



Paclitaxel succinate analogs: Anionic and amide introduction as a strategy to impart blood–brain barrier permeability

Brandon J. Turunen^a, Haibo Ge^a, Jariat Oyetunji^a, Kelly E. Desino^b, Veena Vasandani^b, Sarah Güthe^c, Richard H. Himes^d, Kenneth L. Audus^b, Anna Seelig^c, Gunda I. Georg^{a,*}

^a Department of Medicinal Chemistry, University of Kansas, Malott Hall 4070, 1251 Wescoe Hall Drive, Lawrence, KS 66045, USA

^b Department of Pharmaceutical Chemistry, University of Kansas, 226 Simons, 2095 Constant Avenue, Lawrence, KS 66047, USA

^c Department of Biophysical Chemistry, Biocenter of the University of Basel, Klingelbergstrasse 70, 4065 Basel, Switzerland

^d Department of Molecular Biosciences, University of Kansas, Haworth Hall, 1200 Sunnyside Avenue, Lawrence, KS 66045, USA

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ABSTRACT

A focused library of TX-67 (C10 hemi-succinate) analogs has been prepared, including C7 regioisomers, esters, amides, and one-carbon homologs. These were prepared to investigate whether the lack of TX-67 interaction with P-glycoprotein (Pgp) is due to the presence of the carboxylic acid moiety and whether this phenomenon was restricted to C10 analogs. Tubulin stabilization ability, cytotoxicity, and Pgp interactions were evaluated. All carboxylic acid analogs and several of the amides had no apparent interactions with Pgp at the concentrations used, whereas the ester variants displayed characteristics of Pgp substrates. Furthermore, it was demonstrated that hydrogen-bonding properties were significant with respect to Pgp interactions. Calculations of log*D* and cross-sectional areas revealed that these analogs are predicted to partition into the membrane and can compete for Pgp binding sites. The anionic and amide introduction strategy may allow for delivery of paclitaxel into the CNS and may be a potential approach for the delivery of other, structurally complex and lipophilic non-CNS permeable drugs.

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Paclitaxel (Taxol, **1**, Fig. 1), a structurally complex natural product derived from the bark of the Pacific Yew, is one of the most studied and active anti-cancer agents known.^{1–3} Although its clinical success is remarkable, the efficacy of the parent compound has limitations.^{4,5}

One such shortcoming is paclitaxel's inability to cross the blood–brain barrier (BBB).^{6,7} Accordingly, paclitaxel is not an effective treatment for primary or metastatic brain cancer despite its potent anti-proliferative activity. In addition to paclitaxel's well known anti-tumor properties, it has been shown to protect primary cortical neurons from beta amyloid (A β)-induced cell death as well as being non-toxic to primary cortical neurons.⁸ Indeed, a paclitaxel derivative that could permeate the CNS is highly desirable from both the standpoint of chemotherapy as well as an investigational therapy for neurofibrillary pathology.

A primary mechanism limiting the distribution of paclitaxel and other highly lipophilic substances into the brain is active efflux by the multidrug resistance gene product 1 (MDR1) P-glycoprotein (Pgp).^{9–11} We have recently described a series of recognition elements required for Pgp interactions based upon the analysis of over one hundred known Pgp substrates.^{12–14} This analysis revealed clusters of spatially distinct hydrogen bond acceptor units, which correlated, in their relative frequency, with the strength of Pgp interaction. We have demonstrated that deletion or modification of these “recognition elements” in paclitaxel reduces interaction with Pgp in bovine brain microvessel endothelial cells.^{15,16} These studies also bolstered our ascertainment that the C10 region of paclitaxel is particularly important for Pgp affinity.

This same examination^{12–14} of known Pgp substrates also revealed that compounds that carry a negative charge at physiological pH, such as those that contain a carboxylic acid, sulphonate, or nitro group, with few exceptions, are not substrates for Pgp efflux.^{12–14} One caveat to this observation was that chemical structures that contain additional recognition elements, maintain their affinity for Pgp.

