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Synthesis and Evaluation of Taxol–Folic Acid Conjugates as Targeted Antineoplastics†

Jae Wook Lee, June Y. Lu, P. S. Low and P. L. Fuchs*

Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA

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Abstract—A series of Taxol derivatives tethered at C2′ and C-7 to glutamate and folate have been synthesized for evaluation as prodrugs which release Taxol via hydrolytic lability of their α-alkoxy and α-amino esters. The half-time for hydrolysis of these materials was determined in pH 7 and pH 5 buffer. The in vitro cytotoxicity has been assessed in cell culture against A-549 lung cancer, MCF-7 breast cancer, and HT-29 colon cancer. Selected agents were further screened for folate binding and competitive binding with free folic acid. One agent (**54**), further evaluated in animal studies was found to increase the lifespan in mice, but was less effective than Taxol itself. © 2002 Elsevier Science Ltd. All rights reserved.

Introduction

Although Taxol (paclitaxel) has demonstrated some extremely encouraging clinical responses,[2] its poor water solubility causes formulation problems and manifests side effects due to the necessity of using cremophor EL (polyethoxylated castor oil) and ethanol as a vehicle. Advances in providing more soluble active derivatives and prodrugs have been recently reported.[3] From structure–activity relationships, it has been established that the 2′- and/or the 7-hydroxyl groups in Taxol are suitable for linking ester derivatives designed to both improve water solubility and ultimately foster the release of Taxol itself.[4]

The current cancer treatment protocols of surgery, radiation, hormone treatment,[5] autologous (self-donor) bone marrow transplant,[6] infusions of colony-stimulating factors,[7] and/or interferon[8] are routinely supplemented by an increasingly aggressive adjuvant program featuring high-dose multiple drug chemotherapy.[9] Details of the high-dose clinical trials have been published over the past 5 or 6 years.[10] Although exciting new drugs, such as Taxol will occasionally continue to be added to the pharmacopoeia, it seems likely that further progress will only occur in small increments in the absence of innovative new strategies.

The successes achieved with high-dose chemotherapy in the refractory metastatic patient group are driving oncologists toward application of highly aggressive treatment regimens at the earliest point of diagnosis. These protocols are limited by drug toxicity and severe physiological effects and patient fatalities are not uncommon. This situation has caused several members in the medical community to question whether the benefit/risk boundary has been exceeded with the agents currently available.[11] Clearly, enhancement of the differential specificity of anticancer agents by selective targeting mechanisms could potentially diminish such toxicity.

One low molecular weight ligand that may avoid many of the limitations associated with antibody-mediated targeting[12] is folic acid. Folic acid and its reduced counterparts enter cells via two unrelated pathways.[13] Covalent conjugation of folic acid to another molecule provides a construct that is strongly favored to enter cells via folate receptor mediated endocytosis. Based on this advantage, we are exploring the use of folic acid as a targeting agent for delivery of therapeutic drugs and imaging agents to tumors. As will be documented below, folate, when attached via its γ-carboxylate to any molecule, retains its ability to bind to its receptor with normal affinity and thereby enter receptor-bearing cell by endocytosis. In vitro and in vivo, the targeting is highly tumor specific with cell binding constants near 10^{-10} M. The number of molecules internalized can be very large ($>10^6$ per h), the pathway is nonharmful to the cell, and entry is via a nondegradative, nonlysosomal

*Corresponding author. Tel.: +1-765-494-5242; fax: +1-765-494-797; e-mail: pfuchs@purdue.edu

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pathway. Furthermore, folate is stable during storage, relatively easy to ligate to other molecules, non-immunogenic, and sufficiently small in size to not interfere with extravasation and intercellular diffusion.

In conjunction with our program directed at the design and synthesis of covalently-tethered folic acid drug conjugates for targeted delivery of chemotherapeutic drugs, we elected to initially prepare reagents for the efficient introduction of tri and tetraallyl esters of EDTA and DTPA mono carboxylic acids. In this paper we report the syntheses of Taxol–EDTA and Taxol–DTPA derivatives and determine their in vitro antineoplastic properties and their aqueous solubility. The latter property was held to be a prime determinant in selecting which agents to affix to folic acid for future testing as targeted anticancer agents.

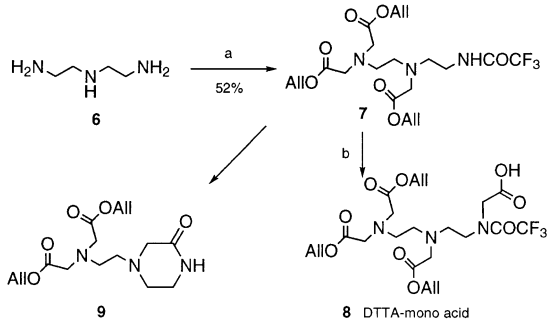
Synthesis of Folate-Linked Taxol Derivatives

As shown Scheme 1, compound **2** was prepared by mono protection of ethylene diamine **1** using $(Boc)_2O$, followed by bisalkylation of the residual amine moiety with allyl bromoacetate in a yield of 86%. Boc deprotection of compound **2** using HCl followed by dialkylation with excess *tert*-butyl bromoacetate gave compound **3** in 58% yield. Alkylation of **2** required the use of DMF and excess alkylating reagent to reduce the formation of piperazin-2-one compounds **5a** and **5b**. When acetonitrile was used as solvent with 2 equiv of *tert*-butyl bromoacetate, **5a** and **5b** were isolated in 20 and 50% yields, respectively. The yield of **5a** is partially reduced from losses in the aqueous wash solution. Fortunately, using DMF with 5 equiv of *tert*-butyl bromoacetate decreases formation of **5a** and **5b** to around 5–10%. The *tert*-butyl groups of compound **3** were removed with TFA and the resulting dicarboxylic acid was reacted with DCC to afford the anhydride intermediate, followed by quenching with allyl alcohol to give the desired EDTA mono acid **4** in 57% yield.

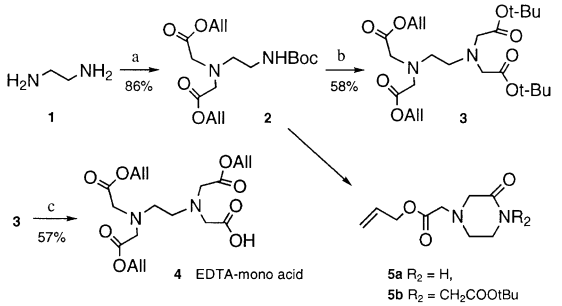
DTTA mono acid **8** was prepared by the trifluoroacetylation and trialkylation of diethylene triamine **6**, followed by alkylation of amide **7** with trimethylsilyl bromoacetate. As expected, deprotection of the trifluoroacetyl group of **7** using basic conditions such as hydrazine and potassium carbonate in allyl alcohol, the cyclization reaction occurred to give piperazinone **9** (Scheme 2).

Because manipulation of **7** was not especially efficient, we prepared DTPA-mono acid **12** employing the same strategy employed for EDTA-mono acid **4** (Scheme 3). Boc mono-protection of diethylene triamine **6** and subsequent trialkylation were carried out to afford compound **10** which was transformed to pentaester **11** by Boc deprotection and dialkylation with *tert*-butyl bromoacetate. When the alkylation reaction was attempted after deprotection of compound **10**, it was again found that DMF and excess alkylating agent were required to reduce the formation of piperazin-2-one derivatives **13a**,**b**. Removal of the *tert*-butyl groups of **11** with TFA and the reaction of the resulting dicarboxylic acid with DCC generated the anhydride intermediate, which was quenched with allyl alcohol to give DTPA-mono acid **12**.

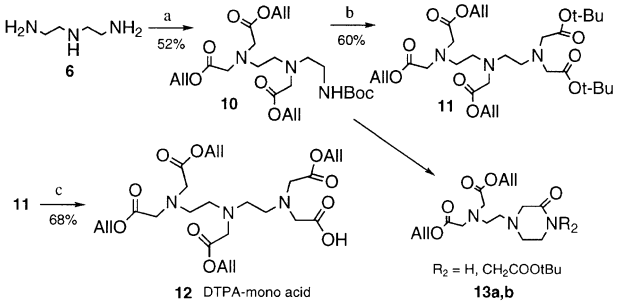
To achieve the preparation of C7-substituted derivatives of Taxol, it was necessary to first protect the more



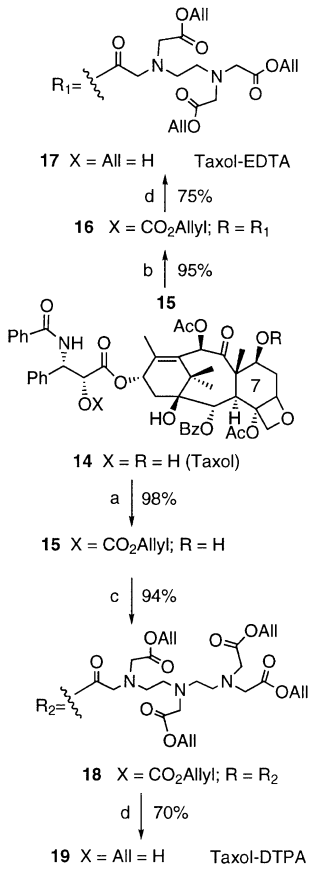
Scheme 2. Synthesis of DTTA mono acid. Reagents and conditions: (a) (i) ethyl trifluoroacetate, CH_2Cl_2, 2 h at 0 °C and then 5 h at 25 °C; (ii) allyl bromoacetate (3.3 equiv), DIEA, CH_3CN, 25 °C, 20 h, 52%; (b) NaH, DMF, trimethylsilyl bromoacetate, 25 °C, 12 h, 70% (98% BRSM).



Scheme 1. Synthesis of EDTA mono acid. Reagents and conditions: (a) (i) $(Boc)_2O$, MeOH, 2 h at 0 °C and then 2 h at 25 °C; (ii) allyl bromoacetate (2 equiv), DIEA, CH_3CN, 25 °C, 20 h, 86%; (b) (i) HCl, EtOAc, 25 °C, 10 h; (ii) *t*-butyl bromoacetate (>5 equiv), DIEA, DMF, 25 °C, 12 h, 58%; (c) (i) TFA, CH_2Cl_2, 25 °C, 10 h; (ii) DCC, CH_2Cl_2, 25 °C, 5 h; (iii) allyl alcohol, Et_3N, DMAP (cat.), 25 °C, 15 h, 57%.



Scheme 3. Synthesis of DTPA mono acid. Reagents and conditions: (a) (i) $(Boc)_2O$, MeOH, 2 h at 0 °C and then 10 h at 25 °C; (ii) allyl bromoacetate (3.3 equiv), DIEA, CH_3CN, 25 °C, 20 h, 52%; (b) (i) HCl, EtOAc, 25 °C, 10 h; (ii) *t*-butyl bromoacetate (>5 equiv), DIEA, DMF, 25 °C, 12 h, 60%; (c) (i) TFA, CH_2Cl_2, 25 °C, 10 h, (ii) DCC, CH_2Cl_2, 25 °C, 5 h; (iii) allyl alcohol, Et_3N, DMAP (cat.), 25 °C, 15 h, 68%.

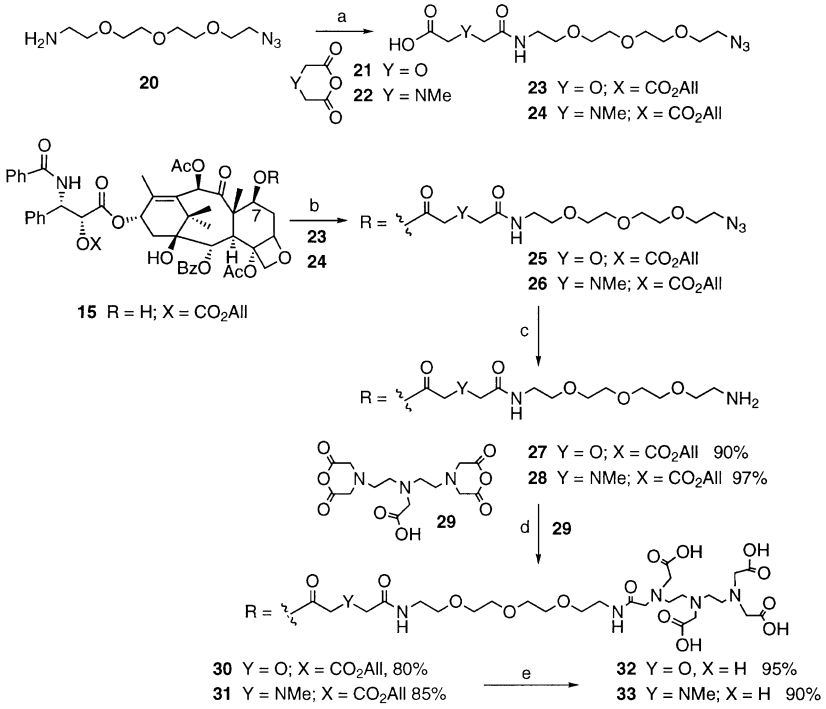


Scheme 4. Reagents and conditions: (a) allyl chioroformate, DIEA, CH_2Cl_2, 25 °C, 10 h, 98%; (b) EDTA mono acid **4**, DCC, DMAP, CH_2Cl_2, 25 °C, 10 h, 95%; (c) DTPA mono acid **12**, DCC, DMAP, CH_2Cl_2, 25 °C, 10 h, 94%; (d) $PhSiH_3$, $Pd(PPh_3)_4$, CH_2Cl_2, 25 °C, 1 h.

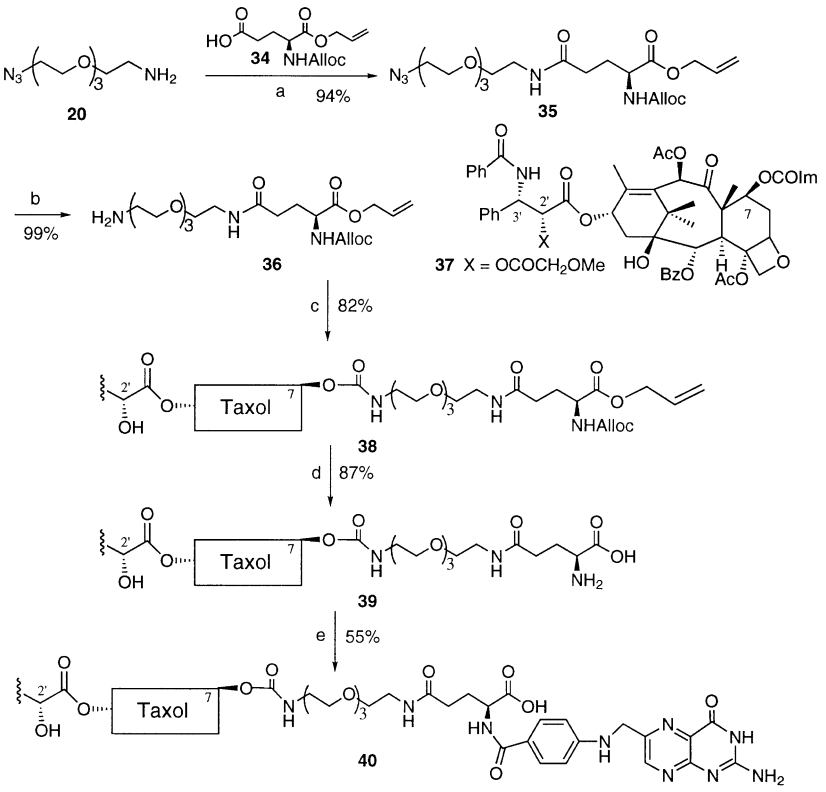
reactive C-2′ hydroxyl moiety. The alloc group was selected as protecting group for the 2′-OH of Taxol which was projected to be deprotected at the end of the synthesis using Pd [0] catalyzed cleavage of a collection of allyl esters. 2′-Alloc Taxol **15** was obtained by the reaction of Taxol **14** with allyl chloroformate in methylene chloride in the presence of DIEA in 98% yield (Scheme 4).

