Anti-Cancer Drugs 2001, **12,** pp. 117–128

Report

Hydrolyzable hydrophobic taxanes: synthesis and anti-cancer activities

Shaukat Ali,[1] Imran Ahmad,[1] Andrew Peters,[1] Gregg Masters,[1] Sharma Minchey,[1] Andrew Janoff[1] and Eric Mayhew[1]

[1]The Liposome Company, Inc., One Research Way, Princeton, NJ 08540, USA.

A series of taxane prodrugs with 2-bromoacyl chains attached at the 2′-position of the paclitaxel side chain, varying from six, eight, 12, 14 to 16 carbons in length, were synthesized, characterized and evaluated against human breast MCF-7 cancer cell line for their growth inhibitory (GI_{50}) activities. The GI_{50} is the drug concentration required to inhibit cell growth by 50%. For comparison, hydrophobic taxanes varying in acyl chain lengths from six to 16 carbons were also synthesized and compared for their $G0_{50}$s with taxanes having equivalent bromoacyl chain lengths. The bromoacyl taxanes bearing six, eight and 12 carbon acyl chain lengths had GI_{50} values very similar to parent paclitaxel. The GI_{50} was 3 nM for three taxanes versus 1 nM for paclitaxel on the MCF-7 cell line. Increasing the acyl chain length to 14 or 16 carbons resulted in a significant decrease in cytotoxicity and an increase in the GI_{50} to 20 or 70 nM, respectively. In general, the GI_{50} values were directly related to the bromoacyl chain lengths in cultured tumor cells. Unlike bromoacyl taxanes, the taxanes lacking bromine in their acyl chain composition were 50- to 250-fold less active, suggesting that the heteroatom facilitated the hydrolysis of acyl chains to yield free paclitaxel. These differences in growth inhibitory activities may indirectly reflect differences in the susceptibility of the acyl chain to bromine-induced hydrolysis after association of the derivative with cell membranes. Liposome formulations of 2-bromoacyl taxanes bearing six, eight, 12 and 16 carbons were prepared and tested in SCID mice against a xenografted human ovcar-3 ovarian tumor. *In vivo* results showed that bromoacyl taxanes with a longer chain were therapeutically more efficacious than those with a short chain, presumably due to slow hydrolysis of the prodrug followed by sustained delivery of paclitaxel to the tumor. [© 2001 Lippincott Williams & Wilkins.]

Key words: Cancer, cytotoxicity, formulation, paclitaxel, prodrug, synthesis.

Correspondence to S Ali, The Liposome Company, Inc., One Research Way, Princeton, NJ 08540, USA.
Tel: (+1) 609 580-3411; Fax: (+1) 609 520-8250;
E-mail: sali@lipo.com

Introduction

Taxol®, paclitaxel formulated in Cremophor EL/ethanol, is an important anticancer agent clinically active against ovarian, breast, lung and other cancers.[1] It interacts with microtubules, leading to their stabilization, which in turn leads to inhibition of cell proliferation.[2] Taxol has clinical activity against several human cancers and has serious but manageable side-effects. Similar to most cancer chemotherapeutic agents, paclitaxel is not entirely cancer selective, can be cross-resistant with other agents and is difficult to formulate. In view of these problems there have been intense ongoing efforts to synthesize and develop new taxane analogs, derivatives or related prodrugs with improved therapeutic profiles compared to the parent compound.[3] Most of these studies have been directed to forming more water-soluble compounds to simplify formulation and to reduce excipient-related side-effects. Our aim was to design hydrophobic taxane derivatives, including prodrugs, which can be stably associated in relatively non-toxic lipid carrier systems.[4] Recently, we have demonstrated that taxane association with membrane bilayer varies with the acyl chain composition of both taxanes and lipids.[5] The long-chain taxanes associated with lipid bilayers more effectively compared with short-chain derivatives. Using these lipid carriers as excipients will enable a more selective delivery of the taxane prodrug to tumors where it is either directly active or is hydrolyzed to paclitaxel. Slow release of the drug from the carrier and/or relatively slow hydrolysis of the acyl chain at the tumor may result in prolonged local anticancer activity with reduced systemic toxicity.

A prodrug is defined as a pharmaceutically inactive compound, which on exposure to chemical or biological conditions could be activated.[6] Drugs that are insoluble, are usually ligated by esterification of the

OH group(s) with a hydrophilic or hydrophobic residue, as shown in (1):

$$\underset{\textit{insoluble drug}}{\text{Drug–OH}} \xrightarrow{\textit{coupling}} \underset{\textit{prodrug (inactive)}}{\text{Drug–O–ligand}} \xrightarrow{\textit{hydrolysis}} \underset{\textit{active drug (in vivo)}}{\text{Drug–OH}} \quad (1)$$

When the prodrug is administered the drug is generated slowly by spontaneous chemical or esterase-assisted hydrolysis. The release of drug at its action site may have equal or enhanced therapeutic effect, and may thereby avoid dose-associated toxicity and serious side-effects. Recently, we have shown that a bromoacyl taxane when used in Cremophor EL formulation is much less toxic than paclitaxel (administered as Taxol) and yet therapeutically as efficacious as the parent molecule against human ovarian tumor, suggesting that prodrug is being hydrolyzed to the parent molecule *in vivo*.[7]

Here we describe the synthesis, characterization and *in vitro* growth inhibitory properties of taxane prodrugs modified with 2-bromoacyl chains at the 2′-position of the *n*-benzoyl-*β*-phenylisoserine side chain and/or at the 7-position of the baccatin III ring of paclitaxel. The *in vivo* efficacy of liposomal bromacyl taxanes, evaluated against ovcar-3 ovarian tumor, is also described. A preliminary report of these studies has been reported previously.[8]

Materials and methods

Paclitaxel (purity >99%) was purchased from Hauser Chemical Research, (Boulder, CO). Bristol Myers Squibb Taxol was purchased from Sigma (St Louis, MO). All the fatty acids were obtained from Aldrich (Milwaukee, WI). 1,3-Dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were obtained from Fluka (Ronkonkoma, NY). The elemental and low-resolution fast atom bombardment mass spectral (FABMS) analyses were performed at the Oneida Research Services, (Whitesboro, NY) and Desert Analytics (Tucson, AZ). Dry CH_2Cl_2 was used as the reaction solvent, unless otherwise stated. Preparative thin-layer chromatography (TLC) was performed on a silica gel GF plate (1000 μM; Analtech, Newark, DE). The compounds were stored in alcohol-free chloroform or in powder form. It is to be noted that the storage of 2-bromoacyl taxanes in methanol resulted in hydrolysis of the chain. ^{1}H-NMR spectra were recorded on a high field Bruker Instrument operating at 300 MHz frequency.