* Corresponding author. Tel.: +1 612 626 6320; fax: +1 612 626 6318.
E-mail address: georg239@umn.edu (G.I. Georg).

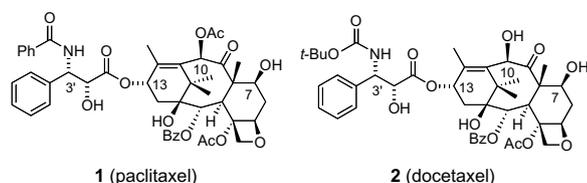


Figure 1. Paclitaxel and docetaxel.

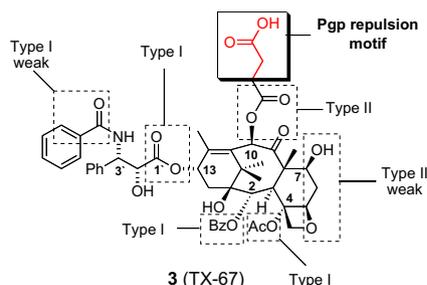
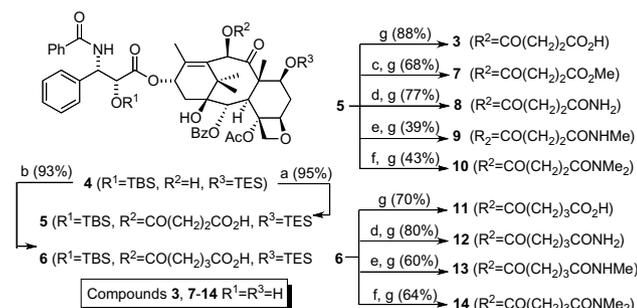


Figure 2. TX-67 recognition and repulsion elements.

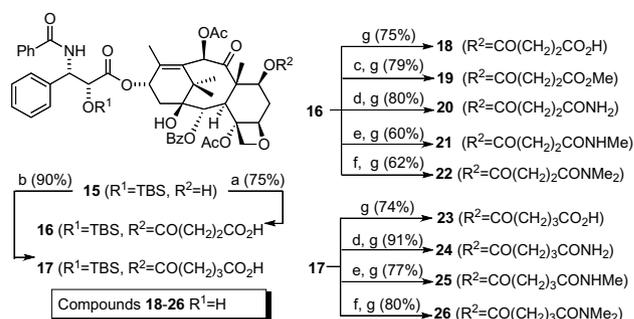
With this in mind we prepared a C10-modified taxane, TX-67 (**3**, Fig. 2), which only differs from paclitaxel by the addition of an acetic acid unit to the terminus of the C10 acetyl ester.¹⁷ TX-67 (**3**) contains all of the recognition elements of paclitaxel, however, our studies suggest Pgp efflux mechanisms are substantially reduced or absent completely.^{17,18} Compound **3** demonstrates improved distribution across the BBB without co-administration of Pgp inhibitors both in vitro and in situ.¹⁷

Herein, we describe the synthesis and biological evaluation of a focused library of TX-67 analogs that include C7 regioisomers, esters, amides, and one-carbon homologs. These were made to determine if the acid functionality was essential for Pgp evasion and if this phenomenon was restricted to only C10 analogs.

The C10 functional group analogs were prepared from common intermediate **4** (Scheme 1). TX-67 (**3**) was prepared as previously described¹⁷ while compounds **7–10** were accessed via acid **5**. Acid **5** is cleanly and quantitatively converted to the methyl ester via treatment with diazomethane (Scheme 1), which, after treatment with HF-pyridine solution provides methyl ester **7** in good yield. The primary amide analog **8** is prepared via mixed anhydride formation with BOC₂O followed by amide formation via ammonium bicarbonate (NH₄CO₃) under basic conditions. The *N*-methyl and *N,N*-dimethyl, amides (**9** and **10**) are generated via standard coupling procedures (Scheme 1). The described functional group transformations were each followed by fluoride anion assisted



Scheme 1. Synthesis of C10-modified TX-67 analogs. Reagents and conditions: (a) succinic anhydride, DMAP, toluene 85 °C; (b) glutaric anhydride, DMAP, toluene 85 °C; (c) CH₂N₂, THF; (d) NH₄HCO₃, THF, BOC₂O, pyridine; (e) NH₂Me·HCl, EDCI, NMM, CH₂Cl₂; (f) NHMe₂·HCl, EDCI, NMM, CH₂Cl₂; (g) HF-pyridine, pyridine.