In order to evaluate the previously prepared polyaminocarboxylates as cleavable linkers, we undertook the reaction of 2′-alloc Taxol **15** with EDTA-mono acid **4** and DTPA-mono acid **12**. Reaction in methylene chloride in the presence of DCC with catalytic DMAP provided the fully protected Taxol–EDTA **16** and Taxol–DTPA **18** in yields of 94–95%. Deprotection of the entire collection of allyl groups was initially assessed with compound **16** using 10% $Pd(PPh_3)_4$ and Et_2NH (40 equiv)[14] in CH_2C1_2. Unfortunately, these conditions provided large amounts of transamidated materials resulting from the excess diethylamine. Substitution of $PhSiH_3$[15] (1–2 equiv) for the diethylamine afforded excellent results. Taxol–EDTA **17** and Taxol–DTPA **19** were smoothly obtained in 70 and 75% yield, respectively, from deprotection of compounds **16** and **18** using $Pd(PPh_3)_4$ and $PhSiH_3$ in CH_2Cl_2. The aqueous solubility of Taxol–EDTA **17** and Taxol–DTPA **19** was estimated using HPLC methodology[16] and was calculated to be 0.19 and 0.23 mg/mL, respectively.

In order to provide substantially increased water solubility and to anticipate the need for the hydrolytic release of the drug, we elected to investigate inductively



Scheme 5. Reagents and conditions: (a) anhydride **21** or **22**, CH_2Cl_2, 25 °C, 36 h; (b) PEG-carboxylic acid **23** or **24**, DCC, DMAP, CH_2Cl_2, 25 °C, 10 h; (c) $SnCl_2$, PhSH, Et_3N, CH_3CN, 25 °C, 30 min; (d) DTPA dianhydride **29**, DMSO, 25 °C, 3 h; (e) Et_2NH, $Pd(PPh_3)_4$, CH_2C1_2, 25 °C, 30 min.



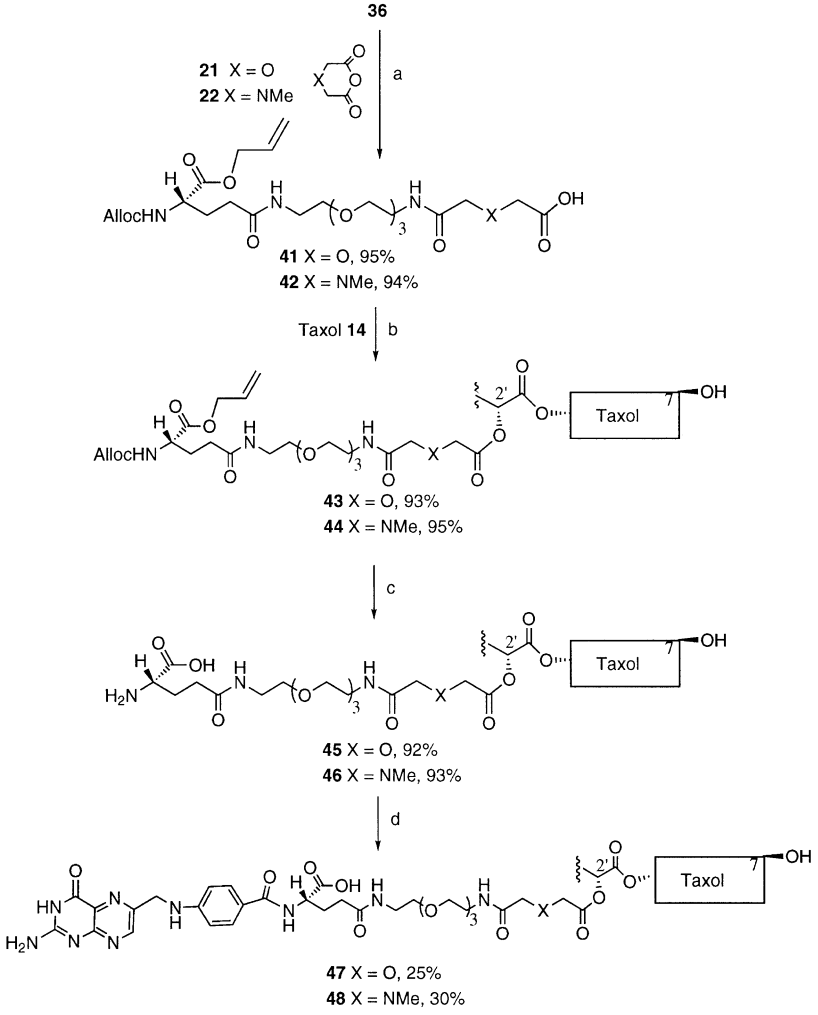
Scheme 6. Reagents and conditions: (a) DCC, DMAP, CH_2Cl_2, 15 h, 25 °C; (b) PPh_3, THF (H_2O), 20 h, 25 °C; (c) **36** (10 equiv), isopropanol, 5 h, reflux; (d) $Pd(PPh_3)_4$, CH_2Cl_2, 15 mm, 25 °C; (e) $PteN_3$, MTBD, DMSO, 2 h, 25 °C.

activated α-alkoxy and α-amino esters as the C-7 connecting function. We also added a short amine terminated PEG linker to improve the water solubility and facilitate amide bond formation. The easily prepared azido-PEG-amine **20**[17,18] was reacted with anhydrides **21** and **22** to provide PEG-carboxylic acids **23** and **24** in >98% yield. Coupling these acids with 2′-alloc Taxol **15** in the presence of DCC and DMAP afforded 2′-alloc Taxol–PEG azides **25** and **26** in >94% yield. Reduction of azides **25** and **26** was smoothly performed using $SnCl_2/PhSH/Et_3N$[18] to give primary amines **27** and **28** in 90 and 97% yields, respectively. These substrates were reacted with excess DTPA dianhydride **29** to afford 2′-alloc Taxol–PEG-DTPA esters **30** and **31** in 80 and 85% yield. Deprotection of the 2′-alloc groups on **30** and **31** using $Pd(PPh_3)_4$ and Et_2NH in CH_2Cl_2 yielded Taxol–PEG-DTPA polyacids **32** and **33** in 95 and 90% yields, respectively. The aqueous solubility of Taxol–PEG-DTPA **32** and **33** were determined to be 25 and 27 mg/mL, respectively (Scheme 5)

Based upon the above preliminary results, we decided to prepare C-7 linked conjugate **40** by coupling of the versatile (and water soluble) amino-PEG-azide **20**[17,18] with protected glutamic acid **34** in the presence of DCC and DMAP (cat.), followed by reduction of the azide moiety of **35** to afford **36** in an overall yield of 93%. Reaction of 2′-methoxyacetyl-protected-Taxol bearing an acylimidazole at C-7 **37**[19] requires a large excess of amino-PEG-glutamate **36** in anhydrous isopropyl alcohol at reflux, but delivers Taxol–7-carbamoyl-PEG-glutamate **38** in 82% yield with concomitant deprotection of the C2′ methoxyacetyl functionality. Taxol–7-carbamoyl-PEG-glutamic acid **39** was obtained from the deprotection of **38** using $Pd(PPh_3)_4$ and Et_2NH in CH_2Cl_2 in 87% yield. Taxol–7-carbamoyl-PEG-Folate **40** was generated in 55% yield by reaction of Taxol–7-carbamoyl-PEG-glutamic acid **39** and pteroyl azide[20] in DMSO in the presence of MTBD. Unfortunately, compounds **39** and **40** showed no activity (see Table 2) and the C-7 carbonate group was impervious to in situ hydrolysis such that Taxol was not released in the aqueous environment (see Table 1). While there are C-7 derivatives of Taxol that are active without release,[21] folate-tethered compound **40** is not among them (Table 2, Scheme 6).

In an effort to introduce linkers capable of releasing Taxol under the hydrolytic conditions, it was decided to employ ester groups as cleavable linkers at the C2′ and C7 alcohols of Taxol. Since the C-2′ methoxyacetyl moiety was readily hydrolyzed, it was decided to retain similar inductive activation in the tethers to be employed. Therefore, α-alkoxy and α-amino esters were utilized as the connecting function in anticipation of ultimate hydrolytic release of the drug itself.

Preparation of C2′-tethered Taxol–folates are detailed in Scheme 7. As a point of departure, amino-PEG-glutamate **36** was reacted with anhydrides **21** and **22** to provide glutamate-PEG-carboxylic acids **41** and **42** in 95 and 94% yields, respectively. Coupling these of



Scheme 7. Reagents and conditions: (a) DMAP (cat.), CH_2Cl_2, 36 h, 25 °C; (b) DIPC, DMAP (cat.), CH_2Cl_2, 12 h, 25 °C; (c) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, 1 h, 25 °C; (e) $PteN_3$, $(i\text{-Pr})_2NEt$, DMSO, 44 h, 25 °C.

Table 1. Hydrolysis rate of folates and glutamates at 37 °C

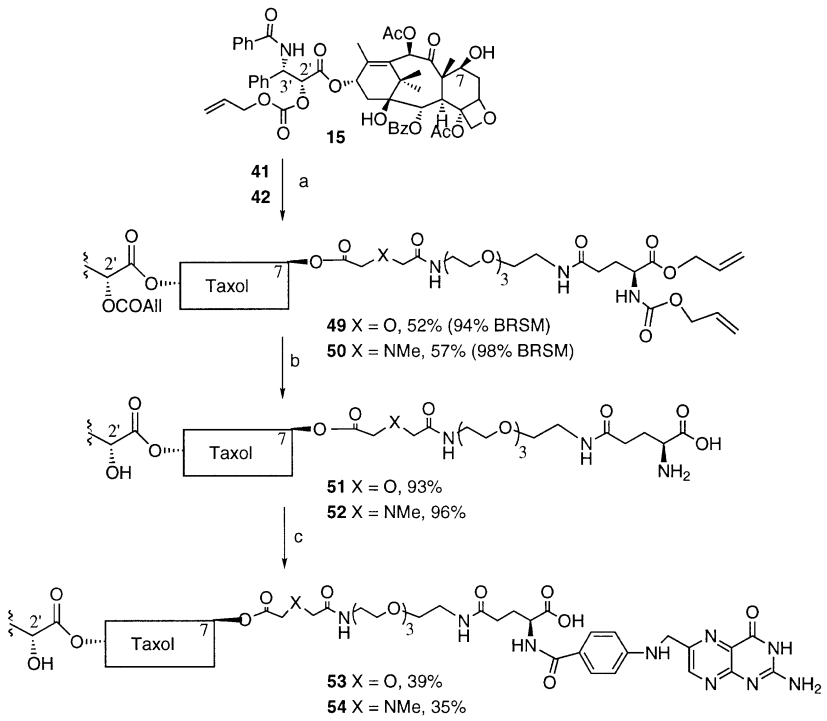
Compd	Compd type	Linker type	Linked at	$t_{1/2}$ at 37 °C	
				pH 7	pH 5
39	Glu	PEG-3	C-7	130 (none)	300 (none)
40	Fol	PEG-3	C-7	130 (none)	300 (none)
45	Glu	PEG-3 + O anyd	C-2′	0.8	19
46	Glu	PEG-3 + NMe anhyd	C-2′	3.3	20
47	Fol	PEG-3 + O anhyd	C-2′	1	17
48	Fol	PEG-3 + NMe anhyd	C-2′	9	38
51	Glu	PEG-3 + O anhyd	C-7	32	> 300
52	Glu	PEG-3 + NMe anhyd	C-7	38	60
53	Fol	PEG-3 + O anhyd	C-7	40	> 300
54	Fol	PEG-3 + NMe anhyd	C-7	109	197
61	Fol	Y PEG-3 azide	C-7	144	> 260
63	Fol	Y PEG-3 tetraacid	C-7	104	170

Substrate dissolved in phosphate buffer solution (pH 7 or pH 5) by the aid of DMSO was incubated at 37 °C and analyzed using HPLC.

glutamate-PEG-carboxylic acids with taxol **14** in the presence of DIPC and DMAP afforded Taxol–PEG glutamates **43** (93%) and **44** (95%). Universal deprotection[15] of **43** and **44** using $Pd(PPh_3)_4$ and $PhSiH_3$ in CH_2Cl_2 yielded Taxol–PEG-glutamic acids **45** and **46** in 92 and 93% yields, respectively. Taxol–2′-PEG-Folates **47** and **48** were obtained by the reaction of Taxol–PEG-glutamic acids **45** and **46** with pteroyl azide in DMSO in the presence of DIEA in 25 and 30% yields, respectively. The low yields in the coupling/purification sequence were partially a reflection of the desired lability.

As a complement and comparison to the C2′ derivatives, it was deemed prudent to also prepare the corresponding C-7 folate derivatives (Scheme 8). Preparation of C7-substitued derivatives of Taxol required blocking the more reactive C-2′ hydroxyl moiety. The selected alloc group was projected to be deprotected at the end of the synthesis during Pd(0) catalyzed cleavage of all allyl esters.

In order to further evaluate glutamate-PEG-carboxylic acids **41** and **42** as cleavable linkers, we undertook the reaction of 2′-alloc Taxol **15** (Scheme 4) with glutamate-PEG-carboxylic acids **41** and **42** in methylene chloride in the presence of DIPC with catalytic DMAP. This provided 2′-alloc Taxol–7-PEG glutamates **49** and **50** in 52% (94% based upon recovered starting material) and 57% (98% BRSM) yields, respectively (Scheme 8). Deprotection of the entire collection of allyl groups of



Scheme 8. Reagents and conditions: (a) DIPC, DMAP (cat.), CH_2Cl_2, 12 h, 25 °C; (b) $Pd(PPh_3)_4$, Et_2NH, CH_2C1_2, 1 h, 25 °C; (c) $PteN_3$, *i*-Pr_2NEt, DMSO, 44 h, 25 °C.