Preparation of 2′,2-bromohexanoyl paclitaxel (**2A**) (Chart 1)

To a 10 min stirred solution of (±)-2-bromohexanoic acid (229 mg, 1.17 mmol) and 1,3-dicyclohexyl carbodiimide (241 mg, 1.17 mmol) in 30 ml of dry methylene chloride was added paclitaxel (500 mg, 0.586 mmol) and the catalyst 4-dimethylaminopyridine (71.5 mg, 0.586 mmol). The reaction mixture was allowed to proceed at room temperature only for 5 min. The white precipitate of dicyclohexyl urea was filtered through a Celite pad. The resultant filtrate was evaporated under vacuo and the residue obtained was purified by preparative TLC in $CHCl_3$:MeOH (95:5) to give the desired product (R_f = 0.58 in $CHCl_3$:MeOH, 95:5). After passing through a Metricel filter (0.1 μm) to remove the silica gel from the $CHCl_3$ solution, the product was solidified by addition of cyclohexane to give 507 mg (84% yield) as the white powder. ^{1}H-NMR ($CDCl_3$, 300 MHz) chemical shifts of some of the characteristic peaks at δ (in p.p.m.): 8.14 (d, *J*=7.3 Hz, 2H), 7.72 (d, *J*=7.3 Hz, 2H), 7.61 (t, *J*=7.3 Hz, 1H), 7.54–7.36 (m, 10H), 6.87 (dd, *J*=3.4 Hz, 2.4 Hz, 1H), 6.29 (m, 2H), 6.0 (m, 1H) 5.68 (d, *J*=6.9 Hz, 1H), 5.50 (dd, *J*=1.4 Hz, 1.0 Hz), 4.97 (d, *J*=7.8 Hz, 1H), 4.45 (m, 1H), 4.32 (d, *J*=7.3 Hz, 1H), 4.28 (m, 1H), 4.20 (d, *J*=8.3 Hz, 1H), 4.0 (br, 1H), 3.81 (d, *J*=6.9 Hz, 1H), 0.86 (app. t, 3H, ω-CH_3). MS *m/z* 1030 $(M.H)^+$. Calcd for $C_{53}H_{60}NO_{15}Br$: C, 61.81; H, 5.83; N, 1.36. Found: C, 61.46; H, 6.12; N, 1.35.

2′-Hexanoyl paclitaxel (**2**)

Compound **2** was prepared the same as described for **2A** except hexanoic anhydride was used. The yield of the product was 80–90%.

^{1}H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.15 (d, *J*=7.3 Hz, 2H), 7.74 (d, *J*=7.3 Hz, 2H), 7.61 (t, *J*=7.3 Hz, 1H), 7.54–7.33 (m, 10H), 6.88 (d, *J*=8.8 Hz, 1H), 6.29 (s, 1H), 6.26 (t, *J*=8.8 Hz, 1H), 5.95 (dd, *J*=8.8 Hz, 2.9 Hz, 1H), 5.69 (d, *J*=6.8 Hz, 1H), 5.50 (d, *J*=3.4 Hz, 1H), 4.99 (d, *J*=7.8 Hz, 1H), 4.45 (bt, 1H), 4.33 (d, *J*=8.3 Hz, 1H), 4.21 (d, *J*=8.3 Hz, 1H), 3.82 (d, *J*=7.3 Hz, 1H), 0.89 (app. t, 3H). MS *m/z* 974 $(M.Na)^+$. Calcd for $C_{53}H_{61}NO_{15}.H_2O$: C, 65.63; H, 6.50; N, 1.44. Found: C, 65.51; H, 6.52; N, 1.45.

Taxanes bearing 2-bromo and non-bromo acyl chains at 2′-position were synthesized and characterized as described before.[4]

2′-Octanoyl paclitaxel (**3**)

^{1}H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.14 (d, *J*=7.3 Hz, 2H), 7.74 (d, *J*=7.3 Hz, 2H), 7.61 (t,

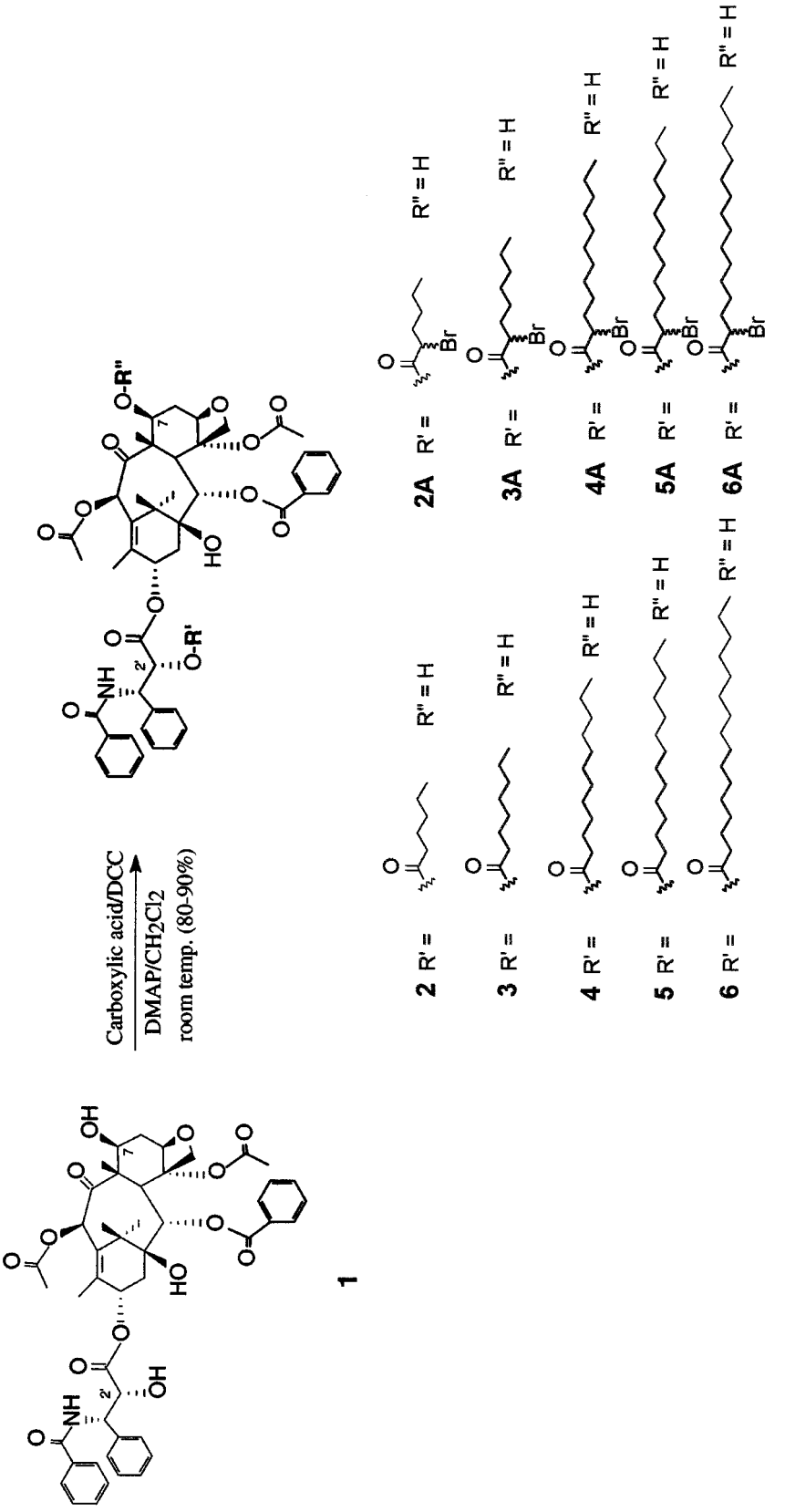

Chart 1. Synthesis of 2′-bromo and non-bromoacylated taxanes.

