rideout, J.L.; Freeman, G.A.; Lehrman, S.N.; Bolognesi, D.P.; Broder, S.; Mitsuya, H.; Barry, D.W. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **1986**, 83, 8333–8337.
3. a) Lee, H.; Hanes, J.; Johnson, K.A. Toxicity of nucleoside used to treat AIDS and the selectivity of the mitochondrial DNA polymerase. *Biochemistry* **2003**, 42, 14711–14719; b) Hobbs, G.A.; Keilbaugh, S.A.; Rief, P.M.; Simpson, M.V. Cellular targets of 3′-azido-3′-deoxythymidine: an early (non-delayed) effect on oxidative phosphorylation. *Biochem. Pharm.* **1995**, 50, 381–390; c) Simons, C. *Nucleoside Mimetics: Their chemistry and Biological Properties.* Gordon and Breach Science Publishers, Amsterdam; 2001; d) Seeberger, P.H.; Caruthers, M.H. Modified oligonucleotides as antisense therapeutics. in *Applied Antisense Oligonucleotide Technology*, ed. C.A. Stein and A.M. Krieg, Wiley-Liss, New York; 1998, ch. 3.
4. a) Lynx, M.D.; Bentley A.T.; McKee, E.E. 3′-Azido-3′-deoxythymidine (AZT) inhibits thymidine phosphorylation in isolated rat liver mitochondria: A possible mechanism of AZT hepatotoxicity. *Biochem Pharmacol.* **2006**, 71, 1342–1348; b) Balzarini, J.; Herdewijn P.; De Clercq, E. Differential patterns of intracellular metabolism of 2′,3′-didehydro-2′,3′-dideoxythymidine and 3′-azido-2′,3′-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. *J. Biol. Chem.* **1989**, 264, 6127–6133; c) Olivero O.A.; Poirier, M.C. Preferential incorporation of 3′-azido-2′,3′-dideoxythymidine into telomeric DNA and Z-DNA-containing regions of Chinese hamster ovary cells. *Mol. Carcinogenesis* **1993**, 8, 81–88.
5. Pereira, L.F.; Oliviera, M.B.; Carnieri, E.G. Mitochondrial sensitivity to AZT. *Cell Biochem. Funct.* **1998**, 16, 173–181.
6. a) Perry, P.J.; Kellard, K.R. Telomeres and telomerase: targets for cancer chemotherapy? *Expert Opin. Ther. Patents* **1998**, 8, 1567–1586; b) Ji, H.J.; Rha, S.Y.; Jeung, H.C.; Tang, S.H.; An, S.W.; Chung, H.C. Cyclic induction of senescence with intermittent AZT treatment accelerates both apoptosis and telomere loss. *Breast Can. Res. Treat.* **2005**, 93, 227–236; c) Melana, S.M.; Holland, J.F.; Pogo, B.G.-T. Inhibition of cell growth and telomerase activity of breast cancer cells *in vitro* by AZT. *Clin. Cancer. Res.* **1998**, 4, 693–696.
7. Feng, J.Y.; Johnson, A.A.; Johnson, K.A.; Anderson, K.S. Insights into the molecular mechanism of meitochondrial toxicity by AIDS drugs. *J. Biol. Chem.* **2001**, 276, 23832–23837.
8. a) Becher, F.; Pruvost, A.G.; Schlemmer, D.D.; Creminon, C.A.; Goujard, C.M.; Delfraissy, J.F.; Benech, H.C.; Grassi, J.J. Significant levels of intracellular stavudine triphosphate are found in HIV-infected zidovudine-treated patients. *AIDS* **2003**, 17, 555–561; b) Benech, B.; Becher, F.; Pruvost, A.; Grassi, J.J. Is stavudine triphosphate a natural metabolite of zidovudine? *Antimicrob. Agents Chemother.* **2006**, 50, 2899–2901.
9. Abraham, N.G.; Bucher, D.; Niranjan, U.; Brown, A.C.; Lutton, J.D.; Distenfeld, A.; Ahmed, T.; Levere, R.D. Microenvironmental toxicity of azidothymidine: partial sparing with hemin. *Blood* **1989**, 74, 139–44.