Table 2. In vitro cell culture ED_{50} (μg/mL) of selected compounds

Compd	A-549 lung	MCF-7 breast	HT-29 colon
15	5.5×10^{-2}	6.6	3.2×10^{-2}
17	1.3×10^{-4}	8.2×10^{-1}	1.8×10^{-3}
19	9.3×10^{-1}	30	24
32	6.5×10^{-2}	1.6	4.1×10^{-2}
33	1.9×10^{-1}	10	4.3×10^{-1}
39	>100,000	>100,000	>100,000
40	>100,000	>100,000	>100,000
45	1.7×10^{-3}	7.5×10^{-3}	1.3×10^{-3}
46	1.5×10^{-3}	9.0×10^{-3}	1.1×10^{-3}
47	1.6×10^{-3}	3.1×10^{-3}	1.2×10^{-3}
48	1.2×10^{-3}	2.4x 10^{-3}	1.0×10^{-3}
51	1.4×10^{-3}	4.9×10^{-3}	1.2×10^{-3}
52	2.5×10^{-3}	8.0×10^{-3}	3.3×10^{-3}
53	1.7×10^{-3}	3.3×10^{-3}	2.9×10^{-3}
54	1.6×10^{-3}	2.6×10^{-3}	1.1×10^{-3}
61	6.5×10^{-2}	1.6	4.0×10^{-2}
63[a]	120	930	110
14	1.4×10^{-2}	7.0×10^{-2}	8.0×10^{-2}
(Taxol)[b]	$\pm2.0\times10^{-2}$	$\pm5.0\times10^{-2}$	$\pm3.0\times10^{-2}$

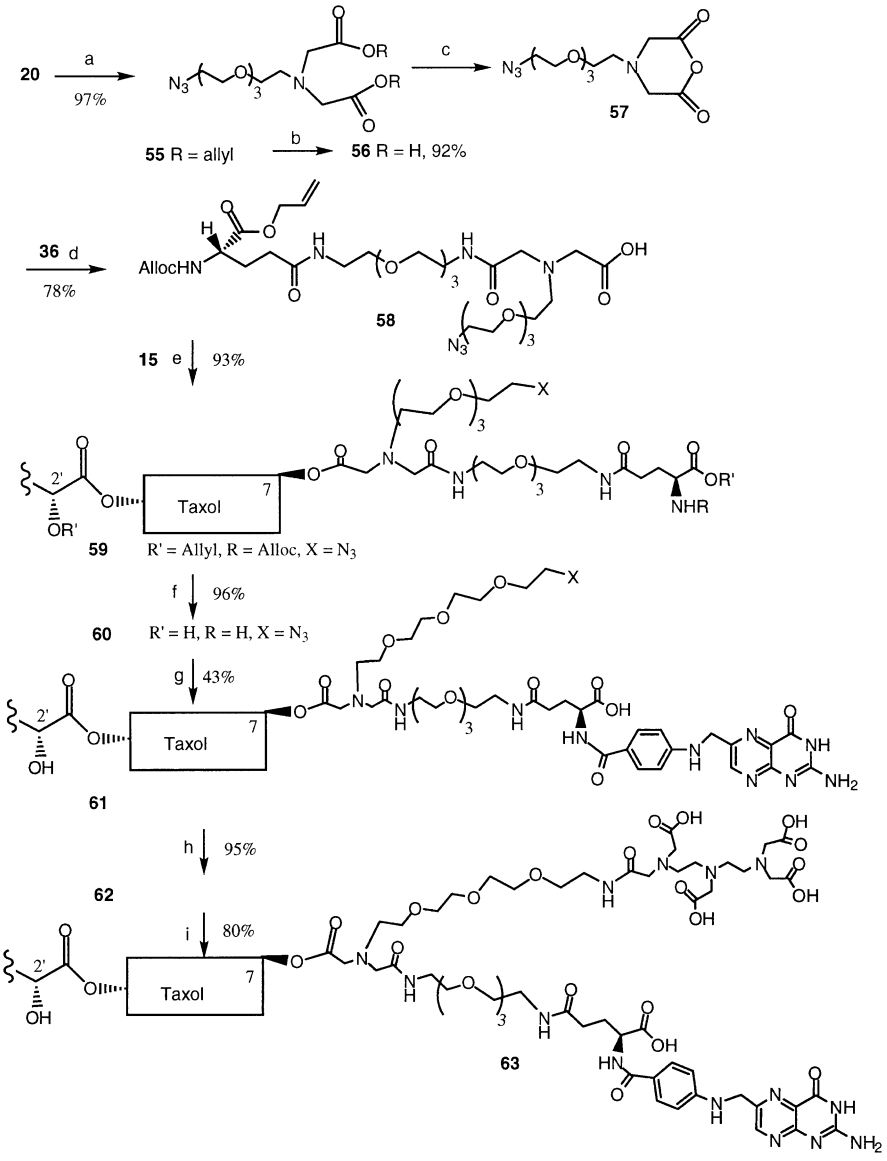
Determined by the Purdue Cancer Center Cell Culture Laboratory.
[a]Average of three independent runs.
[b]Average of six independent runs.

49 and **50** using $Pd(PPh_3)_4$ and Et_2NH in CH_2Cl_2 yielded Taxol–7-PEG-glutamic acids **51** and **52** in 93 and 96% yields, respectively. Taxol–7-PEG-Folates **53** and **54** were obtained by the reaction of Taxol–7-PEG-glutamic acids **51** and **52** with pteroyl azide in DMSO in the presence of DIEA in 39 and 35% yields, respectively.

An additional set of constructs bearing a short, amine-terminated PEG linker was prepared with a view toward with increased water solubility and facilitated amide bond formation with DTPA dianhydride. This series involved synthesis of a Y-shaped intermediate connected with Taxol, folate, and the polycarboxylic acid DTPA. For this purpose, the triply linked system azido-PEG-iminodiacetic acid anhydride **57** was prepared (Scheme 9). Azido-PEG-amine **20** was reacted with allyl bromoacetate in acetonitrile in the presence of K_2CO_3 to give iminodiallylester **55** which was subsequently deprotected using $Pd(PPh_3)_4$ and $PhSiH_3$ in CH_2Cl_2 to afford azido-PEG-iminodiacetic acid **56** in 92% yield. Azido-PEG-iminodiacetic acid **56** was initially treated with DCC at 25 °C to generate anhydride **57**. Addition of amino-PEG-glutamate **36** to preformed anhydride **57** followed by reaction for an additional 12 h at 25 °C smoothly provided monoacid **58**. Reaction of 2′-alloc Taxol **15** with carboxylic acid **58** in the presence of DCC and a catalytic amount of DMAP in methylene chloride for 12 h at 25 °C afforded Y-shaped intermediate **59** in 95% yield after purification. Global deprotection of the allyl groups of glutamate **59** using $Pd(PPh_3)_4$ and Et_2NH in CH_2Cl_2 generated Y-shaped-glutamic acid **60** in 96% yield. Y-Shaped-folate **61** was obtained in 43% yield by the reaction of **60** with pteroyl azide in DMSO in the presence of MTBD. Reduction of azide **61** was effectively performed with $SnCl_2$/PhSH/Et_3N in DMF to give amine **62** in 95% yield. Installation of the poly-acid functionality was accomplished by treatment of **62** with 7 equiv of DTPA dianhydride in DMSO for 3 h at 25 °C. Purification by MPLC followed by lyopholization afforded **63** as a yellow solid in 80% yield.

Hydrolysis and In Vitro Biological Activity

The Taxol–folate conjugates and their glutamate precursors were surveyed for hydrolysis at 37 °C both at pH 7 and pH 5 (Table 1). As can be seen in the table,



Scheme 9. Reagents and conditions: (a) allyl bromoacetate (2.2 equiv), K_2CO_3, CH_3CN, 24 h, 25 °C; (b) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, 1 h, 25 °C; (c) DCC, CH_2Cl_2, 5 h, 25 °C; (d) **36**, CH_2Cl_2, 12 h, 25 °C; (e) **15**, DCC, DMAP (cat.), CH_2Cl_2, 12 h, 25 °C; (f) $Pd(PPh_3)_4$, Et_2NH, CH_2Cl_2, 1 h, 25 °C; (g) $PteN_3$, MTBD, DMSO, 2 h, 25 °C; (h) $SnCl_2$, PhSH, Et_3N, DMF, 1 h, 25 °C; (i) excess DTPA dianhydride **62**, 3 h, 25 °C.

attachment of glutamate (**39**) or folate (**40**) at C-7 via a urethane moiety gives derivatives that, as expected, do not suffer hydrolysis even after extended reaction times. Hydrolytically stable, C-7 functionalized Taxol derivatives have been shown to retain high bioactivity,[21] as exemplified by simple tri and tetracarboxylic acids **17** and **19**, yet both glutamate (**39**) and folate (**40**) are essentially inactive (Table 2).

We next turned to C-2′ for attachment of inductively-activated α-alkoxy and α-methylamino esters **45**–**48** (Scheme 7). While these materials underwent facile hydrolysis (Table 1) and exhibited high in vitro activity (Table 2), the very ease of hydrolysis severely detracted from our ability to isolate acceptable yields of folates **47** and **48** from the DMSO reaction mixture.

Although C-2′ study did not generate any useful drug species, we were pleased with the PEG-3 glutamate-α-alkoxy and α-methylamino reagents **41**, **42** which were prepared in the course of our investigation. Simply attachment of these materials to C-2′ alloc-protected Taxol **15** followed by standard processing (Scheme 8) provided the C-7 tethered glutamates and folates **51**–**54** in sufficient quantities for extensive testing. By this time, we had developed our intuition to the point that we expected folates **53** and **54** to only have importance as structural controls for Y-shaped folate-tetracarboxylic acid **63** (Scheme 9).

Therefore, it came as a surprise that Y-shaped folate-tetracarboxylic acid **63** (three separate runs) was about 105 less active than I-shaped folates **53** and **54**, which do not bear the pendant tetracarboxylic acid (Table 2). It should be noted that Y-shaped folate PEG-3 azide **61** (the precursor of **63**) is far more active than **63**, but still ~50× less active than **53** and **54**. Faced with data that did not reveal any clear trends, we elected to deepen our

testing environment in an attempt to define whether the folate was selectively targeting any of the agents to the tumor site.

Selection of an Agent for an In Vivo Trial

Based upon our initial rationale in conjunction with the hydrolysis and cell culture studies (Tables 1 and 2), we elected to compare the abilities of four C-7 linked folates (**53**, **54**, **61**, and **63**) with that of free folic acid to displace ^{3}H folic acid from cell surface folate receptors in a competitive binding study. In comparison to non-radioactive folate in receptor-positive murine M109 tumor cells, the target folate tetraacid **63** and its truncated *N*-methyl analogue **54** (lacking the PEG-3 spacer and the tetraacid) were far superior to **61** (the azide precursor to **63**) in their relative affinities for the folate receptor (Fig. 1A). It was also interesting to note that replacement of the *N*-methyl unit in **54** with an oxygen atom (**53**) decreased the ability to displace folic acid by at least an order of magnitude. Similarly, in another folate receptor-positive human KB tumor cell line, **54** required a $\sim$4$\times$ greater concentration than free folate to achieve a similar degree of competition with ^{3}H folic acid for receptor binding, indicating that **54** retained most of the native affinity of folic acid for the folate receptor (Fig. 1B).

Although both **63** and **54** demonstrated their strong ability to compete with ^{3}H folic acid for receptor binding (Fig. 1A), previous in vitro bioanalysis yielded a high ED_{50} value ($1–10\times10^{-4}$) with **63** in all cell lines tested (Table 2). Because of this apparently poor cytotoxicity, a large quantity of **63** would be required for animal trials. We therefore chose to first evaluate the 'control' analogue **54** prior to proceeding further with **63**. To look for any receptor-mediated specific cytotoxicity, in vitro comparison of **54** with Taxol **14** was undertaken in the folate-receptor positive human KB tumor cell line. At equivalent molar concentrations, Taxol **14** was 50-fold more toxic than **54** (Fig. 2). A concurrent competition experiment with **54** plus 500-fold molar excess of free folic acid did not decrease the toxicity of **54**, suggesting that the folate receptor was not responsible for the cellular entry of **54**.

Comparison of Taxol **14** and **54** at a constant drug concentration (2×10^{-7} M) was next undertaken in three tumor cell lines (KB, M109, A549). The first two lines express high levels of the folate receptor.[22] The A549 cell line, however, expresses negligible levels of functional folate receptors.[23] With the exception of Taxol **14** being uniformly more potent, little difference was seen with respect to folate receptor involvement (Fig. 3). Therefore, there is no evidence of selective cytotoxicity of **54** towards folate receptor positive tumor cell lines in vitro.

The first note of encouragement came from a comparative general toxicity study in healthy tumor-free mice. At an equivalent Taxol **14** dosage of 25 mg/kg/day ($\sim$46 mg/kg/day for **54**), Taxol-treated mice experienced a maximal body weight loss of 20%, while no weight loss was detected in mice treated with prodrug **54**

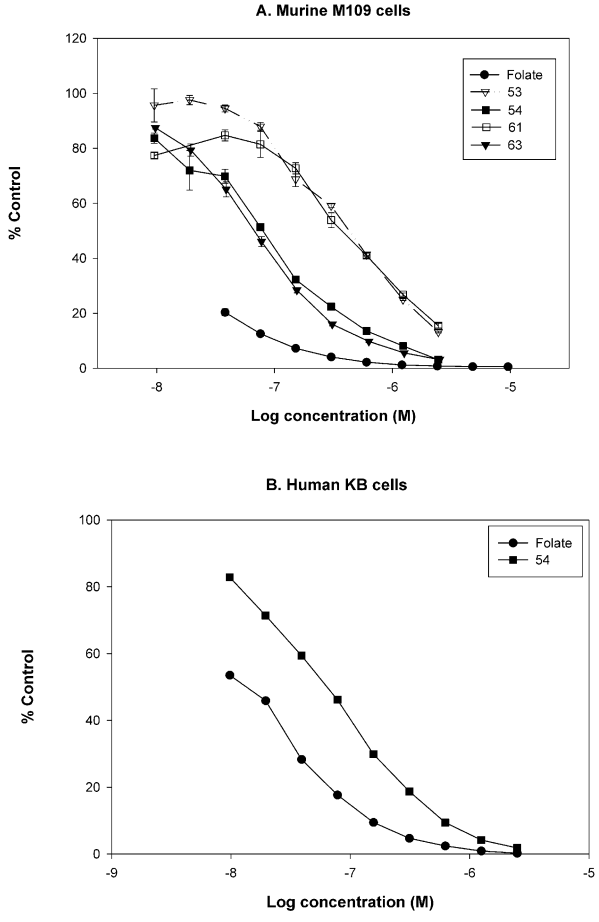


Figure 1. Comparison of the abilities of Taxol–folate conjugates and folate to inhibit ^{3}H folic acid binding to cell-surface folate receptors. Murine M109 (A) or human KB (B) cells were incubated at 4 °C for 1 h in FEMEM containing 10 Nm ^{3}H folic acid and various concentrations of **53** (▽), **54** (■), **61** (□), **63** (▼), or folic acid (●). After thorough washing, cell-associated radioactive folates were stripped by acid saline and measured with a liquid scintillation counter.

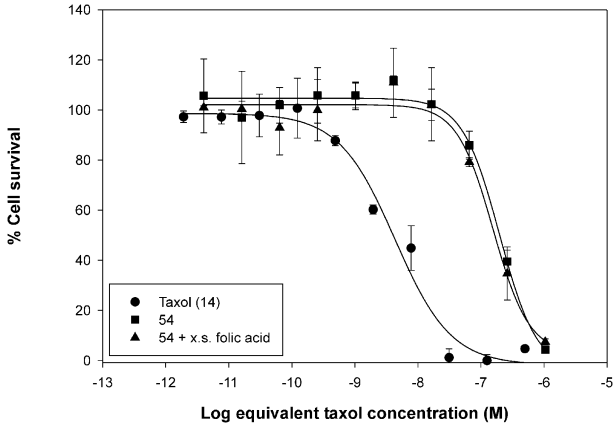


Figure 2. Cytotoxity of **54** and Taxol **14** in folate receptor positive KB tumor cell line. Cells were incubated with Taxol (●), **54** (■), or **54** plus $\sim$500$\times$ molar excess of free folic acid (▲) for 18 h, washed, and incubated further in fresh medium. The percentage of cell survival compared to untreated control 24 h later was then determined. Data are presented as the mean $\pm$ SD of three independent measurements.

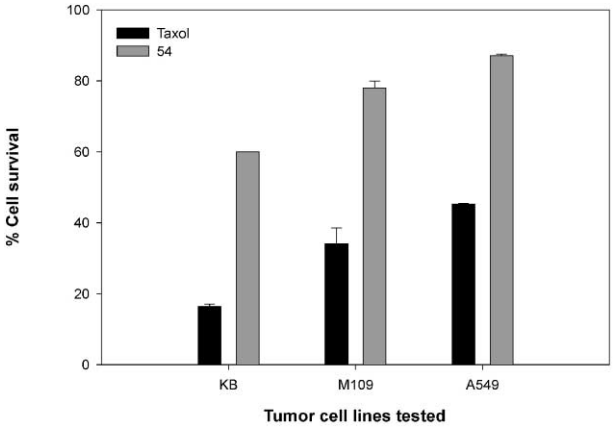


Figure 3. Comparison of the cytotoxicity of **54** and Taxol **14** towards various tumor cell lines. Receptor-positive KB or M109, and receptor-negative A549 cells were incubated with Taxol and **54** at 2×10^{-7} M for 18 h, washed, and incubated further in fresh medium. The percentage of cell survival compared to untreated control cells at confluence was then determined. Data are presented as the mean±SD of three independent measurements.