J=7.3 Hz, 1H), 7.54–7.33 (m, 10H), 6.88 (d, *J*=9.3 Hz, 1H), 6.28 (s, 1H), 6.26 (t, *J*=9.3 Hz, 1H), 5.95 (dd, *J*=9.0 Hz, 2.9 Hz, 1H), 5.69 (d, *J*=7.3 Hz, 1H), 5.49 (d, *J*=2.8 Hz, 1H), 4.99 (d, *J*=8.3 Hz, 1H), 4.44 (dd, *J*=8.6 Hz, 6.8 Hz, 1H), 4.33 (d, *J*=8.3 Hz, 1H), 4.20 (d, *J*=8.3 Hz, 1H), 3.82 (d, *J*=6.8 Hz, 1H), 0.89 (app. t, 3H). MS *m/z* 1002 $(M.Na)^+$. Calcd for $C_{55}H_{65}NO_{15}$-H_2O: C, 66.20; H, 6.72; N, 1.40. Found: C, 66.82; H, 6.67; N, 1.63.

2′-(2-Bromooctanoyl) paclitaxel (**3A**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.13 (d, *J*=7.3 Hz, 2H), 7.75 (d, *J*=7.3 Hz, 2H), 7.62 (t, 1H), 7.54–7.40 (m, 10H), 6.85 (dd, 3.4 *J*=Hz, 2.4 Hz, 1H), 6.29 (bt, 2H), 6.00 (m, 1H), 5.69 (d, *J*=7.3 Hz, 1H), 5.51 (s, 1H), 4.99 (d, *J*=8.3 Hz, 1H), 4.47 (dd, *J*=10.7, 6.8 Hz, 1H), 4.33 (t, 1H), 4.21 (d, *J*=8.3 Hz, 1H), 3.82 (d, *J*=6.8 Hz, 1H), 0.88 (app. t, 3H). MS *m/z* 1058 $(M.H)^+$. Calcd for $C_{55}H_{64}NO_{15}Br$: C, 62.44; H, 6.05; N, 1.32. Found: C, 62.47; H, 6.52; N, 2.43.

2′-Dodecanoyl paclitaxel (**4**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.14 (d, *J*=6.8 Hz, 2H), 7.74 (d, *J*=6.8 Hz, 2H), 7.61 (t, *J*=7.3 Hz, 1H), 7.54–7.31 (m, 10H), 6.88 (d, *J*=8.8 Hz, 1H), 6.28 (s, 1H), 6.26 (t, *J*=8.8 Hz, 1H), 5.95 (dd, *J*=8.8 Hz, 2.9 Hz, 1H), 5.69 (d, *J*=7.3 Hz, 1H), 5.49 (d, *J*=3.4 Hz, 1H), 4.99 (d, *J*=8.3 Hz, 1H), 4.43 (m, 1H), 4.33 (d, *J*=8.3 Hz, 1H), 4.20 (d, *J*=8.3 Hz, 1H), 3.82 (d, *J*=6.8 Hz, 1H), 0.89 (app. t, 3H). MS *m/z* 1058 $(M.Na)^+$. Calcd for $C_{59}H_{73}NO_{15}H_2O$: C, 67.11; H, 7.12; N, 1.33. Found: C, 67.19; H, 6.94; N, 1.37.

2′-(2-Bromododecanoyl) paclitaxel (**4A**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.15 (d, *J*=7.8 Hz, 2H), 7.75 (d, *J*=7.3 Hz, 2H), 7.62 (t, *J*=7.3 Hz, 1H), 7.55–7.40 (m, 10H), 6.87 (d, *J*=9.3, 1H), 6.29 (bs, 2H), 6.01 (m, 1H), 5.69 (d, *J*=6.8, 1H), 5.51 (s, 1H), 4.99 (d, *J*=8.3 Hz, 1H), 4.47 (dd, *J*=8.5 Hz, 6.3 Hz, 1H), 4.33 (t, 1H), 4.21 (d, *J*=8.3 Hz, 1H), 3.80 (m, 1H), 0.89 (app. t, 3H). MS *m/z* 1114 $(M.H)^+$. Calcd for $C_{59}H_{72}NO_{15}Br$: C, 63.61; H, 6.47; N, 1.26. Found: C, 63.34; H, 6.64; N, 2.62.

2′-Tetradecanoyl paclitaxel (**5**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.14 (d, *J*=7.3 Hz, 2H), 7.74 (d, *J*=7.3 Hz, 2H), 7.61 (t, *J*=7.3 Hz, 1H), 7.59–7.33 (m, 10H), 6.88 (d, *J*=8.8 Hz, 1H), 6.29 (s, 1H), 6.26 (t, *J*=8.8 Hz, 1H), 5.95 (dd, *J*=9.0 Hz, 2.4 Hz, 1H), 5.69 (d, *J*=6.8 Hz, 1H), 5.49 (d, *J*=2.9 Hz, 1H), 4.99 (d, *J*=8.3 Hz, 1H), 4.45 (m, 1H), 4.33 (d, *J*=8.3 Hz, 1H), 4.20 (d, *J*=8.3 Hz, 1H), 3.82 (d, *J*=6.8 Hz, 1H), 0.89 (app. t, 3H). MS *m/z* 1086 $(M.Na)^+$. Calcd for $C_{61}H_{77}NO_{15}H_2O$: C, 67.71; H, 7.30; N, 1.30. Found: C, 67.92; H, 7.03; N, 1.39.

2′-(2-Bromotetradecanoyl) paclitaxel (**5A**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.15 (d, *J*=7.8 Hz, 2H), 7.75 (d, *J*=6.8 Hz, 2H), 7.62 (t, *J*=7.3 Hz, 1H), 7.55–7.38 (m, 10H), 6.86 (dd, *J*=11.1 Hz, 2.9 Hz, 1H), 6.29 (bs, 2H), 5.99 (m, 1H), 5.69 (d, *J*=7.3 Hz, 1H), 5.50 (app. t, 1H), 4.99 (d, *J*=8.3 Hz, 1H), 4.45 (dd, *J*=8.5 Hz, 6.3 Hz, 1H), 4.33 (app. t, 1H), 4.21 (d, *J*=8.3 Hz, 1H), 3.82 (d, *J*=6.8 Hz, 1H), 0.89 (app. t, 3H). MS *m/z* 1142 $(M.H)^+$. Calcd for $C_{61}H_{76}NO_{15}Br$: C, 64.15; H, 6.66; N, 1.23. Found: C, 64.78; H, 7.36; N, 2.98.

2′-Hexadecanoyl paclitaxel (**6**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.14 (d, *J*=7.3 Hz, 2H), 7.76 (d, *J*=6.8 Hz, 2H), 7.61 (t, *J*=7.3 Hz, 1H), 7.54–7.33 (m, 10H), 6.91 (d, *J*=9.3 Hz, 1H), 6.29 (s, 1H), 6.26 (t, *J*=9.3 Hz, 1H), 5.95 (dd, *J*=9.3 Hz, 2.9 Hz, 1H), 5.69 (d, *J*=6.8 Hz, 1H), 5.49 (d, *J*=2.9 Hz, 1H), 4.99 (d, *J*=8.3 Hz, 1H), 4.45 (m, 1H), 4.33 (d, *J*=8.3 Hz, 1H), 4.21 (d, *J*=8.8 Hz, 1H), 3.82 (d, *J*=6.8 Hz, 1H), 0.89 (app. t, 3H). MS *m/z* 1114 $(M.Na)^+$. Calcd for $C_{63}H_{81}NO_{15}$: C, 69.29; H, 7.42; N, 1.28. Found: C, 68.55; H, 7.32; N, 1.37.