10. Leamon C.P.; Low P.S. *Receptor-Mediated Drug Delivery. Drug Delivery Principles and Application.* John Wiley & Sons, Hoboken; 2005.
11. a) Diezel, W.; Weber, H.A.; Maciejewski, J.; Volk, H.D. The effect of splenopentin (DA SP-5) on in vitro myelopoiesis and on AZT-induced bone marrow toxicity. *Int. J. Immunopharm.* **1993**, 15, 269–273; b) Lutton, J.D.; Mathew, A.; Levere R.D.; Abraham, N.G. Role of heme metabolism in AZT-induced bone marrow toxicity. *Am. J. Hematology* **1990**, 35, 1–5; c) Sommadossi, J.P.; Carlisle R.; Zhou Z. Cellular pharmacology of 3′-azido-3′-deoxythymidine with evidence of incorporation into DNA of human bone marrow cells. *Mol. Pharmacol.* **1989,** 36, 9–14.
12. Calverley, D.; Haley, L.; Ballem, P. Red blood cell alloimmunization in transfused patients on AZT. *Brit. J. Haematology* **1991**, 78, 248–250.
13. a) Guo W.; Lee, R.L. Receptor-targeted gene delivery via folate-conjugated polyethylenimine. *AAPS PharmSci*, **1999,** 1, E19; b) Bagnato, J.D.; Eilers, A.L.; Horton, R.A.; Grissom, C.B. Synthesis and characterization of a cobalamin-colchicine conjugates as a novel tumor-targeted cytotoxin. *J. Org. Chem.* **2004,** 69, 8987–8996; c) Hogenkamp, H.P.C.; Collins, D.A.; West, F.G.; Grissom, C.B. The

pharmacological uses of cobalamin bioconjugates. In *B-12*, Banerjee, R., Ed., Wiley: New York; 1999, 385–410.

14. a) Gabizon, A.; Horowitz, A.T.; Goren, D.; Tzemach D.; Mandelbaum-Shavit, F.; Qazen M.M.; Zalipsky, S. Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: in vitro studies. *Bioconj. Chem.* **1999,** 10, 289–298; b) Low P.S.; Antony, A.C. Folate receptor-targeted drugs for cancer and inflammatory diseases. *Adv. Drug. Delivery Rev.* **2004,** 56, 1055–1058; c) Yoo, H.S.; Park, T.G. Folate receptor targeted biodegradable polymeric doxorubicin micelles. *J. Control. Release*, **2004**, 96, 273–283.
15. a) Boerman, O.C.; Niekerk, C.C.; Makkink, K.; Hanselaar, T.G.; Kenemans P.; Poels, L.G. Comparative immunohistochemical study of four monoclonal antibodies directed against ovarian carcinoma-associated antigens. *Int. J. Gynecol. Pathol.* **1991**, 10, 15–25; b) Weitman, S.D.; Lark, R.H.; Coney, L.R.; Fort, D.W.; Frasca, V.; Zurawski R.; Kamen, B.A. Distribution of the folate receptor GP38 in normal and malignant cell lines and tissues. *Cancer Res.*, **1992**, 52, 3396–3401; c) Ross, J.F.; Chaudhuri, P.K.; Ratnam, M. Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. physiologic and clinical implications. *Cancer* **2000**, 73, 2432–2443; d) Toffoli, G.; Cernigoi, C.; Russo, A.; Gallo, A.; Bagnoil M.; Boiocchi, M. Overexpression of folate binding protein in ovarian cancers. *Int. J. Cancer* **1997**, 74, 193–198.