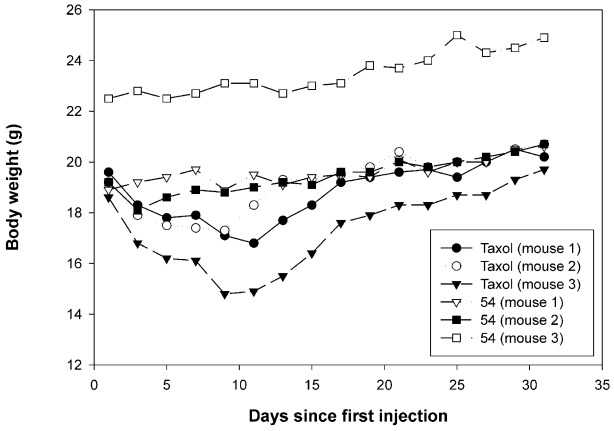


Figure 4. Comparison of the toxicity of **54** and Taxol **14** in nontumor bearing mice. Female Balb/c mice were given four ip injections of 25 mg/kg/day Taxol **14** (●, ▼, ■) or 46 mg/kg/day **54** (○, ▽, □) at 48-h intervals. The mice were weighed every other day to record their body weight changes.

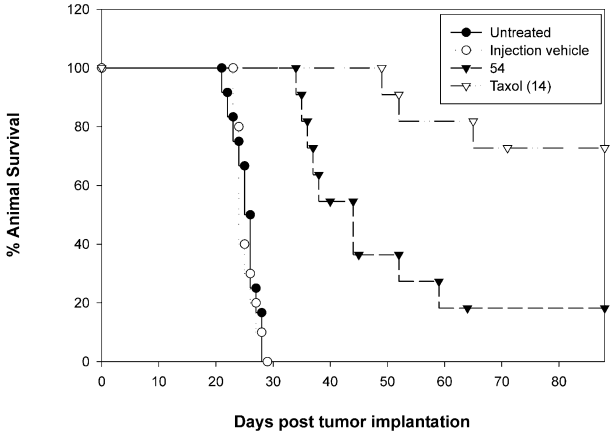


Figure 5. Antitumor effect of **54** and Taxol **14** in tumor-bearing mice. Balb/c mice were inoculated ip with 5×10^5 M109 cells on day 0. Drugs were administered from day 4 according to the schedule of q2d×8 for untreated (●), injection vehicule (○), Taxol **14** at 17.2 mg/kg/day (▽), and **54** at 32.4 mg/kg/day (▼). Each group consists of at least 10 mice.

(Fig. 4). Therefore, **54** is far less toxic than the parent drug Taxol.

Comparative Antitumor Activity In Vivo

While **54** was far from an ideal candidate, we decided to proceed further in vivo to compare the antitumor activity of **54** with Taxol **14** in tumor-bearing mice, since in vitro tests for comparing drug cytotoxicity may not always predict in vivo therapeutic efficacy due to differences in drug stability, biodistribution and metabolism, as well as tumor microenvironment. Mice implanted ip with folate receptor positive M109 tumors were treated twice a day for 8 days with equimolar amounts of Taxol **14** and **54** starting on day 4 post tumor implantation. While the control mice and the mice treated with the injection vehicle survived approximately 26 days, Taxol **14** and prodrug **54** caused >177 and 73% increase in life span and yielded cure rates of 73 and 18%, respectively, at an equivalent Taxol **14** dose of 17.2 mg/kg/day (~32 mg/kg/day for **54**) (Fig. 5).

Conclusion

Our effort to enhance the pharmacologic efficacy of Taxol through covalently linking the drug to a tumor-targeting folate ligand was not successful in the form of **54**. Although the Taxol–folate conjugate **54** retained most of the receptor binding affinity of the folate ligand and was far less toxic than Taxol in normal mice, it failed to demonstrate selective killing of folate receptor-expressing tumor cells in vitro or enhanced in vivo antitumor activity over Taxol when administered in an equimolar quantity formulated in the same injection vehicle.

There are a number of possible reasons to explain the ineffectiveness of **54** in treating folate receptor-positive tumors. First of all, **54** still has limited water solubility despite a ~20× (molar ratio) improvement over Taxol. The low water solubility prevented the administration of a higher, more effective dose of **54** in tumor-bearing mice. Secondly, both **54** and Taxol are large hydrophobic molecules that probably enter cells non-specifically. Thus, the attachment of folate via a short ester linker may not be enough to prevent nonspecific binding and uptake in a receptor-independent manner. Thirdly, the ester linkage was designed to hydrolyze in vivo. Based on hydrolysis data at 37 °C, **54** has a slower hydrolysis rate ($t1/2=197$ h) at an acidic pH (pH 5) than the hydrolysis rate ($t1/2=109$ h) at neutral pH. Upon binding of **54** to the cell surface receptor, it will likely be internalized into endosomes where the pH value is around 5.[24] This acidic pH would certainly slow down the hydrolysis of **54** to free Taxol inside cells. However, once Taxol is released, the drug should have no problem of diffusing out of endosomes and binding to tubulin to exert its cytotoxic effect.

The future of targeting Taxol to folate receptor-positive tumor types via the folate ligand may require the design

of new water-soluble linkers that are stable in the blood, but hydrolyze quickly upon entering acidic endosomal compartments to allow release of the cytotoxic parent drug. The water solubility of any new folate–Taxol derivative should also be high enough to avoid any need for drug-solubilizing agents, so that an optimal drug dosage may be achieved in vivo. Most importantly, in order to achieve selective toxicity against receptor-positive tumors, the mechanism of drug entry should be governed mainly by binding of the folate moiety to cell surface receptors and not by simple membrane diffusion.

Experimental

General methods

Unless otherwise stated, reactions were carried out under argon in flame-dried glassware. Flash chromatography on silica gel was carried out as described by Still (230–400 mesh silica gel was used), and reversed-phase LC was used for preparative purposes (LiChroprep C-18, 310×25 mm). ^{1}H and ^{13}C NMR spectra were obtained using GE QE-300 NMR and Varian Gemini 200 NMR spectrometers at 300 or 200 MHz and 75 or 50 MHz respectively. Mass spectral data were obtained on a Finnigan 4000 mass spectrometer (low resolution) and a CEC 21 110 B high-resolution mass spectrometer, with the molecular ion designated as M. All the chemicals were supplied by Aldrich Chemical Co., Inc, Milwaukee, WI, USA, unless otherwise indicated.

Preparation of *N*-Boc-ethylenediamine allyl ester (2). $(Boc)_2O$ (2.2 g, 10 mmol) in 20 mL of MeOH was added dropwise to a solution of ethylenediamine **1** (6 g, 100 mmol) in 80 mL of MeOH at 0 °C and the resulting solution was stirred for 2 h at 0 °C and then 2 h at 25 °C. The reaction mixture was concentrated. The residue was dissolved with EtOAc/CH_2Cl_2/ether and insoluble material was filtered and the filtrate was concentrated to afford the pure crude *N*-Boc-ethylenediamine as a quantitative yield which is used to the next reaction without purification. ^{1}H NMR ($CDCl_3$) δ 3.13 (td, 6.0, J=5.7 Hz, 2H), 2.75 (t, J=6.0 Hz, 2H), 1.40 (s, 9H), 1.15 (br s, 2H); LRMS (CI) m/z 161 (M+H), 105; HRMS (FAB) calcd for $C_7H_{17}N_2O_2$; (M+H) 161.1290, found 161.1288. Hünig base (4.4 mL, 25 mmol) and a solution of allyl bromoacetate (3.8 g, 22 mmol) in 5 mL of CH_3CN were added to a solution of *N*-Boc-ethylenediamine (1.6 g, 10 mmol) in 35 mL of CH_3CN at 0 °C. The resulting solution was stirred for 20 h at 25 °C and concentrated. EtOAc was added to the residue and the organic phase was washed with water and brine, dried, and concentrated to give a crude product which was chromatographed from EtOAc/hexane (1:3) to afford the desired product **2** (3.1 g, 86%). ^{1}H NMR ($CDCl_3$) δ 5.97–5.84 (m, 2H), 5.49 (br s, 1H), 5.34–5.21 (m, 4H), 4.60 (d, J=5.7 Hz, 4H), 3.57 (s, 4H), 3.15 (td, J=5.7, 5.4 Hz, 2H), 2.85 (t, J=5.7 Hz, 2H), 1.43 (s, 9H); LRMS (CI) m/z 357 (M+H); HRMS (FAB) calcd for $C_{17}H_{29}N_2O_6$; (M+H) 357.2026, found 357.2024.

Preparation of tetraalkylated ethylenediamine derivative (3). A solution of *N*-Boc protected amine **2** (0.8 g, 2.25 mmol) in 20 mL of EtOAc was saturated with HCl (g) and then stirred for 10 h at 25 °C. The resulting solution was concentrated to give the deprotected HCl salt. This crude material was dissolved in 10 mL of DMF and then *tert*-butyl bromoacetate (1.7 mL, 11.3 mmol) and Hünig base (2 mL, 11.3 mmol) was added to the above crude solution at 0 °C. The reaction mixture was stirred for 12 h at 25 °C. EtOAc (100 mL) was added and then the mixture was washed with water and brine. The organic layer was dried and concentrated. The residue was chromatographed with EtOAc/hexane (1:3) to afford the desired product **3** (0.63 g, 58%). ^{1}H NMR ($CDCl_3$) δ 5.97–5.84 (m, 2H), 5.34–5.20 (m, 4H), 4.59 (d, J=5.7 Hz, 4H), 3.66 (s, 4H), 3.45 (s, 4H), 2.88 (t, J=4.2 Hz, 2H), 1.43 (s, 18H); LRMS (CI) m/z 485 (M+H); HRMS (FAB) calcd for $C_{24}H_{41}N_2O_8$; (M+H) 485.2863, found 485.2868.

Preparation of EDTA monoacid (4). A solution of compound **3** (0.5 g, 1 mmol) in 6 mL of CH_2Cl_2 and 4 mL of trifluoroacetic acid was stirred for 10 h at 25 °C. The resulting solution was concentrated and dried under high pressure to give deprotected diacid as a quantitative yield. ^{1}H NMR ($CDCl_3$) δ 5.94–5.81 (m, 2H), 5.33–5.21 (m, 4H), 4.58 (d, J=4.8 Hz, 4H), 4.21 (s, 4H), 3.64 (s, 4H), 3.47 (s, 2H), 3.15 (s, 2H); LRMS (FAB) m/z 372.8 (M+H); HRMS (FAB) calcd for $C_{16}H_{25}N_2O_8$; (M+H) 373.1611, found 373.1610. A solution of DCC (0.2 g, 1 mmol) in 2 mL of CH_2Cl_2 was added to a solution of the deprotected diacid in 8 mL of CH_2Cl_2 at 0 °C and stirred for 5 h at 25 °C. The precipitate was filtered and the filtrate was concentrated to give the crude anhydride. ^{1}H NMR ($CDCl_3$) δ 5.95–5.86 (m, 2H), 5.36–5.25 (m, 4H), 4.60 (d, J=6.0 Hz, 4H), 3.66 (s, 4H), 3.61 (s, 4H), 2.97 (t, J=6.0 Hz, 2H), 2.70 (t, J=6.0 Hz, 2H). This anhydride was dissolved with 8 mL of allyl alcohol and one drop of triethyl amine and 5 mg of DMAP was added to a solution. The resulting solution was stirred for 15 h at 25 °C and concentrated. The residue was chromatographed using EtOAc/MeOH (5:1) to afford the desired product **4** (0.24 g, 57%). ^{1}H NMR ($CDCl_3$) δ 5.97–5.84 (m, 3H), 5.35–5.23 (m, 6H), 4.60 (d, J=5.4 Hz, 6H), 3.61 (s, 4H), 3.55 (s, 2H), 3.50 (s, 2H), 2.88 (s, 4H); LRMS (CI) m/z 413 (M+H); HRMS (FAB) calcd for $C_{19}H_{29}N_2O_8$; (M+H) 413.1924, found 413.1924.

Synthesis of di-allyl 3-((Allylcarbonyl)methyl)-6-(2-(trifluoroacetyl)amino)ethyl-3,6-diazaoctanedioate (7). Ethyl trifluoroacetate (1.46 g, 10.3 mmol) in 12 mL of CH_2Cl_2 was added dropwise to a solution of diethylenetriamine **6** (1.06 g, 10.3 mmol) in 8 mL of CH_2Cl_2 at 0 °C. After stirring 2 h at 0 °C, the reaction mixture was stirred for 5 h at 25 °C. The resulting solution was concentrated and the residue was dissolved in 50mL of acetonitrile. Hünig base (6.3 mL, 36 mmol) and allyl bromoacetate (6.2 g, 36 mmol) added to the above solution at 0 °C and stirred for 20 h at 25 °C. The resulting solution was concentrated and the residue was dissolved in ethyl acetate (100 mL). The organic solution was washed with water and brine, dried, and concentrated. The crude was

chromatographed from EtOAc/hexane (1:2) to afford the desired product **7** (2.6 g, 52%). ^{1}H NMR ($CDCl_3$) δ 8.37 (br s, 1H), 5.96–5.83 (m, 3H), 5.34–5.22 (m, 6H), 4.59 (d, J = 5.7 Hz, 2H), 4.58 (d, J = 6.0 Hz, 4H), 3.61 (s, 4H), 3.43 (s, 2H), 3.35 (td, J = 6.0, 4.2 Hz, 2H), 2.88 (t, J = 6.6 Hz, 2H), 2.86 (t, J = 5.7 Hz, 2H), 2.78 (t, J = 5.4 Hz, 2H); LRMS (CI) m/z 494 (M + H); HRMS (FAB) calcd for $C_{21}H_{31}F_3N_3O_7$; (M + H) 494.2114, found 494.2111.

Synthesis of DTTA monoacid (8). A solution of compound **7** (0.25 g, 0.5 mmol) in 2 mL of DMF was added dropwise to a suspension of 60% NaH (24 mg, 0.6 mmol) in 1 mL of DMF and stirred for 15 min followed by adding trimethylsilyl bromoacetate (0.16 g, 0.75 mmol) to the above solution at 0 °C. The resulting solution was stirred for 12 h at 25 °C and EtOAc (30 mL) was added. The solution was washed with saturated sodium bicarbonate, brine, dried, concentrated, and chromatographed from EtOAc/MeOH (10:1) to afford the desired product 8 (0.2 g, 70%: 98% BRSM). ^{1}H NMR ($CDCl_3$) δ 5.96–5.83 (m, 3H), 5.36–5.23 (m, 6H), 4.60 (d, J = 5.7 Hz, 2H), 4.59 (d, J = 6.0 Hz, 4H), 4.25 (s, 2H), 3.71 (t, J = 6.3 Hz, 2H), 3.59 (s, 2H), 3.07–2.97 (m, 4H), 2.95–2.84 (m, 2H); LRMS (CI) m/z 552 (M + H); HRMS (FAB) calcd for $C_{23}H_{33}F_3N_3O_9$; (M + H) 552.2169, found 552.2167.

Preparation of *N*-Boc-diethylenetriamine derivative (10). $(Boc)_2O$ (1.6 g, 7.5 mmol) in 15 mL of MeOH was added dropwise to a solution of diethylenetriamine **6** (0.7 g, 6.8 mmol) in 25 mL of MeOH at 0 °C, stirred for 2 h at 0 °C and then 10 h at 25 °C. The reaction mixture was concentrated, dissolved with CH_3CN, insoluble material filtered, and the filtrate was concentrated and dissolved with 30 mL of CH_3CN. Hünig base (4.8 mL, 27 mmol) and allyl bromoacetate (4.18 g, 25 mmol) were added at 0 °C. The resulting solution was stirred for 12 h at 25 °C and concentrated. EtOAc was added and the organic phase was washed with water and brine, dried, and concentrated to give a crude product which was chromatographed using EtOAc/hexane (1:3) to afford the desired product **10** (1.8 g, 52%). ^{1}H NMR ($CDCl_3$) δ 5.98–5.85 (m, 3H), 5.62 (br s, 1H), 5.35–5.22 (m, 6H), 4.61–4.58 (m, 6H), 3.61 (s, 4H), 3.44 (m, 2H), 2.86–2.73 (m, 6H), 1.44 (s, 9H); LRMS (CI) m/z 498 (M + H); HRMS (FAB) calcd for $C_{24}H_{40}N_3O_8$; (M + H) 498.2815, found 498.2818.