2′-(2-Bromohexadecanoyl) paclitaxel (**6A**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.15 (d, *J*=7.8 Hz, 2H), 7.75 (d, *J*=6.8 Hz, 2H), 7.64(t, *J*=7.3 Hz, 1H), 7.55–7.34 (m, 10H), 6.87 (dd, *J*=8.8 Hz, 2.5 Hz, 1H), 6.29 (bs, 2H), 6.02 (m, 1H), 5.69 (d, *J*=7.3 Hz, 1H), 5.50 (d, 1H), 4.98 (d, *J*=8.3 Hz, 1H), 4.47 (dd, *J*=8.5 Hz, 6.3 Hz, 1H), 4.33 (app. t, 1H), 4.21 (d, *J*=8.3 Hz, 1H), 3.82 (d, *J*=6.8 Hz, 1H), 0.89 (app. t, 3H). MS *m/z* 1170 $(M.H)^+$. Calcd for $C_{63}H_{80}NO_{15}Br$: C, 64.67; H, 6.84; N, 1.20. Found: C, 64.09; H, 7.09; N, 1.77.

Preparation of 7-hexanoyl paclitaxel (**7**) (Chart 2)

2′-Troc paclitaxel (162 mg, 0.14 mmol), prepared from paclitaxel **1** by protection of 2′-OH group with Troc-chloride/pyridine and then acylation with hexanoic anhydride and DMAP, was reacted with Zn dust (201 mg) in 10 ml of methanol:acetic acid (9:1). After 30 min, the reaction mixture was diluted with $CHCl_3$

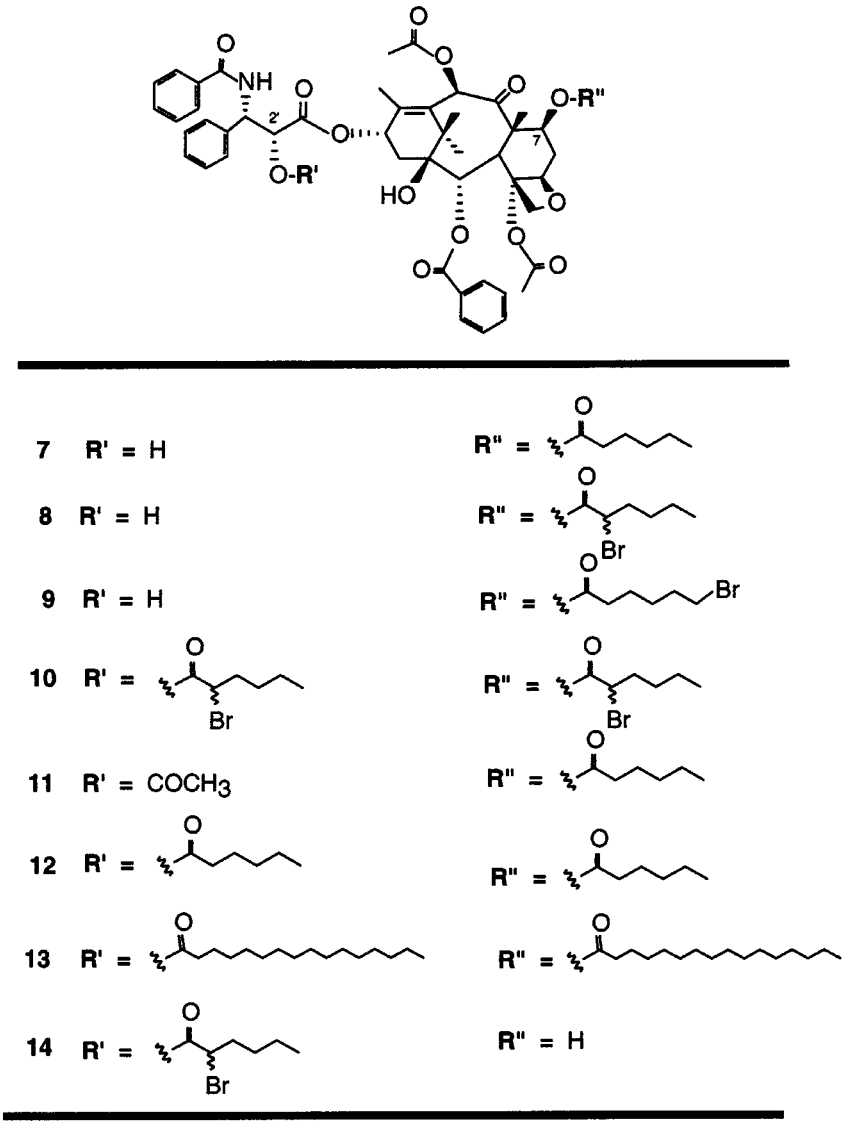

Chart 2. Structures of taxanes **7–14**.

(10 ml). The inorganic precipitate was filtered through a Celite bed and the filtrate was dried under vacuo. The residue obtained was resuspended in EtOAc to remove the inorganics. After evaporation under reduced pressure the product was purified on a preparative TLC using $CHCl_3$:MeOH (96:4) to yield 91 mg (67%) of white solid. ^1H NMR ($CDCl_3$, 300 MHz) chemical shifts of some of the characteristic peaks at δ (in p.p.m.): 8.15 (d, J=7.3 Hz, 2H), 7.75 (d, J=7.3 Hz, 2H), 7.61 (t, J=7.3 Hz, 1H), 7.59–7.35 (m, 10H), 6.8 (d, J=8.8 Hz, 1H), 6.29 (s, 1H), 6.26 (t, J=8.8 Hz, 1H), 5.82 (dd, J=9.3 Hz, 2.4 Hz, 1H), 5.65 (d, J=7.3 Hz, 1H), 5.55 (dd, J=6.9 Hz, 7.4 Hz, 1H), 4.95 (d, J=8.3 Hz, 1H, H-5), 4.80 (d, 2.43, 1H), 4.33 (d, J=8.3 Hz, 1H), 4.19 (d, J=8.3 Hz, 1H), 3.90 (d, J=6.9 Hz, 1H), 3.8 (d, J=7.3 Hz, 1H), 0.88 (app. t, 3H). MS m/z 952 $(M.H)^+$. Calcd for $C_{53}H_{61}NO_{15}$: C, 66.80; H, 6.51; N, 1.47. Found: C, 66.17; H, 6.40; N, 1.46.

7-(2-Bromohexanoyl) paclitaxel (**8**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.12 (d, J=7.3 Hz, 2H), 7.78 (d, J=7.3 Hz, 2H), 7.60 (t, J=7.3 Hz, 1H), 7.53–7.32 (m, 10H), 7.06 (d, J=8.8 Hz, 1H), 6.20 (bt, J=8.8 Hz, 2H), 5.82 (d,

J=8.8 Hz, 1H), 5.67 (d, *J*=6.8 Hz, 1H), 5.55 (dd, *J*=10.3, 7.3 Hz, 1H), 5.09 (bs, 1H), 4.96 (d, *J*=8.3 Hz, 1H), 4.81 (m, 1H), 4.33 (d, *J*=8.3 Hz, 1H), 4.20 (m, 2H), 3.92 (m, 4H), 3.57 (d, *J*=4.9 Hz, 1H), 0.90 (t, 3H). MS *m/z* 1030 $(M.H)^+$. Calcd for $C_{53}H_{60}NO_{15}Br6H_2O$: C, 55.94; H, 6.33; N, 1.23. Found: C, 55.91; H, 5.43; N, 1.15.