Scheme 2. Synthesis of C7 TX-67 analogs.¹⁹ Reagents and conditions: (a) succinic anhydride, DMAP, toluene 85 °C; (b) glutaric anhydride, DMAP, toluene 85 °C; (c) MeOH, EDCI, DMAP; (d) NH₄HCO₃, THF, BOC₂O, pyridine; (e) NH₂Me·HCl, EDCI, NMM, CH₂Cl₂; (f) NHMe₂·HCl, EDCI, NMM, CH₂Cl₂; (g) HF-pyridine, pyridine.

protecting group removal. Compounds **11–14** were prepared in the same manner from glutaric acid derivative **6**.

The desired C7 analogs were accessed in short synthetic sequences from common intermediate **15** (Scheme 2). The C7 succinic acid analog **18** was prepared by acylating the C7 hydroxyl group with succinic anhydride, giving **16**, followed by removing the 2'OTBS protecting group. The methyl ester analog **19** was generated from a coupling between acid **16** and MeOH followed by the same deprotection. The remaining compounds **21–26** were synthesized as described for the C10 analogs.

All compounds were first evaluated for tubulin assembly ability and their effectiveness against the MCF7 breast cancer cell line and the drug resistant breast cancer cell line NCI/ADR-RES (Table 1).

In the tubulin assembly assay, all analogs maintained similar activity to the parent compound (entries 2–19, Table 1). Against the MCF7 breast cancer cell line most analogs maintained effectiveness. The carboxylic acid analogs (entries 2–5), as a functional group class, showed the largest drop in activity (approx. 20-fold). The methyl ester variants (entries 6 and 7) maintained similar potency as compared to paclitaxel while the amides (entries 8–19) generally experienced an approximate 5-fold reduction in cytotoxicity. Most analogs were similar to paclitaxel against the NCI/ADR-RES cancer cell line. We had originally hypothesized an increase in effectiveness of these analogs against the Pgp over-

Table 1

ED₅₀ ratios (compound/paclitaxel) for in vitro tubulin assembly and cytotoxicity.

Entry	Compound	Tubulin assembly	MCF7	NCI/ADR-RES
1	Paclitaxel (1)	1.0	1.0	1.0
2	TX-67 (3 – C10 CO ₂ H)	1.7	13.3	1.3
3	C10 CH ₂ CO ₂ H (11)	1.8	27.9	5.8
4	C7 CO ₂ H (18)	3.8	>30.0	5.8
5	C7 CH ₂ CO ₂ H (23)	1.0	8.8	13.6
6	C10 CO ₂ Me (7)	1.0	2.5	0.4
7	C7 CO ₂ Me (19)	1.2	0.54	0.5
8	C10 CONH ₂ (8)	0.4	10	6.0
9	C10 CH ₂ CONH ₂ (12)	1.8	10	5.3
10	C7 CONH ₂ (20)	0.6	3.2	0.6
11	C7 CH ₂ CONH ₂ (24)	0.6	5.0	6.5
12	C10 CONHMe (9)	0.8	7.9	6.0
13	C10 CH ₂ CONHMe (13)	1.8	2.3	>6.3
14	C7 CONHMe (21)	0.8	2.2	1.0
15	C7 CH ₂ CONHMe (25)	0.8	3.7	3.2
16	C10 CONMe ₂ (10)	1.6	2.1	2.7
17	C10 CH ₂ CONMe ₂ (14)	2.2	5.6	>6.3
18	C7 CONMe ₂ (22)	1.1	10.8	0.8
19	C7 CH ₂ CONMe ₂ (26)	0.7	3.9	3.0

Paclitaxel has a mean ED₅₀ of 3.2 nM ± 1.8 and 1.5 μM ± 1.3 in the MCF7 and NCI/ADR-RES lines, respectively.