Preparation of Pentaalkylated-diethylenetriamine derivative (11). A solution of *N*-Boc protected amine **10** (0.75 g, 1.5 mmol) in 15 mL of EtOAc was saturated with HCl (g) and then stirred for 10 h at 25 °C. The resulting solution was concentrated to give deprotected product. This crude material was dissolved with 7.5 mL of DMF and then *tert*-butyl bromoacetate (1.1 mL, 7.5 mmol) and Hünig base (1.6 mL, 9 mmol) were added to the solution at 0 °C. The mixture was stirred for 12 h at 25 °C. EtOAc (100 mL) was added and then the mixture was washed with water and brine. The organic layer was dried and concentrated. The residue was chromatographed from EtOAc/hexane (1:3) to afford the desired product **11** (0.56 g, 60%). ^{1}H NMR ($CDCl_3$) δ 5.97–5.84 (m, 3H), 5.34–5.19 (m, 6H), 4.60–4.55 (m, 6H), 3.61 (s, 4H), 3.53 (s, 2H), 3.43 (s, 4H), 2.86–2.80 (m, 8H), 1.44 (s, 18H); LRMS (CI) m/z 626 (M + H); HRMS (FAB) calcd for $C_{31}H_{52}N_3O_{10}$; (M + H) 626.3653, found 626.3656.

Synthesis of DTPA monoacid (12). A solution of compound **11** (0.5 g, 0.8 mmol) in 6 mL of CH_2Cl_2 and 4 mL of trifluoroacetic acid was stirred for 10 h at 25 °C. The solution was concentrated and dried under high pressure to give deprotected diacid as a quantitative yield. ^{1}H NMR ($CDCl_3$) δ 5.95–5.81 (m, 3H), 5.40–5.21 (m, 6H), 4.61–4.57 (m, 6H), 4.07 (s, 4H), 3.72 (s, 6H), 3.50 (m, 2H), 3.15 (m, 2H), 2.96 (m, 4H); LRMS (FAB) m/z 514 (M + H); HRMS (FAB) calcd for $C_{23}H_{36}N_3O_{10}$; (M + H) 514.2401, found 514.2399. A solution of DCC (0.17 g, 0.8 mmol) in 2 mL of CH_2Cl_2 was added to a solution of the diacid in 6 mL of CH_2Cl_2 at 0 °C and stirred for 5 h at 25 °C. The precipitate was filtered and the filtrate concentrated to give the crude anhydride. ^{1}H NMR ($CDCl_3$) δ 5.97–5.84 (m, 3H), 5.35–5.22 (m, 6H), 4.60–4.57 (m, 6H), 3.65 (s, 4H), 3.61 (s, 4H), 3.54 (s, 2H), 2.92–2.86 (m, 6H), 2.70 (t, J = 5.1 Hz, 2H). This crude anhydride was dissolved in 8 mL of allyl alcohol and one drop of triethylamine and 5 mg of DMAP was added to a solution. The solution was stirred for 15 h at 25 °C and concentrated. The residue was chromatographed from EtOAc/MeOH (5:1) to afford the desired product 12 (0.3 g, 68%). ^{1}H NMR ($CDCl_3$) δ 5.96–5.83 (m, 4H), 5.35–5.21 (m, 8H), 4.61–4.57 (m, 8H), 3.63 (s, 2H), 3.57 (s, 4H), 3.56 (s, 2H), 3.48 (s, 2H), 2.94–2.86 (m, 8H); LRMS (CI) m/z 554 (M + H); HRMS (FAB) calcd for $C_{26}H_{40}N_3O_{10}$; (M + H) 554.2714, found 554.2711.

Preparation of 2′-Alloc-Taxol (15). Hünig base (0.26 mL, 1.46 mmol) and allyl chloroformate (0.17 mL, 1.6 mmol) was added to a solution of Taxol (500 mg, 0.59 mmol) in 10 mL of methylene chloride at 0 °C and stirred for 10 h. 20 mL of CH_2Cl_2 added and the mixture washed with 0.1N HCl (20 mL), dried, and concentrated to give solid which was recrystallized from CH_2Cl_2–ether to afford the pure product **15** (540 mg, 98%). ^{1}H NMR ($CDCl_3$) δ 8.13 (dd, J = 8.5, 1.2 Hz, 2H, Bz), 7.74 (dd, J = 8.2, 1.2 Hz, 2H, Bz), 7.62 (t, J = 7.5 Hz, 1H, Bz), 7.52–7.32 (m, 10H, Ar), 6.93 (d, J = 9.3 Hz, 1H, NH), 6.29 (s, 1H, 10-H), 6.28 (t, J = 9.0 Hz, 1H, 13-H), 5.99 (dd, J = 9.3, 2.4 Hz, 1H, 3′-H), 5.92∼5.86 (m, 1H, =CH), 5.69 (d, J = 6.9 Hz, 1H, 2-H), 5.43 (d, J = 2.7 Hz, 1H, 2′-H), 5.39–5.28 (m, 2H, $=CH_2$), 4.98 (b d, J = 8.0 Hz, 1H, 5-H), 4.64 (dd, J = 3.9, 1.8 Hz, 2H, allylic CH_2), 4.44 (m, 1H, 7-H), 4.33 (A of AB, d, J = 8.4 Hz, 1H, 20-H), 4.21 (B of AB, d, J = 8.4 Hz, 1H, 20-H), 3.82 (d, J = 7.0 Hz, 1H, 3-H), 2.56 (m, 1H, 6-H), 2.47 (s, 3H, OAc), 2.40 (dd, 1H, 14-CH_2), 2.23 (s, 3H, OAc), 2.19 (JJ, 1H, 14-CH_2), 1.93 (s, 3H, 18-CH_3), 1.89 (m, 1H, 6-H), 1.69 (s, 3H, 19-CH_3), 1.25 (s, 3H, 16-CH_3), 1.14 (s, 3H, 17-CH_3); LRMS (FAB) m/z 937.8 (M).

General procedure for the preparation of fully protected Taxol derivatives (16 and 17). A solution of DCC (0.2 mmol) and 5 mg of DMAP in 0.5 mL of CH_2Cl_2 was

added to a solution of monoacid **4** (or **12**) (0.15 mmol) and 2′-alloc-Taxol **15** (0.1 mmol) in 1.5 mL of CH_2Cl_2 at 0 °C. The resulting solution was allowed to warm to 25 °C and stirred for 10 h. The reaction mixture was filtered, concentrated, and chromatographed from EtOAC/hexane (1:1 and/or 2:1) to afford the desired product **16** or **18**.

16. 95% yield; ^{1}H NMR ($CDCl_3$) δ 8.13 (d, J = 7.2 Hz, 2H), 7.75 (d, J = 7.2 Hz, 2H), 7.61 (t, J = 7.2 Hz, 1H), 7.53–7.45 (m, 4H), 7.43–7.35 (m, 6H), 6.94 (d, J = 9.3 Hz, 1H, NH), 6.25 (t, J = 9.0 Hz, 1H), 6.23 (s, 1H), 5.99 (dd, J = 9.3, 2.4 Hz, 1H), 5.97–5.84 (m, 4H), 5.68 (d, J = 6.9 Hz, 1H), 5.60 (dd, J = 7.2, 3.3 Hz, 1H), 5.43 (d, J = 2.4 Hz, 1H), 5.39–5.20 (m, 8H), 4.95 (d, J = 9.3 Hz, 1H), 4.64 (dd, 4.5, 1.2 Hz, 2H), 4.58 (d, J = 5.7 Hz, 6H), 4.33 (A of AB, d, J = 8.4 Hz, 1H), 4.18 (B of AB, d, J = 8.4 Hz, 1H), 3.94 (d, J = 6.6 Hz, 1H), 3.67–3.55 (m, 8H), 2.89 (s, 4H), 2.61 (m, 1H), 2.45 (s, 3H), 2.39 (m, 1H), 2.22 (m, 1H), 2.12 (s, 3H), 1.98 (s, 3H), 1.88 (m, 1H), 1.79 (s, 3H), 1.20 (s, 3H), 1.14 (s, 3H); LRMS (PDMS) m/z 1333 (M + H).

18. 94% yield; ^{1}H NMR ($CDCl_3$) δ 8.13 (d, J = 6.9 Hz, 2H), 7.75 (d, J = 6.9 Hz, 2H), 7.62 (t, J = 6.6 Hz, 1H), 7.54–7.46 (m, 4H), 7.43–7.34 (m, 6H), 6.92 (d, J = 9.3 Hz, 1H, NH), 6.26 (t, J = 9.9 Hz, 1H), 6.23 (s, 1H), 5.99 (dd, J = 9.3, 2.7 Hz, 1H), 5.97–5.84 (m, 5H), 5.68 (d, J = 6.9 Hz, 1H), 5.60 (dd, J = 7.2, 3.3 Hz, 1H), 5.43 (d, J = 2.4 Hz, 1H), 5.40–5.21 (m, 10H), 4.96 (d, J = 8.1 Hz, 1H), 4.64 (dd, 4.5, 1.2 Hz, 2H), 4.60 (d, J = 5.7 Hz, 8H), 4.33 (A of AB, d, J = 8.4 Hz, 1H), 4.18 (B of AB, d, J = 8.4 Hz, 1H), 3.94 (d, J = 6.9 Hz, 1H), 3.64–3.52 (m, 10H), 2.84–2.80 (m, 8H), 2.63 (m, 1H), 2.46 (s, 3H), 2.40 (m, 1H), 2.23 (m, 1H), 2.12 (s, 3H), 1.98 (s, 3H), 1.84 (m, 1H), 1.79 (s, 3H), 1.21 (s, 3H), 1.14 (s, 3H); LRMS (PDMS) m/z 1476 (M + H).

General procedure for the preparation of Taxol EDTA/DTPA (17 and 19). $PhSiH_3$ (0.5 mmol) and $Pd(PPh_3)_4$ (0.004 mmol) was added to a solution of protected Taxol **16** (or **18**) (0.08 mmol) in 3 mL of methylene chloride and stirred for 1 h at 25 °C. 1 mL of MeOH was added and the reaction solution stirred for 10 min. The resulting solution was concentrated and the residue was recrystallized from CH_2Cl_2/EtOAc/ether system to afford the desired product **17** (or **19**) in good yield. **17**: 75% yield; LRMS (PDMS) m/z 1129 (M + H). And **19**. 70% yield; LRMS (PDMS) m/z 1230 (M + H).

Preparation of PEG-carboxylic acid (23 and 24). A solution of amino-PEG-azide **20** (0.64 g, 2.90 mmol) and anhydride **21** (or **22**) (2.24 mmol) in 25 mL of CH_2Cl_2 was stirred for 36 h at 25 °C. Water (1 mL) was added to destroy excess anhydride and left to stir overnight. The reaction solution was dried and concentrated to afford the desired product **23** or **24** (∼98% yield). **23**: ^{1}H NMR ($CDCl_3$) δ 7.63 (brs, 1H), 4.18 (s, 2H), 4.14 (s, 2H), 3.74–3.62 (m, 10H), 3.56 (m, 4H), 3.40 (t, J = 5.4 Hz, 2H). **24**: ^{1}H NMR ($CDCl_3$) δ 3.69–3.61 (m, 12H), 3.55 (t, J = 4.5 Hz, 2H), 3.42 (s, 4H), 3.40 (t, J = 5.4 Hz, 2H), 2.62 (s, 3H).

Preparation of azido-PEG Taxol (25 and 26). A solution of DCC (78 mg, 0.38 mmol) and 5 mg of DMAP in 1 mL of CH_2Cl_2 was added to a solution of PEG-acid **23** (or **24**) (0.4 mmol) and 2′-alloc-Taxol **15** (250 mg, 0.27 mmol) in 3 mL of CH_2Cl_2 at 0 °C. The resulting solution was allowed to warm to 25 °C and stirred for 10 h. The reaction mixture was filtered, concentrated, and chromatographed from EtOAc to afford the desired product (**25** or **26**).

25. 94% yield. ^{1}H NMR ($CDCl_3$) δ 8.13 (d, J = 8.7 Hz, 2H), 7.74 (d, J = 8.4 Hz, 2H), 7.62 (t, J = 7.5 Hz, 1H), 7.54–7.34 (m, 10H), 7.22 (br s, 1H), 6.95 (d, J = 9.6 Hz, 1H, NH), 6.26 (t, J = 8.1 Hz, 1H), 6.19 (s, 1H), 5.98 (dd, J = 9.3, 2.7 Hz, 1H), 5.94–5.83 (m, 1H), 5.68 (d, J = 6.9 Hz, 1H), 5.66 (dd, J = 5.5, 3.3 Hz, 1H), 5.43 (d, J = 2.7 Hz, 1H), 5.39–5.27 (m, 2H), 4.97 (d, J = 9.3 Hz, 1H), 4.64 (m, 2H), 4.35–4.03 (m, 6H), 3.95 (d, J = 6.9 Hz, 1H), 3.69–3.47 (m, 14H), 3.38 (t, J = 5.4 Hz, 2H), 2.62 (m, 1H), 2.48 (s, 3H), 2.40 (m, 1H), 2.21 (m, 1H), 2.16 (s, 3H), 1.97 (s, 3H), 1.90 (m, 1H), 1.79 (s, 3H), 1.22 (s, 3H), 1.14 (s, 3H); LRMS (PDMS) m/z 1254 (M).

26. 95% yield. ^{1}H NMR ($CDCl_3$) δ 8.12 (d, J = 8.7 Hz, 2H), 7.73 (d, J = 8.7 Hz, 2H), 7.61 (t, J = 7.5 Hz, 1H), 7.58 (br s, 1H), 7.53–7.45 (m, 4H), 7.42–7.34 (m, 6H), 6.94 (d, J = 9.3 Hz, 1H, NH), 6.24 (t, J = 9.3 Hz, 1H), 6.21 (s, 1H), 5.97 (dd, J = 9.3, 2.7 Hz, 1H), 5.92–5.83 (m, 1H), 5.68 (d, J = 6.9 Hz, 1H), 5.60 (dd, 6.9, 3.3 Hz, 1H), 5.42 (d, J = 2.7 Hz, 1H), 5.38–5.26 (m, 2H), 4.95 (d, J = 8.4 Hz, 1H), 4.63 (m, 2H), 4.32 (A of AB, d, J = 8.1 Hz, 1H), 4.17 (B of AB, d, J = 8.7 Hz, 1H), 3.94 (d, J = 6.9 Hz, 1H), 3.67–3.55 (m, 12H), 3.48 (t, 6.6 Hz, 2H), 3.45–3.24 (m, 4H), 3.21 (d, J = 2.7 Hz, 2H), 2.62 (m, 1H), 2.45 (s, 3H), 2.41 (m, 1H), 2.38 (s, 3H), 2.22 (m, 1H), 2.12 (s, 3H), 1.97 (s, 3H), 1.89 (m, 1H), 1.79 (s, 3H), 1.20 (s, 3H), 1.13 (s, 3H); LRMS (PDMS) m/z 1268 (M + H).

Preparation of amino-PEG–Taxol (27 and 28). PhSH (1.2 mmol) and Et_3N (0.9 mmol) were added to a solution of $SnCl_2$ (0.3 mmol) in 4 mL of CH_3CN. After 5 min, azido-compound **25** (or **26**) (0.2 mmol) was added and stirred for 1 h at 25 °C. 30 mL of CH_2Cl_2 and 30 mL of saturated $NaHCO_3$ solution was added. The organic layer was separated and the separated aqueous layer was extracted with methylene chloride twice. The combined organic solution was washed with brine, dried, and concentrated. The residue was dissolved in 5 mL of MeOH, stirred for 30 min at 25 °C and concentrated. The purification by column chromatography afforded the desired product amine.