7-(6-Bromohexanoyl) paclitaxel (**9**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.12 (d, *J*=7.6 Hz, 2H), 7.75 (d, *J*=7.3 Hz, 2H), 7.65 (t, *J*=7.3 Hz, 1H), 7.55–7.35 (m, 10H), 7.09 (d, *J*=8.8 Hz, 1H), 6.21 (s, 1H), 6.16 (t, *J*=9.3 Hz, 1H), 5.81 (d, *J*= 8.8 Hz, 1H), 5.67 (d, *J*=6.8 Hz, 1H), 5.55 (dd, *J*=10.3 Hz, 7.3 Hz, 1H), 4.95 (d, *J*=8.8 Hz, 1H), 4.79 (bs, 1H), 4.33 (d, *J*=8.3 Hz, 1H), 4.19 (d, *J*=8.3 Hz, 1H), 3.92 (d, *J*=6.8 Hz, 1H), 3.62 (d, *J*=4.4 Hz, 1H), 3.40 (t, 2H). MS *m/z* 1030 $(M.H)^+$. Calcd for $C_{53}H_{60}NO_{15}Br$: C, 60.74; H, 5.92; N, 1.34. Found: C, 60.34; H, 6.21; N, 1.58.

2′,7-Di-(2-bromohexanoyl) paclitaxel (**10**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.14 (d, *J*=7.3 Hz, 2H), 7.76 (d, *J*=7.3 Hz, 2H), 7.62 (t, *J*=7.3 Hz, 1H), 7.54–7.36 (m, 10H), 6.88 (dd, *J*=9.0 Hz, 2.9 Hz, 1H), 6.21 (bm, 2H), 5.99 (m, 1H), 5.69 (d, *J*=6.8 Hz, 1H), 5.60 (t, 1H), 5.52 (s, 1H), 4.99 (d, *J*=8.8 Hz, 1H), 4.31 (d, *J*=2.9 Hz, 1H), 4.18 (m, 2H), 3.94 (d, *J*=6.8 Hz, 1H), 0.90 (m, 6H). MS *m/z* 1208 $(M.H)^+$. Calcd for $C_{59}H_{69}NO_{16}Br_2$: C, 58.66; H, 5.72; N, 1.16. Found: C, 60.36; H, 6.21; N, 1.58.

2′, *O*-Acetyl-7-hexanoyl paclitaxel (**11**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.14 (d, *J*=7.8 Hz, 2H), 7.76 (d, *J*=7.3 Hz, 2H), 7.61 (t, *J*=7.3 Hz, 1H), 7.54–7.36 (m, 10H), 6.94 (d, *J*=8.8 Hz, 1H), 6.27 (s, 1H), 6.21 (t, *J*=8.8 Hz, 1H), 5.94 (dd, *J*=9.0 Hz, 2.9 Hz, 1H), 5.69 (d, *J*=6.8 Hz, 1H), 5.59 (dd, *J*=10.9 Hz, 7.4 Hz, 1H), 5.55 (d, *J*=2.9 Hz, 1H), 4.97 (d, *J*=8.8 Hz, 1H), 4.34 (d, *J*=8.3 Hz, 1H), 4.19 (d, *J*=8.3 Hz, 1H), 3.95 (d, *J*=6.8 Hz, 1H), 0.89 (app. t, 3H). MS *m/z* 1016 $(M.Na)^+$. Calcd for $C_{55}H_{63}NO_{16}7$-H_2O: C, 58.96; H, 6.88; N, 1.25. Found: C, 58.51; H, 6.19; N, 1.58.

2′,7-Dihexanoyl paclitaxel (**12**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.14 (d, *J*=7.3 Hz, 2H), 7.76 (d, *J*=7.3 Hz, 2H), 7.62 (t, *J*=7.3 Hz, 1H), 7.49–7.35 (m, 10H), 6.92 (d, *J*=9.3 Hz, 1H), 6.28 (s, 1H), 6.22 (t, *J*=8.8 Hz, 1H), 5.95 (dd, *J*=9.0 Hz, 3.4 Hz, 1H), 5.69 (d, *J*=6.8 Hz, 1H), 5.59 (dd, *J*=10.3 Hz, 7.3 Hz, 1H), 5.54 (d, *J*=2.9 Hz, 1H), 4.98 (d, *J*=8.8 Hz, 1H), 4.34 (d, *J*=8.8 Hz, 1H), 4.19 (d, *J*=8.3 Hz, 1H), 3.94 (d, *J*=6.8 Hz, 1H), 0.89 (m, 6H). MS *m/z* 1072 $(M.Na)^+$. Calcd for $C_{59}H_{71}NO_{15}Br2H_2O$: C, 67.50; H, 6.77; N, 1.33. Found: C, 67.04; H, 5.83; N, 1.34.

2′-(6-Bromohexanoyl) paclitaxel (**13**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.15 (d, *J*= 6.8 Hz, 2H), 7.75 (d, *J*=7.3 Hz, 2H), 7.61 (t, *J*=7.3 Hz, 1H), 7.58–7.35 (m, 10H), 6.88 (d, *J*=9.3 Hz, 1H), 6.29 (s, 1H), 6.26 (t, *J*=8.8 Hz, 1H), 5.98 (dd, *J*=9.3 Hz, 2.9 Hz, 1H), 5.69 (d, *J*=7.3 Hz, 1H), 5.51 (d, *J*=2.9 Hz, 1H), 4.99 (d, *J*=8.3 Hz, 1H), 4.45 (dd, *J*=10.7 Hz, 6.8 Hz, 2H), 4.33 (d, *J*=8.3 Hz, 1H), 4.21 (d, *J*=8.3 Hz, 1H), 3.82 (d, *J*=6.8 Hz, 1H), 3.42 (d, *J*=6.3 Hz, 1H), 3.36 (t, *J*=6.8 Hz, 2H). MS *m/z* 1030 $(M.H)^+$. Calcd for $C_{53}H_{60}NO_{15}Br2H_2O$: C, 59.72; H, 6.01; N; 1.31. Found: C, 59.02; H, 6.11; N, 1.38.

2′,7-Di-hexadecanoyl paclitaxel (**14**)

^1H ($CDCl_3$, 300 MHz): the characteristic peaks at δ: 8.14 (d, *J*=7.3 Hz, 2H), 7.76 (d, *J*=6.8 Hz, 2H), 7.61 (t, *J*=7.3 Hz, 1H), 7.54–7.33 (m, 10H), 6.91 (d, *J*=9.3 Hz, 1H), 6.27 (s, 1H), 6.22 (t, *J*=8.8 Hz, 1H), 5.94 (dd, *J*=9.3 Hz, 2.9 Hz, 1H), 5.69 (d, *J*=6.8 Hz, 1H), 5.58 (t, *J*=7.3 Hz, 1H), 5.53 (d, *J*=2.9 Hz, 1H), 4.97 (d, *J*=8.8 Hz, 1H), 4.34 (d, *J*=8.8 Hz, 1H), 4.19 (d, *J*=8.8 Hz, 1H), 3.96 (d, *J*=6.8 Hz, 1H), 0.89 (app. t, 6H). MS *m/z* 1330 $(M.H)^+$. Calcd for $C_{79}H_{111}NO_{16}H_2O$: C, 70.38; H, 8.38; N, 1.04. Found: C, 70.84; H, 8.12; N, 1.19.