16. a) Leamon, C.P.; Parker, M.A.; Vlahov, I.R.; Xu, L.; Reddy, J.A.; Vetzel, M.; Douglas, N. Synthesis and biological evaluation of ec20: a new folate-derived, ^{99m}Tc-based radiopharmaceutical. *Bioconj. Chem.* **2002**, 13, 1200–1210; b) Jayaram, H.N.; Zhang, Y.; Guo, L.; Roeske R.W.; Antony, A.C. Pteroyl-γ -glutamate-cysteine synthesis and its application in folate receptor-mediated cancer cell targeting using folate-tethered liposomes. *Anal. Biochem.* **2004,** 322, 168–177; c) Leamon, C.P.; Reddy, J.A.; Vlahov, I.R.; Vetzel, M.; Parker, N.; Nicoson, J.S.; Xu L.; Westrick, E. Synthesis and biological evaluation of EC72: a new folate-targeted chemotherapeutic. *Bioconj. Chem.* **2005**, 16, 803–811; d) Leamon, C.P.; Low, P.S. Delivery of macromolecules into living cells: a method that exploits folate receptor endocytosis. *Proc. Natl. Acad. Sci. USA* **1991**, 88, 5572–5576.
17. Sun, X.-L.; Jayaram, H.N.; Gharehbaghi, K.; Li, Q.-J.; Xiao, X.; Antony, A.C. Modulation of the cytotoxicity of 3′-azido-3′-deoxythymidine and methotrexate after transduction of folate receptor cDNA into human cervical carcinoma: identification of a correlation between folate receptor expression and thymidine kinase activity. *Cancer Res.* **1999**, 59, 940–946.
18. a) Wang, S.; Lee, R.J.; Mathias, C.J.; Green M.A.; Low, P.S. Synthesis, purification, and tumor cell uptake of 67Ga-deferoxamine-folate, a potential radiopharmaceutical for tumor imaging. *Bioconj. Chem.* **1996**, 7, 56–62; b) Liu, M.; Xu, W.L.; Zhong, G.; Che, S.; Lu, W. Synthesis and biological evaluation of diethylenetriamine pentaacetic acid-polyethylene glycol-folate: a new folate-derived, ^{99m}Tc-based radiopharmaceutical. *Bioconj. Chem.* **2005**, 16, 1126–1132.
19. a) Park, T.G.; Yoo, H.S. Folate-receptor targeted delivery of doxorubicin nano-aggregates stabilized by doxorubicin-peg-folate conjugate. *J. Contr. Release* **2004**, 100, 247–256; b) Wei, W.-H.; Fountain, M.; Magda, D.; Wang, Z.; Lecane, P.; Meslin, M.; Miles, D.; Sessler, J.L. Gadolinium texaphrin-methotrexate conjugates. Towards improved cancer themotherapeutic agents.*Org. Biomol. Chem.* **2005**, 3, 3290–3296.
20. Ciapetti, G.; Granchi, D.; Verri, E.; Savarino, L.; Cavedagna, D. Application of a combination of neutral red and amido black staining for rapid, reliable cytotoxicity testing of biomaterials. *Biomaterials* **1996**, 17, 1259–1264.
21. http://www4.gelifesciences.com/aptrix/upp00919.nsf/Content/LabSep_AKTA~design~Auto
22. Guo, W.; Hinkle, G.H.; Lee, R.J. ^{99m}Tc-HYNIC-Foate: A novel receptor-based targeted radiopharmaceutical for tumor imaging. *J. Nucl. Med.* **1999**, 40, 1563–1571.
23. a) Tadayoni, B.M.; Friden, P.M.; Walus, L.R.; Musso, G.F. Synthesis, in Vitro Kinetics, and in Vivo Studies of Protein Conjugates of AZT: Evaluation as a Transport System To Increase Brain Delivery. *Bioconj. Chem.* **1993**, 4, 139–145; b) Berezovskaya, Y.V.; Chudinov, M.V.; Yurkevich, A.M. Optimization of antiviral prodrug properties using combinatorial methods. *Nucleosides, Nucleotides Nucleic Acids* **2003**, 22, 837.
24. Arano, Y.; Wakisaka, K.; Ohmono, Y.; Uezono, T.; Akizawa, H.; Nakayama, M.; Sakahara, H.; Tanaka, C.; Konishi, J.; Yokoyama, A. Assessment of radiochemical design of antibodies using an ester bond as the metabolizable linkage: Evaluation of maleimidoethyl-3-(tri-n-butylstannyl)hippurate as a radioiodination reagent of antibodies for diagnostic and therapeutic applications. *Bioconj. Chem.* **1996**, 7, 628–637.