27. 90% yield; ^{1}H NMR ($CDCl_3$) δ 8.13 (d, J = 8.7 Hz, 2H), 7.74 (d, J = 8.4 Hz, 2H), 7.62 (t, J = 7.5 Hz, 1H), 7.54–7.34 (m, 10H), 7.22 (br s, 1H), 6.95 (d, J = 9.6 Hz, 1H), 6.26 (t, J = 8.1 Hz, 1H), 6.19 (s, 1H), 5.98 (dd, J = 9.3, 2.7 Hz, 1H), 5.94–5.83 (m, 1H), 5.68 (d, J = 6.9 Hz, 1H), 5.66 (dd, J = 5.5, 3.3 Hz, 1H), 5.43 (d, 2.7 Hz, 1H), 5.39–5.27 (m, 2H), 4.97 (d, J = 9.3 Hz, 1H), 4.64 (m, 2H, allylic CH_2), 4.35–4.03 (m, 6H), 3.95 (d, J = 6.9 Hz, 1H), 3.69–3.47 (m, 14H), 2.90 (t, J = 5.1 Hz, 2H), 2.62 (m, 1H), 2.48 (s, 3H), 2.40 (m, 1H), 2.21 (m, 2H),

2.16 (s, 3H), 1.97 (s, 3H), 1.90 (m, 1H), 1.79 (s, 3H), 1.22 (s, 3H), 1.14 (s, 3H); LRMS (PDMS) *m*/*z* 1228.4 (M + H).

28. 97% yield; ^{1}H NMR ($CDCl_3$) δ 8.12 (d, *J* = 8.7 Hz, 2H), 7.73 (d, *J* = 8.4 Hz, 2H), 7.61 (t, *J* = 7.5 Hz, 1H), 7.58 (br s, 1H), 7.53–7.45 (m, 4H), 7.42–7.34 (m, 6H), 6.99 (d, *J* = 9.3 Hz, 1H), 6.24 (t, *J* = 9.3 Hz, 1H), 6.21 (s, 1H), 5.97 (dd, *J* = 9.3, 2.7 Hz, 1H), 5.92–5.83 (m, 1H), 5.68 (d, *J* = 6.9 Hz, 1H), 5.60 (dd, *J* = 6.9, 3.3 Hz, 1H), 5.42 (d, 2.7 Hz, 1H), 5.38–5.26 (m, 2H), 4.95 (d, *J* = 8.4 Hz, 1H), 4.63 (m, 2H, allylic CH_2), 4.32 (A of AB, d, *J* = 8.1 Hz, 1H), 4.03 (B of AB, d, *J* = 8.7 Hz, 1H), 3.94 (d, *J* = 6.9 Hz, 1H), 3.67–3.55 (m, 12H), 3.48 (t, *J* = 6.6 Hz, 2H), 3.45–3.24 (m, 2H), 3.21 (d, *J* = 2.7 Hz, 2H), 2.92 (t, *J* = 5.3 Hz, 2H), 2.62 (m, 1H), 2.45 (s, 3H), 2.41 (m, 1H), 2.38 (s, 3H), 2.22 (m, 2H), 2.12 (s, 3H), 1.97 (s, 3H), 1.89 (m, 1H), 1.79 (s, 3H), 1.20 (s, 3H), 1.13 (s, 3H); LRMS (PDMS) *m*/*z* 1241.5 (M + H).

Preparation of compounds 30 and 31. Taxol-amine **27** (or **28**) (0.066 mmol) was added to a solution of DTPA-dianhydride **29** (0.5 mmol) in 3 mL of DMSO and stirred for 3 h at 25 °C. Water (2 mL) was added and the solution stirred for several hours. The solution was lyophilized and the residue was dissolved with CH_2Cl_2 and filtered. The filtrate was concentrated and recrystallized from CH_2Cl_2/EtOAc/ether (1:2:3) to afford the desired product **30** (or **31**) as a good yield. 30: 80% yield. LRMS (MALDI) *m*/*z* 1604 (M + H). **31**: 85% yield. LRMS (MALDI) *m*/*z* 1617 (M + H).

Preparation of Taxol–PEG multiacid (32 and 33). Et_2NH (0.28 mmol) and $Pd(PPh_3)_4$ (2 mg) was added to a solution of protected glutamate **30** (or **31**) (0.028 mmol) in 4 mL of methylene chloride and stirred for 30 min at 25 °C. The reaction mixture was concentrated and the residue was recrystallized from CH_2Cl_2/EtOAc/ether (1:1:3) system to afford the desired product **32** or **33** as a good yield. **32**: 95% yield. LRMS (MALDI) *m*/*z* 1519 (M + H). **33**: 90% yield. LRMS (MALDI) *m*/*z* 1533 (M + H).

Preparation of glutamate (35). A solution of DCC (3.15 mmol) and 5 mg of DMAP in 8 mL of CH_2Cl_2 was added slowly to a solution of protected glutamic acid **34** (3 mmol) and N_3-PEG-NH_2 **20** (3.3 mmol) in 16 mL of THF at 0 °C. The reaction mixture was stirred for 15 h at room temperature. After filtering to remove solid, the filtrate was concentrated and chromatographed with EtOAc/*n*-hexane to obtain the desired product in 94% yield. ^{1}H NMR ($CDCl_3$) δ 6.36 (br s, 1H, NH), 5.92–5.81 (m, 3H, involving NH), 5.33–5.16 (m, 4H), 4.60 (d, *J* = 5.7 Hz, 2H), 4.53 (d, *J* = 5.4 Hz, 2H), 4.32–4.30 (m, 1H), 3.66–3.57 (m, 10H), 3.52 (t, *J* = 5.1 Hz, 2H), 3.43–3.40 (m, 2H), 3.36 (t, *J* = 4.8 Hz, 2H), 2.30–2.25 (m, 2H), 2.22–2.15 (m, 1H), 2.02–1.95 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.0, 171.8, 156.2, 132.7, 131.6, 119.0, 117.9, 70.8, 70.7, 70.6, 70.3, 70.1, 69.8, 66.1, 65.9, 53.8, 50.7, 39.4, 32.4, 28.2; LRMS (CI) *m*/*z* 472 (M + H), 388, 219, 182, 170; HRMS (CI) calcd for $C_{20}H_{34}N_5O_8$; (M + H) 472.2407, found 472.2412.

Preparation of amino-PEG-glutamate (36). A solution of azido-PEG-glutamate **35** (1.4 g, 3 mmol) and triphenylphosphine (1 g, 3.6 mmol) in 20 mL of THF with a drop of water was stirred for 24 h at 25 °C. The reaction solution was concentrated and chromatographed with EtOAc and EtOAc/MeOH (10:1) to remove triphenylphosphine derivatives and then MeOH/Et_3N (98:2) to obtain the desired product in 99% yield. ^{1}H NMR ($CDCl_3$) δ 7.14 (br s, 1H, NH), 6.08 (d, *J* = 8.4 Hz, 1H, NH), 6.00–5.84 (m, 2H), 5.34–5.19 (m, 4H), 4.63 (d, *J* = 5.7 Hz, 2H), 4.56 (d, *J* = 5.4 Hz, 2H), 4.34 (m, 1H), 3.71–3.50 (m, 12H), 3.44 (t, *J* = 4.8 Hz, 2H), 2.87 (t, *J* = 5.1 Hz, 2H), 2.31 (br s, 2H, NH_2), 2.28 (m, 2H), 2.22–2.17 (m, 1H), 2.07–1.97 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.2, 172.0, 156.3, 132.8, 131.7, 118.9, 117.8, 70.5, 70.2, 70.1, 70.0, 69.9, 69.8, 66.0, 65.8, 53.9, 41.4, 39.4, 32.3, 28.0; LRMS (CI) *m*/*z* 446(M + H), 383; HRMS (CI) calcd for $C_{20}H_{36}N_3O_8$; (M + H) 446.2502, found 446.2488.

Preparation of Taxol-7-carbamoyl-PEG-glutamate (38). A solution of 2′-protected-Taxol–7-OCOIm **37** (40 mg, 0.04 mmol) and amino-PEG-glutamate **36** (175 mg, 0.4 mmol) in 3 mL of anhydrous isopropyl alcohol was heated at reflux for 5 h. After cooling to the room temperature, the reaction mixture was concentrated and the residue was chromatographed from EtOAc to afford the desired product (43 mg, 82%). ^{1}H NMR (DMSO-d_6) δ 8.90 (d, *J* = 7.6 Hz, 1H, NH), 7.95 (d, *J* = 7.2 Hz, 2H), 7.87 (d, *J* = 6.9 Hz, 2H), 7.70 (t, *J* = 6.4 Hz, 1H), 7.68–7.37 (m, 10H), 7.20 (m, 1H), 6.30 (s, 1H), 6.18 (d, *J* = 7.5 Hz, 1H), 5.87 (t, *J* = 7.6 Hz, 1H), 5.91–5.82 (m, 2H), 5.40 (d, *J* = 7.8 Hz, 1H), 5.37 (d, *J* = 8.1 Hz, 1H), 5.30–5.13 (m, 4H), 4.92 (d, *J* = 9 Hz, 1H), 4.75 (s, 1H), 4.57 (t, *J* = 7.8 Hz, 1H), 4.54 (d, *J* = 5.1 Hz, 2H), 4.44 (d, *J* = 4.8HZ, 2H), 4.01 (m, 3H), 3.69 (d, *J* = 6.6 Hz, 1H), 3.47 (m, 4H), 3.40–3.24 (m, 8H), 3.14 (t, *J* = 5.7 Hz, 2H), 3.04 (m, 2H), 2.37 (m, 1H), 2.20 (s, 3H), 2.14 (t, *J* = 7.5 Hz, 2H), 2.05 (s, 3H), 1.96 (m, 2H), 1.79 (s, 3H), 1.76 (m, 3H), 1.61 (s, 3H), 1.00 (s, 3H), 0.98 (s, 3H); LRMS (FAB) *m*/*z* 1325.0 (M).

Preparation of Taxol–7-carbamoyl-PEG-glutamic acid (39). A solution of protected glutamate **38** (95 mg, 0.7 mmol), Et_2NH (100 mg, 1.4 mmol), and $Pd(PPh_3)_4$ (4 mg, 0.004 mmol) in 5 mL of methylene chloride was stirred for 30 min at 25 °C. The reaction mixture was concentrated under reduced pressure. The residue was recrystallized from CH_2Cl_2/ether system to afford the desired product (72 mg, 87%). ^{1}H NMR (DMSO-d_6) δ 9.36 (d, *J* = 8.4 Hz, 1H, NH), 8.02 (br s, 1H), 7.93 (d, *J* = 7.8 Hz, 2H), 7.90 (d, *J* = 10.8 Hz, 2H), 7.70 (t, *J* = 7.2 Hz, 1H), 7.64–7.36 (m, 10H), 7.19 (m, 1H), 7.19 (m, 1H), 6.29 (s, 1H), 5.85 (t, *J* = 7.6 Hz, 1H), 5.39–5.28 (m, 4H), 4.90 (d, *J* = 9 Hz, 1H), 4.74 (s, 1H), 4.63 (t, *J* = 8.1 Hz, 1H), 4.00 (m, 3H), 3.69 (d, *J* = 6.6 Hz, 1H), 3.46–3.14 (m, 14H), 2.98 (m, 2H), 2.24 (m, 3H), 2.14 (s, 3H), 2.01 (s, 3H), 1.87 (m, 3H), 1.78 (s, 3H), 1.70 (m, 2H), 1.60 (s, 3H), 1.00 (s, 3H), 0.97 (s, 3H); LRMS (FAB) *m*/*z* 1200.8 (M), 1223.8 (M + Na^+).

Preparation of Taxol–7-carbamoyl-PEG-folate (40). MTBD (10 mg, 0.063 mmol) was added to a solution

of Taxol–7-carbamoyl-PEG-glutamic acid **39** (25 mg, 0.02 mmol) and pteroyl azide (8.5 mg, 0.025 mmol) in 1 mL of DMSO which the heterogeneous solution became homogeneous within 30 seconds. The solution was left to stir for 2 h at 25 °C and was then loaded directly onto a MPLC column (LiChroprop C18, 310×25 mm) and developed by 20 mL of 50 mM NH_4HCO_3 and then 73:27 50mM NH_4HCO_3/CH_3CN, followed by 60:40 50 mM NH_4HCO_3/CH_3CN for purification. After lyopholization the desired product was obtained (17 mg, 55%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 10.11 (d, J = 7.5 Hz, 1H), 8.53 (s, 1H), 8.09 (br s, 1H), 7.90 (d, J = 7.2 Hz, 4H), 7.71 (t, J = 7.2 Hz, 1H), 7.64–7.34 (m, 10H), 7.58 (d, J = 8.1 Hz, 2H), 7.14 (br s, 1H), 7.08 (br, 1H), 6.95 (br, 1H), 6.81 (br, 1H), 6.63 (d, 8.1 Hz, 2H), 6.28 (s, 1H), 5.81 (t, J = 8.1 Hz, 1H), 5.35 (d, 5.7 Hz, 2H), 5.21 (t, J = 4.5 Hz, 2H), 4.80 (m, 2H), 4.67 (s, 1H), 4.41 (t, J = 4.8 Hz, 2H), 4.12 (m, 1H), 4.00 (s, 2H), 3.66 (d, J = 6.6 Hz, 1H), 3.43–3.04 (m, 18H), 2.25 (m, 2H), 2.10 (s, 3H), 2.04 (s, 3H), 1.91–1.81 (m, 6H), 1.77 (s, 3H), 1.59 (s, 3H), 1.00 (s, 3H), 0.98 (s, 3H); LRMS (FAB) m/z 1494.5 (M), 1517.5 (M + Na^+).

Preparation of glutamyl-PEG-carboxylic acid (41 and 42). A solution of amino-PEG-glutamate 36 (0.9 g, 2 mmol), diglycolic anhydride **21** (or **22**) (3 mmol), and DMAP (20 mg, 0.16 mmol) in 20 mL of CH_2Cl_2 was stirred for 36 h at 25 °C. H_2O (0.5 mL) was added to the solution and stirred for 24 h to destroy excess anhydride and then dried, filtered, and concentrated to afford the desired product.

41. 95% yield; ^{1}H NMR ($CDCl_3$) δ 7.61 (br s, 1H, NH), 6.72 (br s, 1H, NH), 5.96–5.83 (m, 3H, including NH), 5.35–5.19 (m, 4H), 4.62 (d, J = 5.7 Hz, 2H), 4.55 (d, J = 5.4 Hz, 2H), 4.35 (m, 1H), 4.17 (s, 2H), 4.11 (s, 2H), 3.66–3.56 (m, 12H), 3.52 (t, J = 4.8 Hz, 2H), 3.44 (br s, 2H), 2.32 (t, J = 6.9 Hz, 2H), 2.20 (m, 1H), 2.02 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.8, 171.9, 171.7, 170.0, 156.4, 132.7, 131.6, 119.0, 118.0, 71.5, 70.6, 70.5, 70.3, 70.1, 69.9, 69.7, 69.2, 66.2, 66.0, 53.8, 39.4, 38.9, 32.4, 28.2; LRMS (FAB) m/z 562.2 (M + H); HRMS (FAB) calcd for $C_{24}H_{40}N_3O_{12}$; (M + H) 562.2612, found 562.2594.

42. 94% yield; ^{1}H NMR ($CDCl_3$) δ 7.98 (br s, 1H, NH), 6.90 (br s, 1H, NH), 6.05 (d, J = 6.3 Hz, 1H, NH), 5.95–5.82 (m, 2H), 5.34–5.18 (m, 4H), 4.62 (d, J = 5.7 Hz, 2H), 4.55 (d, J = 5.4 Hz, 2H), 4.33 (m, 1H), 3.64–3.56 (m, 12H), 3.48–3.39 (m, 4H), 3.36 (s, 4H), 2.54 (s, 3H), 2.33 (t, J = 6.9 Hz, 2H), 2.19 (m, 1H), 2.03 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.6, 172.0, 156.3, 132.7, 131.6, 119.0, 118.0, 70.6, 70.4, 70.3, 70.1, 69.8, 66.2, 66.1, 66.0, 60.5, 59.2, 53.9, 43.6, 39.3, 39.1, 32.4, 28.2; LRMS (FAB) m/z 575.2(M + H); HRMS (FAB) calcd for $C_{25}H_{43}N_4O_{11}$; (M + H) 575.2928, found 575.2903.