In vitro growth inhibition experiments

The inhibition of *in vitro* proliferation of MCF-7 (human breast carcinoma) cells by the hydrophobic prodrugs and paclitaxel was quantitated using a Sulforhodamine B (SRB) cytotoxicity assay as described previously.[16] Briefly, cells (5000 cells/well) were plated onto 96-well microliter plates in RPMI 1640 medium supplemented with 10% FBS and incubated at 37°C in a humidified 5% CO_2 atmosphere. After 24 h, cells were exposed to various concentrations of drug and cultured for another 72 h. The cells were fixed by addition of 50 μl of cold 5% (w/v) trichloroacetic acid (TCA) per well and incubated at 4°C for 1 h. Plates were rinsed 6 times with tap water and left to air dry overnight. Then 100 μl of SRB (0.4% w/v in 1% acetic acid) was added and incubated at

room temperature for 10–15 min. Unbound SRB was removed by washing 3 times with 1% acetic acid. Plates were air-dried and bound SRB was solubilized with 10 mM Tris buffer. Optical densities were read at 490 nm and concentration (μM) of drug required to inhibit cell growth 50% (GI_{50}) was calculated from $100 \times [(T-T_0)/(C-T_0)]=50$, where control optical density was C, test optical density was T and optical density at time zero was T_0.

Preparation and characterization of liposomes

Stock solutions of taxane prodrugs and phospholipids were prepared in chloroform, and stored at −20°C. For preparation of liposomes, the taxanes were co-dissolved with lipids at a mole ratio of 84 (DSPC):10 (DPPE-GA):6 (bromoacyl taxane) in 10 ml tubes and the $CHCl_3$ was evaporated under stream of nitrogen. The resulting thin film was dried under vacuum for several hours. The lipid film was then hydrated in 150 mM NaCl. The mixture was vortexed and heated (above the phase transition temperatures of the lipids, 58°C) and cooled (0°C) several times. The resulting mainly multilamellar vesicle (MLV) formulations were then extruded through nucleopore filters to a final pore size of 100 nm. The paclitaxel liposomes were characterized for drug content and size before use. The final mole ratios varied in the range 83.9–84.9 DSPC:9.7–9.8 DPPE-GA:5.4–6.5 bromoacyl taxane and the final concentration before dilution into PBS for animal experiments varied between 2.5 and 5.2 mg/ml. The final mean diameter of liposomes varied between 90 and 140 nm (Nicomp).

Toxicity and therapeutics

For acute toxicity determinations CDF1 (five to 10 per group) female mice were administered a single i.p. injection of 12.5–100 mg/kg Taxol, or 300–500 mg/kg liposomal **2A** or **6A**. The dose volume for injection was 25 ml/kg. Mice were observed daily for 14 days and maximum tolerated dose (MTD) was calculated. CB17 female SCID mice (five per group) were inoculated i.p. with 10×10^6 ovcar-3 cells on day 0. Mice were treated with control liposomes (DSPC:DPPE-GA, 90:10 mole ratio), liposomes containing 2′-(±)-2-bromoacyl taxanes (**2A**, **3A**, **4A**, **5A** and **6A**) at 50 mg/kg, Taxol at 12.5 mg/kg i.p. on days 1, 3, 5, 7 and 9. The dose volume used was 25 ml/kg. Observations and mortality were recorded twice per week until day 300. Percent survival of each group was calculated.

Tubulin assay

Tubulin (Sigma), approximately 7.5 mg per vial, was reconstituted in 800 μl of distilled water containing 0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA and 2.5 M glycerol at pH 6.5 (tubulin buffer). The vial was shaken gently at 37°C for 5 min and then aliquoted in four vials (200 μl each). The vials were frozen in liquid nitrogen and stored at −70°C. Immediately before the tubulin assembly assay, a vial of tubulin was thawed and diluted 5-fold with the tubulin buffer, then incubated on ice for 40 min, sonicating every 10 min in a bath sonicator. The solution was centrifuged at 25 000 *g*g at 4°C for 40 min. The supernatant was collected and assayed for total protein content (DC Protein Assay; BioRad, Hercules, CA) using a bovine serum albumin standard. The tubulin solution was diluted to 1.3 mg/ml with the tubulin buffer. Paclitaxel (**1**) and 2′-bromohexadecanoyl (**6A**) taxane were reconstituted in DMSO (JT Baker, Phillipsburg, NJ) to 2.26 mM. Compound **6A**, incubated at 55°C for 75 min (from above), was reconstituted in DMSO to 2.26 mM. The three formulations were serially diluted 1:3 in DMSO for a range of concentrations.

The tubulin assay was modified from that described by Lin *et al.*[14] Briefly, tubulin, 1.06 mg/ml, 0.4 M glutamate (Sigma) reconstituted in tubulin buffer and pH adjusted to 6.6, and the drug formulations, ranging in concentration from 90 to 3.3 μM, were each added to microtubes. The tubes were incubated for 15 min at 37°C (water bath), then centrifuged for 10 min at 25 000 *g*. The supernatant was collected and analyzed for protein content (DC Protein Assay).

Results

Synthesis

The overall scheme for the synthesis of the 2′-acyl taxane derivatives (**2–6** and **2A–6A**) is illustrated in Chart 1. The 2′-acyl derivatives were synthesized in a one-step process where paclitaxel was reacted with the appropriate carboxylic acid anhydride or acid in the presence of DCC using DMAP as a catalyst. The yields ranged from 80 to 90%. Compound **14** was prepared using the procedure same as described in Chart 1, except 6-bromohexanoic acid was used. The 7-acyl taxane derivatives (Chart 2: **7–9**) were synthesized in a three-step process where the 2′-position of paclitaxel was protected by standard procedure[9] using trialkylsilyl chloride or Troc chloride in pyridine before acylation with the appropriate fatty acid anhydride in the presence of DMAP. The protecting group at the 2′-

position was then cleaved by reacting the silyl group with Bu_4NF/THF or reductive cleavage of Troc with Zn dust in methanol/acetic acid, which yielded 60–70% of the product after purification by TLC. The 2′,7-diacyl taxanes (Chart 2: **10**, **12** and **13**) were synthesized by reacting excess of acid anhydride or acid chloride in presence of DMAP as a catalyst at a slightly elevated temperature. Compound **11** was prepared by acetylation of paclitaxel followed by reacting with hexanoic anhydride with the aid of DMAP catalyst.

In vitro growth inhibition

The GI_{50} values of a series of 2′-(±)-2-bromoacyl taxanes (**2A–6A**) varying in their acyl chain compositions were examined and compared with 2′-acyl taxanes (**2–6**) bearing equivalent chain lengths lacking bromine. Likewise, other taxanes bearing mono-acyl chains at the 7-position and di-acyl substituted at the 2′,7-positions were also synthesized, and their growth inhibitions were evaluated in the MCF-7 (human breast cancer) cell line. The results are shown in Tables 1 and 2 where the results are expressed as GI_{50}, the μM concentration required to inhibit 50% cell growth in the MCF-7 cell line. In general, all taxane derivatives bearing or lacking a heteroatom were less active than paclitaxel against MCF-7 cell lines tested but the potency of the various taxanes varied widely. Table 1 shows that the 2′-(±)-2-bromohexanoyl derivative (**2A**) was only about 3-fold less active than paclitaxel (**1**), while the 2′-hexanoyl derivative (**2**) was about 500 times less active than paclitaxel and about 200 times less potent than **2A** against MCF-7. Compound **2A** when compared with **8** having a 2-bromohexanoyl chain at 7-position showed about 1.5-fold more potency against MCF7. It appears that the absence of a hydrolyzable group at the α-position of the acyl chain greatly diminished the growth inhibitory activity. The reduction in activity was also observed with 7-acyl substituted taxanes lacking a hydrolyzable group. For example, 7-hexanoyl taxane **7** was about 5 times less active than compound **8** having the same chain length in MCF-7, while it was about 27-fold less active than **1**. Compound **8** showed a GI_{50} value 4 times lower, and hence more active, than compound **9** under equivalent conditions. This difference in activity may, in part, be due to a faster rate of hydrolysis of the bromoacyl chain induced by bromine at the 2-position compared to the 6-position of the acyl chain to yield paclitaxel **1**. From Table 2 it was also clear that taxane **9** bearing a 6-bromohexanoyl chain at the 7-position was exceptionally 550-fold more potent than compound **14** with an equivalent chain length at the 2′-position. As can be seen from Table 2, the di-acyl taxane derivatives (Table 2: **10–13**) were much less active against MCF-7 than corresponding mono-acyl derivatives (Table 1: **2–6**, **2A–6A**, and Table 2: **7–9**).