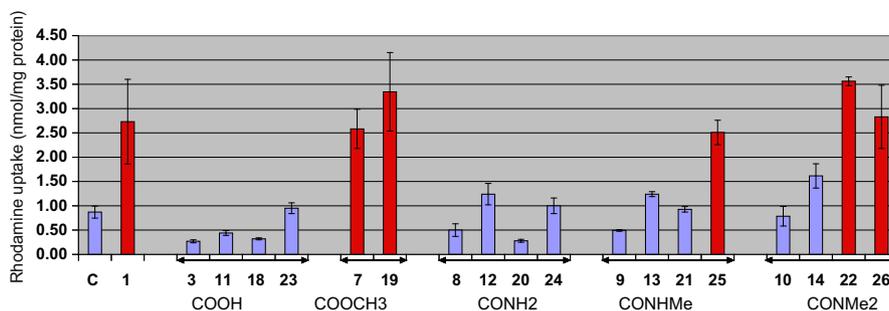


Figure 3. Rhodamine uptake results for compounds **1** (paclitaxel), C10 and C7 acids (**3**, **11**, **18**, and **23**), C10 and C7 esters (**7** and **19**), C10 and C7 primary amides (**8**, **12**, **20**, and **24**), C10 and C7 secondary amides (**9**, **13**, **21**, and **25**), and C10 and C7 tertiary amides (**10**, **14**, **22**, and **26**) in BMECs. Paclitaxel and the derivatives were present at a concentration of 10 μ M. The concentration of rhodamine was 5 μ M. C, control. Compounds that significantly increase rhodamine 123 uptake are shown as red bars. Compounds causing decreased or limited rhodamine 123 uptake are shown in blue.

expressing cancer cell line MCF7-ADR. However, this was not the case as none of the analogues in Table 1 had superior potency compared to paclitaxel.

Our next screen was the rhodamine 123 uptake assay (Fig. 3).^{20,21} This assay is a preliminary screen to evaluate a compound's interaction with Pgp in bovine brain microvessel endothelial cells (BMECs). In this assay, rhodamine 123 is used as a surrogate Pgp substrate. The effect of the test compound on rhodamine 123 is determined by monitoring intracellular fluorescence. If the test compound has higher affinity to Pgp than rhodamine 123, then uptake of the latter will increase relative to the negative control.

All analogs containing a carboxylic acid functionality (**3**, **11**, **18**, and **23**) showed no apparent interaction with Pgp. When the carboxylic acid is capped with a methyl group (methyl esters **7** and **19**), a type I Pgp recognition element, in both the C7 and C10 series, a marked increase in rhodamine accumulation is observed. This is in agreement with our previous results suggesting that the carboxylic acid functionality is advantageous for Pgp evasion.¹⁷

Many members of the amide series (**8**–**10**, **12**–**14**, **20**, **21**, and **24**) did not significantly increase rhodamine accumulation. The weak interaction of secondary and primary amides with Pgp had already been suggested by us in an analysis of Pgp substrates and non-substrates.¹² As the secondary amides are converted from hydrogen bond donors (**8**, **9**, **12**, **13**, **20**, **21**, and **25**) to tertiary amides, hydrogen bond acceptors (**10**, **14**, **22**, and **26**), increased rhodamine uptake is noted indicating that the molecules are interacting more strongly with Pgp. This is in accord with our hypothesis that H-bond donors will not interact significantly with Pgp and that H-bond acceptors will serve as substrates.