Preparation of Taxol–2′-PEG-glutamate (43 and 44). DIPC (16 mg, 0.12 mmol), Taxol **14** (104 mg, 0.12 mmol), and DMAP (10 mg) were added to a solution of gulutamyl-PEG-carboxylic acid **41** (or **42**) (0.1 mmol) in 5 mL of CH_2Cl_2 at 0 °C. The resulting solution was allowed to warm to 25 °C and stirred for 12 h. The reaction mixture was washed with 0.1 N HCl, dried, concentrated, followed by column chromatography (EtOA/MeOH = 10:1) to afford the desired product.

43. 93% yield; ^{1}H NMR ($CDCl_3$) δ 8.14 (d, J = 8.5 Hz, 2H), 7.76 (d, J = 7.5 Hz, 2H), 7.62 (t, J = 7.5 Hz, 1H), 7.54–7.36 (m, 10H), 7.24 (br s, 1H), 7.02 (br s, 1H), 6.49 (br s, 1H), 6.30 (s, 1H), 6.23 (d, J = 9.0 Hz, 1H), 6.02 (dd, J = 9.0, 3.6 Hz, 1H), 5.95–5.81 (m, 3H), 5.68 (d, J = 6.9 Hz, 1H), 5.59 (d, J = 3.6 Hz, 1H), 5.34–5.17 (m, 4H), 4.98 (d, J = 7.8 Hz, 1H), 4.58 (d, J = 6.0 Hz, 1H), 4.53 (d, J = 5.4 Hz, 2H), 4.44 (m, 1H), 4.33–4.18 (m, 5H), 4.02 (s, 2H), 3.81 (d, J = 6.9 Hz, 1H), 3.65–3.56 (m, 10H), 3.50 (m, 4H), 3.38 (t, J = 5.7 Hz, 2H), 2.56 (m, 1H), 2.46 (s, 3H), 2.34–2.21 (m, 4H), 2.23 (s, 3H), 2.14 (m, 1H), 1.98 (m, 1H), 1.93 (s, 3H), 1.89 (m, 1H), 1.69 (s, 3H), 1.23 (s, 3H), 1.14 (s, 3H); LRMS (FAB) m/z 1397.2 (M).

44. 95% yield; ^{1}H NMR ($CDCl_3$) δ 8.21 (d, J = 0.9 Hz, 1H), 8.15 (d, J = 7.2 Hz, 2H), 7.82 (d, J = 7.2 Hz, 2H), 7.62 (t, J = 7.2 Hz, 1H), 7.55–7.36 (m, 10H), 7.32 (m, 1H), 6.73 (d, J = 7.2 Hz, 1H), 6.49 (br s, 1H), 6.31 (s, 1H), 6.22 (d, J = 9.0 Hz, 1H), 6.01 (dd, J = 9.3, 3.9 Hz, 1H), 5.97–5.81 (m, 3H), 5.68 (d, J = 7.5 Hz, 1H), 5.55 (d, J = 3.3 Hz, 1H), 5.36–5.17 (m, 4H), 4.97 (d, J = 8.4 Hz, 1H), 4.58 (d, J = 5.7 Hz, 1H), 4.53 (d, J = 5.4 Hz, 2H), 4.42 (m, 1H), 4.35 (m, 1H), 4.32 (A of AB, d, J = 8.4 Hz, 1H), 4.20 (B of AB, d, J = 8.1 Hz, 1H), 3.81 (d, J = 6.9 Hz, 1H), 3.62–3.43 (m, 16H), 3.37 (s, 2H), 3.25 (s, 2H), 2.56 (m, 1H), 2.48 (s, 3H), 2.38 (s, 3H), 2.34–2.21 (m, 4H), 2.23 (s, 3H), 2.14 (m, 1H), 1.98 (m, 1H), 1.94 (s, 3H), 1.89 (m, 1H), 1.68 (s, 3H), 1.23 (s, 3H), 1.14 (s, 3H); LRMS (FAB) m/z 1410.0 (M).

Preparation of Taxol–2′-PEG-glutamic acid (45 and 46). $PhSiH_3$ (20 mg, 0.18 mmol) and $Pd(PPh_3)_4$ (4 mg, 0.004 mmol) was added to a solution of protected glutamate **43** (or **44**) (0.09 mmol) in 5 mL of methylene chloride and stirred for 1 h at 25 °C. One drop of MeOH was added and the reaction stirred for 5 min. The reaction mixture was concentrated and the residue was recrystallized from CH_2Cl_2/diethyl ether.

45. 92% yield; ^{1}H NMR (DMSO-d_6) δ 9.34 (d, J = 8.1 Hz, 1H, NH), 8.04 (br s, 1H), 7.95 (d, J = 6.9 Hz, 2H), 7.83 (d, J = 7.2 Hz, 2H), 7.75 (t, J = 7.2 Hz, 1H), 7.72–7.37 (m, 10H), 7.15 (m, 1H), 6.27 (s, 1H), 5.79 (t, J = 7.6 Hz, 1H), 5.49 (t, J = 8.4 Hz, 1H), 5.40 (t, J = 4.5 Hz, 1H), 5.38 (d, J = 5.1 Hz, 1H), 4.88 (d, J = 9.6 Hz, 1H), 4.62 (br s, 1H), 4.33 (s, 2H), 4.08 (m, 1H), 3.98 (d, J = 9.0 Hz, 2H), 3.94 (s, 2H), 3.55 (d, J = 7.2 Hz, 1H), 3.48–3.15 (m, 16H), 2.25 (m, 1H), 2.22 (t, J = 6.9 Hz, 2H), 2.19 (s, 3H), 2.08 (s, 3H), 1.88–1.80 (m, 3H), 1.77 (s, 3H), 1.56 (m, 2H), 1.46 (s, 3H), 1.00 (s, 3H), 0.97 (s, 3H); LRMS (FAB) m/z 1273.2 (M).

46. 93% yield; ^{1}H NMR (DMSO-d_6) δ 9.34 (d, J = 8.1 Hz, 1H, NH), 8.02 (t, J = 5.4 Hz, 1H), 7.94 (d, J = 6.9 Hz, 2H), 7.83 (d, J = 6.9 Hz, 2H), 7.77–7.36 (m, 11H), 7.15 (m, 1H), 6.26 (s, 1H), 5.78 (t, J = 9.0 Hz, 1H), 5.49 (t, J = 8.4 Hz, 1H), 5.40 (t, J = 5.1 Hz, 1H), 5.38 (d, J = 9.4 Hz, 1H), 4.88 (d, J = 9.3 Hz, 1H), 4.61 (br s, 1H),

4.08 (m, 1H), 3.99 (d, $J=8.4$ Hz, 2H), 3.96 (d, $J=7.8$ Hz, 2H), 3.55 (d, $J=7.2$ Hz, 1H), 3.51–3.12 (m, 18H), 3.11 (s, 2H), 2.26 (s, 3H), 2.25 (m, 1H), 2.22 (t, $J=6.9$ Hz, 2H), 2.20 (s, 3H), 2.07 (s, 3H), 1.88–1.80 (m, 3H), 1.75 (s, 3H), 1.56 (m, 2H), 1.46 (s, 3H), 0.99 (s, 3H), 0.96 (s, 3H); LRMS (FAB) m/z 1286.0 (M).

Preparation of Taxol–2′-PEG-folate (47 and 48). Hünig base (25 mg, 0.2 mmol) was added to a solution of Taxol-PEG-glutamic acid **45** (or **46**) (0.04 mmol) and pteroyl azide (7 mg, 0.02 mmol) in 1.2 mL of DMSO and stirred for 44 h at 25 °C. After removing the volatile components, the reaction mixture was loaded directly onto a MPLC column (LiChroprop C18, 310×25 mm) and developed by 20 mL of 5mM NH_4HCO_3 and then 80:20 5mM NH_4HCO_3/CH_3CN, followed by 73:27 5mM NH_4HCO_3/CH_3CN for purification. After lyopholization, the desired product was obtained in low yield. **47**: 25% yield as a yellow solid; LRMS (FAB) m/z 1566.8 (M). **48**: 30% yield as a yellow solid; LRMS (FAB) m/z 1579.8 (M).

Preparation of Taxol–7-PEG-glutamate (49 and 50). DIPC (11 mg, 0.08 mmol), 2′-alloc-Taxol **15** (70 mg, 0.075 mmol), and DMAP (10 mg) were added to a solution of gulutamyl-PEG-carboxylic acid **41** (or **42**) (0.082 mmol) in 4 mL of CH_2Cl_2 at 0 °C. The resulting solution was allowed to warm to 25 °C and stirred for 12 h. The reaction mixture was washed with 0.1N HCl, dried, and concentrated, followed by column chromatography (EtOA/MeOH = 10:1) to afford the desired products.

49. 52% yield (92% BRSM); ^{1}H NMR ($CDCl_3$) δ 8.12 (d, $J=8.1$ Hz, 2H), 7.75 (d, $J=8.4$ Hz, 2H), 7.62 (t, $J=7.5$ Hz, 1H), 7.54–7.35 (m, 10H), 7.23 (br s, 1H), 7.02 (d, $J=9.0$ Hz, 1H), 6.54 (br s, 1H), 6.22 (t, $J=8.7$ Hz, 1H), 6.19 (s, 1H), 5.99 (dd, $J=9.3$, 2.4 Hz, 1H), 5.96–5.82 (m, 4H), 5.68 (d, $J=6.9$ Hz, 1H), 5.65 (dd, $J=5.5$, 3.3 Hz, 1H), 5.43 (d, $J=2.4$ Hz, 1H), 5.38–5.17 (m, 6H), 4.96 (d, $J=8.7$ Hz, 1H), 4.61 (m, 4H), 4.53 (d, $J=5.4$ Hz, 2H), 4.35–4.07 (m, 7H), 3.94 (d, $J=6.9$ Hz, 1H), 3.64–3.58 (m, 10H), 3.51 (m, 4H), 3.42 (t, $J=5.7$ Hz, 2H), 2.61 (m, 1H), 2.46 (s, 3H), 2.38 (m, 1H), 2.26 (t, $J=6.6$ Hz, 2H), 2.21 (m, 2H), 2.15 (s, 3H), 2.03 (m, 1H), 1.97 (s, 1H), 1.90 (m, 1H), 1.79 (s, 3H), 1.21 (s, 3H), 1.14 (s, 3H); LRMS (FAB) m/z 1481 (M + H), 1503 (M + Na).

50. 57% yield (98% BRSM); ^{1}H NMR ($CDCl_3$) δ 8.12 (d, $J=8.1$ Hz, 2H), 7.75 (d, $J=8.4$ Hz, 2H), 7.62 (t, $J=7.5$ Hz, 1H), 7.55–7.38 (m, 10H), 7.32 (m, 1H), 7.00 (d, $J=9.3$ Hz, 1H), 6.50 (br s, 1H), 6.23 (t, $J=8.7$ Hz, 1H), 6.22 (s, 1H), 6.00–5.85 (m, 4H), 5.99 (dd, $J=9.3$, 2.4 Hz, 1H), 5.69 (d, $J=6.9$ Hz, 1H), 5.64 (dd, $J=5.5$, 3.3 Hz, 1H), 5.43 (d, $J=2.7$ Hz, 1H), 5.39–5.18 (m, 6H), 4.97 (d, $J=8.7$ Hz, 1H), 4.62 (m, 4H), 4.54 (d, $J=5.4$ Hz, 2H), 4.42 (m, 1H), 4.34 (d, $J=8.1$ Hz, 1H), 4.19 (d, $J=8.4$ Hz, 1H), 3.95 (d, $J=6.9$ Hz, 1H), 3.64–3.42 (m, 16H), 3.44 (s, 2H), 3.23 (s, 2H), 2.61 (m, 1H), 2.46 (s, 3H), 2.41 (s, 3H), 2.37–2.04 (m, 5H), 2.14 (s, 3H), 1.98 (s, 3H), 1.93 (m, 2H), 1.80 (s, 3H), 1.21 (s, 3H), 1.15 (s, 3H); LRMS (FAB) m/z 1494.2 (M + H).

Preparation of Taxol–7-PEG-glutamic acid (51 and 52). Et_2NH (0.12 mL, 1.2 mmol) and $Pd(PPh_3)_4$ (4 mg, 0.004 mmol) was added to a solution of protected glutamate **49** (or **50**) (0.04 mmol) in 3 mL of methylene chloride and stirred for 1 h at 25 °C. The reaction mixture was concentrated and the residue was recrystallized from CH_2Cl_2/ether system to afford the desired product.

51. 93% yield; ^{1}H NMR (DMSO-d_6) δ 9.22 (d, $J=7.9$ Hz, 1H, NH), 8.02 (m, 1H), 7.93 (d, $J=7.8$ Hz, 2H), 7.89 (d, $J=7.2$ Hz, 2H), 7.73–7.37 (m, 11H), 7.29 (m, 1H), 7.20 (m, 1H), 6.02 (s, 1H), 5.87 (t, $J=7.6$ Hz, 1H), 5.45–5.35 (m, 4H), 4.93 (d, $J=8.9$ Hz, 1H), 4.81 (s, 1H), 4.63 (d, $J=8.1$ Hz, 1H), 4.03 (m, 4H), 3.91 (s, 2H), 3.67 (d, $J=7.2$ Hz, 1H), 3.47–3.13 (m, 16H), 2.22 (m, 4H), 2.18 (s, 3H), 2.09 (s, 3H), 1.81 (m, 4H), 1.73 (s, 3H), 1.62 (s, 3H), 1.01 (s, 3H), 0.95 (s, 3H); LRMS (FAB) m/z 1272.8 (M).

52. 96% yield; ^{1}H NMR (DMSO-d_6) δ 9.22 (d, $J=8.7$ Hz, 1H, NH), 8.01 (t, $J=5.4$ Hz, 1H), 7.94 (d, $J=7.2$ Hz, 2H), 7.88 (d, $J=6.6$ Hz, 2H), 7.73–7.59 (m, 4H), 7.55–7.41 (m, 3H), 7.37 (m, 4H), 7.19 (m, 1H), 6.04 (s, 1H), 5.87 (t, $J=7.6$ Hz, 1H), 5.44–5.32 (m, 4H), 4.94 (d, $J=8.9$ Hz, 1H), 4.79 (s, 1H), 4.63 (d, $J=8.9$ Hz, 1H), 4.43 (m, 1H), 4.02 (br s, 2H), 3.70 (d, $J=7.2$ Hz, 1H), 3.47–3.15 (m, 18H), 3.03 (s, 2H), 2.25 (2, 3H), 2.20 (m, 4H), 2.17(s, 3H), 2.08 (s, 3H), 1.80 (m, 4H), 1.73 (s, 3H), 1.63 (s, 3H), 1.01 (s, 3H), 0.95 (s, 3H); LRMS (FAB) m/z 1286.0 (M).

Preparation of Taxol–7-PEG-folate (53 and 54). Hünig base (25 mg, 0.2 mmol) was added to a solution of Taxol–PEG-glutamic acid **51** (or **52**) (0.023 mmol) and Pteroyl azide (5 mg, 0.015 mmol) in 1.2 mL of DMSO and stirred for 44 h at 25 °C. After removing the volatile components, the reaction mixture was loaded directly onto a MPLC column (LiChroprop C18, 310×25 mm) and developed by 20 mL of 5 mM NH_4HCO_3 and then 80:20 5 mM NH_4HCO_3/CH_3CN, followed by 70:30 5 mM NH_4HCO_3/CH_3CN for purification. After lyopholization obtained the desired product. **53**: 39% yield as a yellow solid; LRMS (FAB) m/z 1566.8 (M). **54**: 35% yield as a yellow solid; LRMS (FAB) m/z 1581.2 (M + H).