Like taxane **2A**, the GI_{50} values of 2-bromooctanoyl (**3A**) and 2-bromododecanoyl (**4A**) taxane prodrugs were about 3-fold less than **1**, as shown in Table 1. In contrast, 2-bromtetradecanoyl (**5A**) and bromohexadecanoyl (**6A**) taxane derivatives were about 20 or 70 times less active than paclitaxel **1**, respectively, against MCF7 cell lines investigated. In general, increasing the acyl chain length decreased the *in vitro* potency not only amongst **2A–6A** but also amongst **2–6**. It is worth noting that compound **2** was about 15 times more potent than compound **6** but was approximately as active as compound **3**, and about twice more potent than **4** and **5** under similar conditions. A marked disparity in GI_{50} (about 120-fold) was also apparent against MCF-7 with long acyl chain taxanes **6A** and **6**,

Table 1. Growth inhibition of 2′-bromo and non-bromoacyl taxanes

Taxane		GI_{50} (μM)
Paclitaxel	**1**	<0.001
2′-Hexanoyl (C-6)	**2**	0.50
2′-(2-Bromo)-hexanoyl (C6)	**2A**	0.003
2′-Octanoyl (C-8)	**3**	0.30
2′-(2-Bromo)-octanoyl (C-8)	**3A**	0.003
2′-Dodecanoyl (C-12)	**4**	0.84
2′-(2-Bromo)-dodecanoyl (C-12)	**4A**	0.003
2′-Tetradecanoyl (C-14)	**5**	0.90
2′-(2-Bromo)-tetradecanoyl (C-14)	**5A**	0.020
2′-Hexadecanoyl (C-16)	**6**	8.6
2′-(2-Bromo)-hexadecanoyl (C-16)	**6A**	0.070

GI_{50} (in μM) shows the concentration required for 50% growth inhibition by 2′-acyl-substituted hydrophobic hydrolyzable taxane prodrugs against human MCF7 breast carcinoma cells following 72 h incubation of the cells with drug, using SRB assay.

Table 2. Growth inhibition of mono- and di-acylated taxanes

Taxane		GI_{50} (μM)
7-Hexanoyl (C-6)	**7**	0.027
7-(2-Bromo)-hexanoyl (C-6)	**8**	0.0046
7-(6-Bromo)-hexanoyl (C-6)	**9**	0.018
2′,7-Di-(2-Bromo)-hexanoyl (C-6)	**10**	1.43
2′-*O*-Acetyl-7-hexanoyl (C-6)	**11**	4.46
2′,7-Di-Hexanoyl (C-6)	**12**	>10.0
2′,7-Di-Hexadecanoyl (C-16)	**13**	>20.0
2′-(6-Bromo)-hexanoyl (C-6)	**14**	>10.0

GI_{50} (in μM) shows the concentration required for 50% growth inhibition by 7-substituted mono-acyl and 2′,7-substituted di-acyl hydrophobic hydrolyzable taxane prodrugs against human MCF7 breast carcinoma cells following 72 h incubation of the cells with drug, using SRB assay.

presumably due to ease of hydrolysis of the bromoacyl chain to free paclitaxel.

In vivo therapeutics

We investigated the therapeutic efficacy of liposomal bromoacyl taxanes (**2A–6A**) against i.p. ovarian cancer. The maximum dose of 2′-bromoacyl taxanes (**2A–6A**) was kept at 50 mg/kg for each of a 5-day injection (total dose 250 mg/kg), while of Taxol was kept at 12.5 mg/kg for each of a 5-day injection (total dose 62.5 mg/kg). Figure 1 shows the survival data of mice treated over 300 days with compounds **2A–6A**. All liposomal formulations of taxanes (**2A–6A**) and Taxol had increased the survival time of tumor-bearing mice compared to the control arm with survival of 90 days only. Liposomal formulations of **6A** significantly increased the survival time of tumor-bearing mice compared to the control arm (Figure 1). The *in vivo* efficacy decreased in order: **6A** > **3A** > **4A** > **5A** > **2A**. Compound **6A**, however, was more effective and significantly increased survival time of tumor bearing mice ($p < 0.01$) versus controls than Taxol. Compound **6A** was the most effective, with 100% survival at day 300 with no evidence of tumor recurrence.

Tubulin assembly

An earlier study suggests that substitution at the 2′-position of paclitaxel interferes with taxane ability to assemble tubulin.[10] To examine whether the acyl chain of compound **6A** interferes in tubulin polymerization, binding assays were carried out using a standard procedure.[11] Results are shown in Figure 2. It is clear from Figure 2 that compound **6A** at room temperature did not associate with tubulin polymerization at concentrations as high as 90 μM (*curve a*), whereas, paclitaxel **1** polymerized the tubulin in a dose-dependent manner under similar conditions

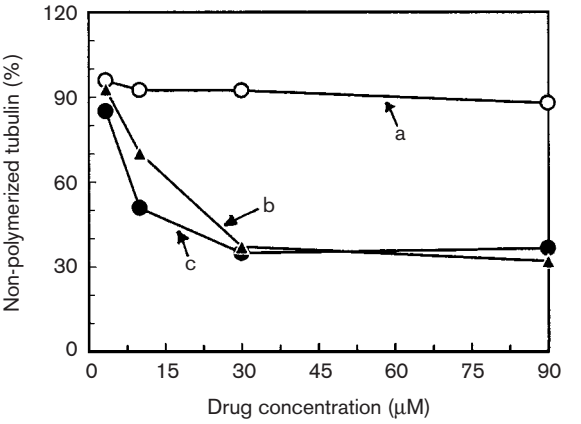

Figure 2. Amount (in %) of non-polymerized tubulin in supernatant with increasing amount of taxanes. Solution contained tubulin (1 mg/ml), 0.4 M glutamate, 4% DMSO and drugs at pH 6.8, **6A** at 25°C (*curve a*), **6A** at 55°C (*curve b*) and **1** (control) at 25°C (*curve c*), details given in Materials and methods.