It is also of note that the succinic amides **8**, **20**, **9**, **21**, and **10** showed less rhodamine uptake than the corresponding one carbon homologs, the glutaric amides **12**, **24**, **13**, **25**, and **14**, indicating that the length of the tether plays a role in binding to Pgp.

Our data further suggest that groups added to decrease Pgp interactions are generally more effective at the C10 position on the paclitaxel structure than on the C7 position. For example, the 10-linked methylamides **9** and **13** showed less rhodamine uptake than the 07-linked methylamides **21** and **25**. This implies that the C10 region on these analogs has a very intimate relationship with Pgp.

Our results suggest that Pgp evasion may not only be achieved by the introduction of a carboxylic acid moiety but also by the introduction of a primary or secondary amide into Pgp substrates.

To compare the different analogs with respect to their ability to partition into the membrane and bind to Pgp we modeled the membrane-binding conformations and calculated the respective cross-sectional areas, A_D . In addition we calculated the $\log D$ values at pH 7.4 using an ionization constant pK_a 4.2 for the carboxylic acid group (Fig. 4).¹¹ The former varied between $\log D$ 0 and 4

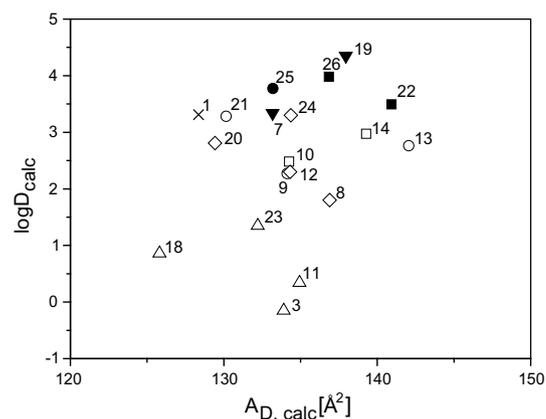


Figure 4. Calculations of cross-sectional areas, A_D and $\log D$ values at pH 7.4 for paclitaxel and analogues. \times = paclitaxel, Δ = CO_2H , \blacktriangledown = CO_2Me , \diamond = CONH_2 , \circ = CONHMe , \bullet = CONHMe , \square = CONMe_2 , \blacksquare CONMe_2 (filled symbols = Pgp substrates).

and the latter between A_D 125 and 143 \AA^2 . The analogs of paclitaxel are thus clearly more hydrophobic and larger than rhodamine 123 with a $\log D$ – 3.13 and an A_D 69 \AA^2 and can therefore be predicted to compete for Pgp binding sites provided they carry the relevant recognition elements.

In summary, we have prepared a focused library of paclitaxel analogs in short synthetic sequences from the parent molecule. Biological evaluation in tubulin stabilization and cytotoxicity assays demonstrated that the designed analogs maintained the desired properties of paclitaxel. The rhodamine assay indicated that the introduction of esters at C10 and C7 increased rhodamine 123 uptake, whereas the placement of a carboxylic acid functionality on either the C7 or C10 position resulted in decreased uptake. Since carboxylic acid transporters are known to exist in the BBB, it should also be considered that the carboxylic acid functionality may be a substrate for an influx pump, in a similar fashion that paclitaxel acts as a substrate for cellular efflux. It is possible that this influx transport system is shuttling TX-67 into the cell, past the Pgp efflux system, allowing for the observed increase in BBB permeation. Furthermore, we have illustrated that positioning an acid or amide at C10 of paclitaxel is generally more effective than C7 attachment and that succinic acid and succinic amides lead to less rhodamine 123 uptake than the corresponding glutaric acid and amide analogs. We have also discovered that the hydrogen-bonding character of the amide analogs plays a significant role and that the primary amides were generally superior compared to the secondary and tertiary amides analogs in evading interaction with Pgp.

The anionic introduction strategy and the introduction of a primary amide into the molecule may allow for delivery of paclitaxel into the CNS. This strategy could also hold promise for the delivery of other, highly complex, non-CNS permeable drugs, provided they are highly lipophilic.

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