Preparation of azido-PEG-iminodiacetic diallylester (55). K_2CO_3 (1.42 g, 10.3 mmol) was added to a solution of azido-PEG-amine **20** (0.9 g, 4 mmol) and allyl bromoacetate (1.5 g, 8.7 mmol) in 40 mL of acetonitrile and stirred for 30 h at room temperature. The reaction mixture was filtered and evaporated. The residue was dissolved in 100 mL of methylene chloride and then washed with brine, dried, and concentrated to afford the desired product **55** (1.65 g, 97%). ^{1}H NMR ($CDCl_3$) δ 5.95–5.86 (m, 2H), 5.34–5.21 (m, 4H), 4.59 (d, $J=4.8$ Hz, 4H), 3.68–3.56 (m, 16H), 3.38 (t, $J=5.1$ Hz, 2H), 2.97 (t, $J=5.7$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.1, 132.1, 118.6, 70.75, 70.73, 70.67, 70.50, 70.41, 70.11, 65.17, 55.85, 53.7, 50.7.

Preparation of azido-PEG-iminodiacetic acid (56). $PhSiH_3$ (0.92 mL, 7.4 mmol) and $Pd(PPh_3)_4$ (80 mg,

0.068 mmol) was added to a solution of diallyl ester **55** (0.7 g, 1.7 mmol) in 17 mL of methylene chloride and stirred for 1 h at 25 °C. Then 0.5 mL of MeOH added and stirred for 5 min. The reaction mixture was concentrated and the residue was washed with EtOAc twice by decantation to afford the desired product **56** (0.52 g, 92%). ^{1}H NMR ($CDCl_3$) δ 3.87 and 3.75 (br s, 4H), 3.65–3.60 (m, 12H), 3.36 (t, J = 5.1 Hz, 4H).

Preparation of azido-PEG-iminodiacetic anhydride (57). A solution of DCC (0.28 g, 1.38 mmol) in 4 mL of CH_2Cl_2 was added to a solution of diacid **56** (0.52 g, 1.56 mmol) in 10 mL of CH_2Cl_2 at 0 °C and stirred for 5 h at 25 °C. 10 mL of hexane was added and then stirred for 5 min. The resulting solution was filtered and concentrated to give the desired product **57** as a crude anhydride, which was used in the next reaction. ^{1}H NMR ($CDCl_3$) δ 3.69–3.59 (m, 16H, including a singlet for two CH_2), 3.39 (t, J = 5.1 Hz, 4H), 2.81 (t, J = 5.1 Hz, 4H).

Preparation of glutamyl-PEG-carboxylic acid (58). A solution of amino-PEG-glutamate **36** (0.45 g, 1 mmol) and azido-PEG-iminodiacetic anhydride **57** (from above reaction) in 10 mL of CH_2Cl_2 was stirred for 12 h at 25 °C. The reaction solution was concentrated and chromatographed with CH_2Cl_2/MeOH (5:1) or EtOAc/MeOH (1:1) to afford the desired product **58** (0.59 g, 78% for two steps). ^{1}H NMR ($CDCl_3$) δ 7.92 (br s, 1H, NH), 6.71 (br s, 1H, NH), 5.95–5.84 (m, 3H including NH), 5.36–5.19 (m, 4H), 4.63 (d, J = 5.7 Hz, 2H), 4.56 (d, J = 5.4 Hz, 2H), 4.34 (m, 1H), 3.70–3.54 (m, 24H), 3.51–3.34 (m, 10H, m which has two singlets and three triplets), 2.91 (t, J = 4.8 Hz, 2H), 2.33 (t, J = 6.6 Hz, 2H), 2.21 (m, 1H), 2.04 (m, 1H).

Preparation of 7-glutamyl-PEG-aminoacetyl–Taxol (59). A solution of DCC (49 mg, 0.24 mmol) and 5 mg of DMAP in 1 mL of CH_2Cl_2 was added to a solution of acid **58** (200 mg, 0.26 mmol) and 2′-alloc-Taxol **15** (154 mg, 0.16 mmol) in 4 mL of CH_2Cl_2 at 0 °C. The resulting solution was allowed to warm to 25 °C and stirred for 12 h. The reaction mixture was filtered, concentrated, and chromatographed from EtOAc and then EtOAc/MeOH (10:1) to afford the desired product **59** (256 mg, 93%). ^{1}H NMR ($CDCl_3$) δ 8.13 (d, J = 6.9 Hz, 2H), 7.90 (br s, 1H), 7.75 (d, J = 6.9 Hz, 2H), 7.63 (t, J = 7.2 Hz, 1H), 7.55–7.48 (m, 4H), 7.46–7.36 (m, 6H), 6.96 (d, J = 9.3 Hz, 1H, NH), 6.51 (br s, 1H, NH), 6.26 (t, J = 8.4 Hz, 1H), 6.21 (s, 1H), 5.98 (dd, J = 9.3, 2.4 Hz, 1H), 5.95–5.83 (m, 4H), 5.69 (d, J = 6.9 Hz, 1H), 5.60 (dd, J = 6.9, 3.0 Hz, 1H), 5.43 (d, J = 2.4 Hz, 1H), 5.39–5.18 (m, 6H), 4.96 (d, J = 8.4 Hz, 1H), 4.66–4.58 (m, 4H, two allylic CH_2), 4.55 (d, J = 5.4 Hz, 2H), 4.43 (m, 1H), 4.34 (A of AB, d, J = 8.4 Hz, 1H), 4.18 (B of AB, d, J = 8.4 Hz, 1H), 3.94 (d, J = 6.6 Hz, 1H), 3.70–3.50 (m, 24H), 3.49–3.36 (m, 10H, m which has two singlets and three triplet), 2.87 (m, 2H), 2.62 (m, 1H), 2.46 (s, 3H), 2.38 (m, 1H), 2.31 (t, J = 6.6 Hz, 2H), 2.23 (m, 2H), 2.14 (s, 3H), 2.02 (m, 1H), 1.98 (s, 3H), 1.83 (m, 1H), 1.80 (s, 3H), 1.21 (s, 3H), 1.14 (s, 3H); LRMS (FAB) m/z 1682.2 (M + H).

Preparation of Taxol–7-aminoacetyl-PEG-glutamic acid (60). Et_2NH (0.28 mL, 2.7 mmol) and $Pd(PPh_3)_4$ (3 mg, 0.003 mmol) was added to a solution of protected glutamate **59** (150 mg, 0.09 mmol) in 5 mL of methylene chloride and stirred for 1 h at 25 °C. The reaction mixture was concentrated and the residue was recrystallized from CH_2Cl_2/ether to afford the desired product **60** (126 mg, 96%). ^{1}H NMR (DMSO-d_6) δ 9.23 (d, J = 8.7 Hz, 1H, NH), 8.03 (t, J = 5.4 Hz, 1H), 7.94 (d, J = 7.2 Hz, 2H), 7.88 (d, J = 6.6 Hz, 2H), 7.73–7.59 (m, 4H), 7.55–7.41 (m, 3H), 7.37 (m, 4H), 7.19 (m, 1H), 6.04 (s, 1H), 5.87 (t, J = 7.6 Hz, 1H), 5.44–5.32 (m, 4H), 4.94 (d, J = 8.9 Hz, 1H), 4.79 (s, 1H), 4.63 (d, J = 8.9 Hz, 1H), 4.43 (m, 1H), 4.02 (br s, 2H), 3.70 (d, J = 7.2 Hz, 1H), 3.48–3.15 (m, 34H), 2.68 (br s, 2H), 2.20 (m, 4H), 2.17 (s, 3H), 2.08 (s, 3H), 1.80 (m, 4H), 1.73 (s, 3H), 1.63 (s, 3H), 1.01 (s, 3H), 0.95 (s, 3H); LRMS (FAB) m/z 1474.0 (M + H).

Preparation of Taxol–azido-PEG-Folate (61). MTBD (24 mg, 0.157 mmol) was added to a solution of Taxol–PEG-glutamic acid **60** (110 mg, 0.075 mmol) and pteroyl azide (28 mg, 0.082 mmol) in 1.5 mL of DMSO which became homogeneous within 30 s. The solution was left to stir for 2 h at 25 °C and then loaded directly onto a MPLC column (LiChroprop C18, 310×25 mm) and developed by 20 mL of 5mM NH_4HCO_3 and then 80:20 5mM NH_4HCO_3/CH_3CN, followed by 70:30 5mM NH_4HCO_3/CH_3CN for purification. After lyopholization the desired product **61** was obtained (57 mg, 43%) as a yellow solid. LRMS (FAB) m/z 1767.2 (M).

Preparation of Taxol–amino-PEG-folate (62). PhSH (0.12 mmol) and Et_3N (0.09 mmol) were added to a solution of $SnCl_2$ (0.03 mmol) in 4 mL of DMF. After 5 min, azide compound (0.02 mmol) was added and stirred for 1 h at 25 °C. MeOH (2 mL) was added and the reaction was stirred for 1 h. The resulting solution was concentrated to leave ca. 1 mL of volume and then EtOAc/CH_2Cl_2 (5:1) added to effect precipitation. The solid was taken after centrifugation by discarding the supernatant and washed with CH_2Cl_2/EtOAc (5:1). 95% yield; LRMS (PDMS) m/z 1742.6 (M + H).

Preparation of Y-shaped Taxol–folate-DTPA (63). Amino-Taxol–folate **62** (8 mg, 0.0037 mmol) was added to a solution of DTPA-dianhydride (5.2 mg, 0.015 mmol) in 2 mL of DMSO and then water (0.0026 mmol) was added after 4 min. The resulting solution was stirred for 3 h at 25 °C and was then loaded directly onto a MPLC column (LiChroprop C18, 310×25 mm) and developed by 50 mL of 5 mM NH_4HCO_3, 150 mL of 90:10 5 mM NH_4HCO_3/CH_3CN, 200 mL of 85:15 5 mM NH_4HCO_3/CH_3CN, and then 80:20 5 mM NH_4HCO_3/CH_3CN for purification. After lyopholization the desired product **63** (80%) was obtained as a yellow solid. LRMS (MALDI) m/z 2117 (M).

Biochemical materials. ^{3}H folic acid was purchased from Amersham (Arlington Heights, NY, USA). Cremophor EL and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). KB (human nasopharyngeal

epidermal carcinoma) and A549 (human lung carcinoma) cell cultures were received as a gift from the Purdue Cancer Center (West Lafayette, IN, USA). M109 (murine lung carcinoma) was provided by Dr. Alberto Gabizon, Sharet Institute of Oncology, Hadassah-Hebrew University Medical Center and Medical School, Jerusalem, Israel[25] All tissue culture products were supplied by Gibco BRL (Grand Island, NY, USA). All other chemicals were obtained from major suppliers.

Cell culture and tumor model

Monolayers of KB and A549 cell lines were grown continuously in folate-deficient Dulbecco's modified Eagles medium supplemented with 10% v/v heat-inactivated fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine (FDMEM) at 37 °C in a humidified atmosphere containing 5% CO_2. M109 cells were cultured in folate-deficient RPMI-1640 (Gibco BRL) supplemented with 10% v/v heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Female Balb/c mice were purchased from Harlan Labs, Indianapolis, IN, USA and used for therapy studies as soon as they reached 5–6 weeks of age. Mice were fed on a folate-deficient diet starting 2 weeks prior to tumor inoculation and ending 1 day after the last drug treatment. All animal experiments were carried out in accordance with procedures approved by the Purdue Animal Care and Use Committee. M109 tumors were serially maintained as subcutaneous tumors in female Balb/c mice at 2–3-week intervals. At appropriate times, subcutaneous tumors were harvested and regenerated according to established procedures.[25] For intraperitoneal (ip) tumor implant, 5×10^5 M109 cells at an early passage (P0 or P1) were implanted in 400 μL volume into each animal.

^{3}H Folic acid competition study. The Taxol–folate conjugates' competition with ^{3}H folic acid for cell surface receptor binding was studied at 4 °C. Briefly, ~90% confluent cells in 24-well plates were incubated with 10 nM ^{3}H folic acid plus various amounts of **53**, **54**, **61**, **63** or non-radioactive folate at a concentration range of 0.01–10 μM. After 1 h incubation and subsequent washing with cold PBS, cell-surface bound ^{3}H folic acid was recovered in 0.5 mL acid saline (150 mM NaCl, 10 mM sodium acetate, pH 3.5) and subjected to liquid scintillation counting. The ligand-stripped cells were lysed in 0.5 mL of 1% Triton X-100/PBS solution and assayed for the protein content by the standard BCA method. Each data point was obtained in triplicate.

MTT assay

The cytotoxicity of **54** and free Taxol **14** towards cultured tumor cells was determined using the MTT cell viability assay.[26] For concentration-dependent cytotoxicity studies, KB cells were plated into 96-well plates at a density of 5×10^4 cells/well in 150 μL of FDMEM. The cells were incubated for 24 h at 37 °C before being exposed to 150 μL of FDMEM containing (a) Taxol, (b) **54**, or (c) **54** plus >500× molar excess of free folic acid at drug concentrations ranging from 1.9×10^{-12} to 5×10^{-7} M for taxol 14 and 4.0×10^{-12} to 1.05×10^{-6} M for **54**. For drug solubility purposes, <0.085% of cremophor/ethanol (1:1) was present in the growth media including the control. After incubation for 18 h at 37 °C, the cells were washed 2× with warm growth medium to remove the unbound drug and incubated further in fresh FDMEM until the control cells became confluent (~24 h later). Thereafter, 15 μL of 5 mg/mL MTT dissolved in PBS, pH 7.4, was added to each well and the cells were stained for 2 h at 37 °C. The formazan crystals that formed were solubilized in 150 μL isopropanol containing 0.01N HCl, and the number of viable cells in each well was determined from the absorbance at 570 nm in a 96-well plate reader. For analyses of cell cytotoxicity at a single-concentration, KB, M109 or A549 cells were plated in 24-well plates at ~30% confluence and treated with 2×10^{-7} M Taxol **14** or **54** for 18 h at 37 °C [all growth media contained <0.034% of cremophor/ethanol (1:1)]. Afterwards, the cells were washed 2× and incubated further until the control cells were confluent. MTT (50 μL of 5 mg/mL) was then added to each well containing 450 μL FDMEM. MTT-stained viable cells were solubilized in 1 mL of isopropanol containing 0.01N HCl. The number of viable cells in each well was determined from the absorbance at 570 nm.

Comparative toxicity and antitumor activity in vivo

Taxol **14** and **54** were first dissolved in 100% cremophor followed by 1:1 dilution with ethanol and stored at 4 °C. The stock solution in 1:1 cremophor/ethanol was further diluted 1:4 with PBS, pH 7.4, and used within 30 min. Thus, the injection vehicle consisted of cremophor/ethanol/PBS at a volume ratio of 1:1:8. For general toxicity studies, four groups of healthy tumor-free mice at 3 mice/group were used. The mice (adapted to folate-deficient diet) were ip injected with (a) PBS, (b) injection vehicle, (c) Taxol **14** or (d) **54** at a Taxol equivalent dose of 25 mg/kg/day (i.e., 46 mg/kg/day of **54**). The dosing schedule used consisted of a total of four injections at 2-day intervals. All mice were monitored and weighed every other day to assess any general toxicity associated with the drug treatment. The antitumor activity study was conducted in four groups (a–d) of mice at 10–12 mice per group. The groups were designated as follows: (a) untreated control; (b) injection vehicle only; (c) Taxol **14** and (d) **54** formulated in the same injection vehicle. Starting on day 4 after ip implantation of M109 tumor cells, mice were either left untreated (a) or treated with 0.5 mL of the injection vehicle (b), or 17.2 mg/kg/day of Taxol **14** (c), or 32.4 mg/kg/day of **54** (d). All treatments were performed ip at a schedule of q2d×8, that is, a total of eight ip injections at 2-day intervals. Daily survival of the animals was recorded as a measure of therapeutic efficacy due to treatment. Animals that survived over 88 days were considered cured when no macroscopic tumors were found in the peritoneal cavity after euthanasia.

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References and Notes

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