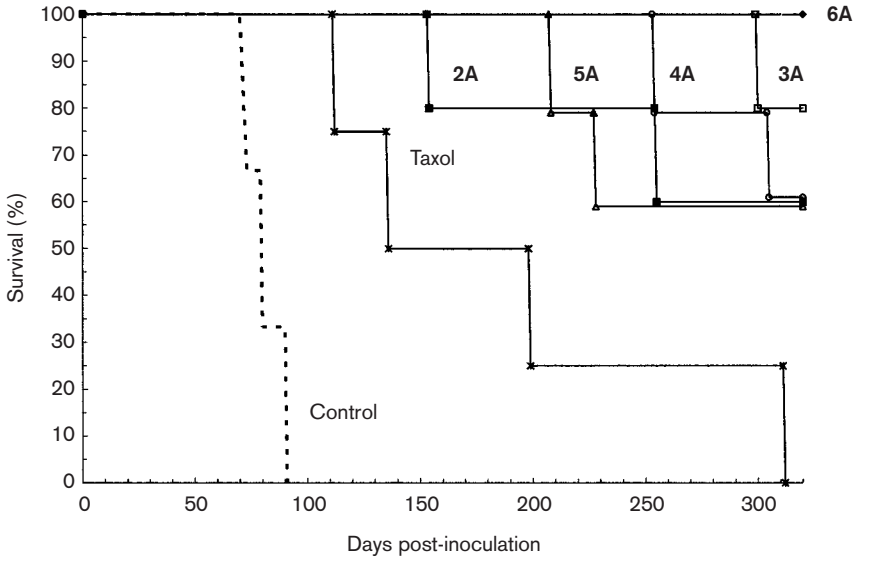

Figure 1. Effect of liposomal taxane prodrugs against ovcar-3 (human ovarian tumor) grown i.p. in SCID mice. Mice were inoculated i.p. with 10×10^6 cells on day 0, and doses of liposomal prodrugs **2A**, **3A**, **4A**, **5A** and **6A** (n= 5/group) at 50 mg/kg, Taxol (n=4) at 12.5 mg/kg or control (empty liposomes n=3) were administered i.p. on days 1, 3, 5, 7 and 9 post-inoculation. Control,- - - -; Taxol, *; **2A** ■; **3A** □; **4A** ○; **5A** △; **6A**, ◆.

(*curve c*). The data thus suggest that the acyl chain at the 2′-position interfered with the paclitaxel association in tubulin polymerization. Compound **6A** when preincubated at 55°C (*curve b*) polymerized the tubulin in a concentration-dependent manner, indicating that hydrolysis product **1** indeed was active in the assemblance of tubulins. The conversion of **6A** to **1** progressed relatively rapidly for approximately 4 days before it slowed down. It was also noted that compounds **6A** and **1** were being simultaneously metabolized in plasma at the rate **6A** was hydrolyzed to **1** (data not shown). It is not yet clear whether the enhanced therapeutic efficacy of **6A** was related to the formation of metabolites or prodrug or/and paclitaxel alone. Although the data suggests the $T_{1/2}$ for hydrolysis of **6A** in human plasma *in vitro* is several days, further work is required to determine the kinetics of acyl taxane hydrolysis in detail.

Discussion

This paper describes the synthesis of hydrophobic taxane prodrugs bearing acyl chains that could be activated to paclitaxel by hydrolysis of the acyl chain assisted by the inductive effect of a 2-substituted bromine atom. *In vitro* data against MCF-7 cells in culture indeed support the fact that the longer the 2-brominated hydrophobic acyl chain, the lower the biological activity and vice versa. In contrast, the *in vivo* data against xenografted ovcar-3 suggest that the longer acyl chain had better therapeutic efficacy in long-term management of the ovarian cancer (Figure 1). This report also shows that paclitaxel prodrug derivatives associated with a lipid carrier system are less acutely toxic than Taxol and have at least as good antitumor activity as Taxol against a human ovarian cancer grown in SCID mice. It is possible that the derivatives are acting as prodrugs as we have demonstrated the hydrolysis in the biological environment to yield paclitaxel for at least one derivative.[7]

It is also possible that the intact 'prodrugs' could have some activity not necessarily involving tubulin assembly stabilization.[12] In the current *in vivo* studies liposomes were used as the carrier system for the bromoacylated taxane but they are not the only potential carrier that may be developed. In particular, carriers with a much higher drug to lipid ratio than can be normally attained for liposomes may be devised. The results also indicate that low μM GI_{50}'s in MCF-7 cells obtained for some bromoacylated taxanes in the standard 72 h SRB *in vitro* growth inhibition studies do not predict superior *in vivo* activity for the type of prodrug we have investigated. It was indeed the case with compound **6A** when examined against human ovcar-3 ovarian tumor. Many analogs and derivatives of paclitaxel, including more aqueous soluble prodrugs, have been synthesized and investigated extensively *in vitro*.[13] Far fewer analogs have been investigated against human and animal tumors *in vivo*, and at present no analog, derivative or novel formulation of paclitaxel has been proven to be superior to Taxol in the clinic, although Taxotere® may have advantages in some circumstances.[14] The results presented here indicate that relatively straightforward modification of paclitaxel at the 2′-position can result in marked changes in the biological properties of paclitaxel. The 2′-(±)-2-bromoacyl series of taxane can be synthesized in high yield and high purity in a one-step process, and scale-up to several grams per preparation has been done in our laboratories and further scale-up is feasible.

The SRB results may indicate indirectly the rate of hydrolysis of the acylated taxane and, if anti-tumor efficacy is inversely related to hydrolysis rate, then slow but sustained delivery to proliferating target cancer cells may be more efficient *in vivo*,[15] although these taxanes would have relatively high *in vitro* GI_{50}'s. The rate of administration of Taxol has been reported to influence clinical outcome and some studies have suggested slow infusion may be more beneficial than faster infusion, but the optimal scheduling of Taxol administration is still work in progress.[1c] Pharmacological studies may direct more optimal dosing schedules for the bromacyl taxanes.

Recently, we have demonstrated that taxane concentrations varied with acyl composition of the lipid bilayers.[5a] For paclitaxel prodrugs with an acyl chain, the drug:lipid ratio could increase to about 1:15 (6.3 mol%), a 2-fold increase compared to **1** under similar physical conditions, suggesting that the taxane ring is not encapsulated in the bilayer membranes, in part, due to its bulky skeleton, yielding low incorporation in liposomes.[15c] This increase in prodrug concentration in liposomes may help reduce the toxicity of drugs in the long-term survival of mice. The data in Figure 1 shows that compounds **2A–6A** when formulated in liposomes or cremophor EL (data not shown) had better therapeutic efficacy than 1 with reduced toxicity.

Conclusion

Hydrophobic 2′-2-bromoacyl taxanes have been synthesized and evaluated for their biological activities against 2′-acyl taxanes lacking a bromine atom. It is obvious from the GI_{50} data that a taxane bearing the 2-

substituted bromine was at least several fold more potent *in vitro* than its equivalent taxane lacking bromine. Furthermore, other taxanes bearing 7-substituted mono-acyl and 2′,7-substituted di-acyl chains with or without bromine were significantly less potent than 2′-2-brominated acyl taxanes when tested *in vitro* for their GI_{50}'s against MCF-7 cells. The therapeutic efficacy data of liposomal bromoacylated taxanes in the treatment of human ovcar-3 xenografted in SCID mice showed that compound **6A** was more effective than other taxane prodrugs in the treatment of ovarian tumor and may possibly be effective against other tumors. The hydrolysis data further supports the hypothesis that the cleavage of the fatty acid chain possibly assisted by a bromine results from the sustain release of paclitaxel in the enhancement of activity of **6A** *in vivo*.

Acknowledgments

The authors thank Dr Donna Lilly for helpful discussion and Dr George Thomas for help with HPLC methodology. The authors also indebted to Tom Baldoni and Lou Zhang for providing help with mass spectrometry, Bhavana Arya for determining the cytotoxicity assays, and Judy Pattassery and Cathy Herbert for their technical assistance.

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(Received 31 October 2000; revised form accepted 11 November 2000)