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Survivin Is Not Induced by Novel Taxanes

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Abstract: Taxanes are a critical component of chemotherapy for breast, prostate, lung and other cancers. Initial or acquired tumor resistance to taxanes is therefore one of the most important issues in oncology. Survivin is a prosurvival gene whose expression is a poor prognostic feature. Survivin is induced acutely upon exposure to taxanes and coordinates resistance to taxane-mediated cell death, although the exact mechanism of taxane-mediated survivin induction is not clear. Here, we describe the synthesis of a series of novel taxanes, with modifications on the 7- or 10-position of the taxane backbone, as well as the side chain. We found that the novel taxanes with modifications at the 10-position have robust tubulin binding and tubulin polymerization activity. Gene expression profiling and quantitative PCR of cells treated with the 10-position conjugates reveals that the effect of treatment with a subset of these novel taxanes lacks a gene expression signature, including survivin induction, which is characteristically induced with paclitaxel treatment. Furthermore, we show that this gene expression signature is not due to differences in G2/M arrest. Cell sensitivity studies suggest that the inability to induce survivin is associated with increased drug cytotoxicity and apoptosis. This work suggests that taxanes that effectively bind tubulin need not invariably induce survivin as a mechanism of drug resistance.

Keywords: Taxanes; chemotherapy; survivin; paclitaxel; prostate cancer; breast cancer; lung cancer

Introduction

The taxanes paclitaxel and docetaxel serve as the chemotherapy backbone for multiple malignancies,[1] including breast, prostate, lung and ovarian cancers.[2−5] Resistance to taxane chemotherapy is therefore one of the most pressing problems in oncology. Survivin, a member of the inhibitor of apoptosis (IAP) family, is differentially expressed in cancer cells, antagonizes apoptosis and promotes tumor-associated angiogenesis.[6,7] Survivin expression is rapidly induced by paclitaxel within 4 h, in a manner that is

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independent of G2/M arrest and induces resistance to taxane-mediated cell death.[8] Survivin expression is a poor prognostic feature that may be due in part to inherent drug resistance and is associated with a more aggressive clinical course in breast[9] and prostate cancer.[10] Furthermore, the inhibition of survivin induction by various means sensitizes cancer cells to chemotherapy.[11,12] The critical importance of chemotherapy resistance due to survivin has led to the pursuit of strategies and the clinical development of agents designed to downregulate survivin expression.[7,13−15] An alternative approach is to instead design equally efficacious drugs which are incapable of inducing survivin as a mode of chemoresistance. Our aim is to describe the design, synthesis and activity of novel taxanes that have equivalent tubulin binding activity to paclitaxel, yet lack the ability to induce survivin, and which may thus lead to better and more effective cancer therapy.

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Materials and Methods

Chemistry. Three series of paclitaxel derivatives were investigated, with substituents in the 7- and 10-positions and on the N-3′ position on the side chain. All compounds contained the cyanonilutamide residue, which was incorporated so as to target the androgen receptor, since cyanonilutamide is an androgen receptor ligand. The detailed synthetic procedures used are provided as Supporting Information, and the structures of all the compounds tested are shown in Figures 1A−1C. In brief, conjugates of cyanonilutamide at the 7-position of paclitaxel were prepared from 2′-(*tert*-butyldimethylsilyl)paclitaxel (2′-TBS-paclitaxel), which was then reacted with a selected bifunctional linker (either bis(paranitrophenyl)carbonate, succinic anhydride, or glutaric anhydride) by treatment with *N*,*N*-dimethylaminopyridine (DMAP) and 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDCI) in dichloromethane (DCM) at room temperature. The resulting monofunctional acyl derivatives were then used to esterify the cyanonilutamide derivative **S2** to generate the corresponding cyanonilutamide analogues. Removal of the TBS protecting group provided a series of C-7 paclitaxel conjugates **1**−**4**.

Conjugates of docetaxel with cyanonilutamide derivatives at the 10-position were synthesized from 2′-(*tert*-butyldimethylsilyl)-7-(triethylsilyl)docetaxel (2′-TBS-7-TES-docetaxel), which was first treated with succinic anhydride or glutaric anhydride and DMAP in toluene at 85−90 °C for up to 4 days. The hemiester products were then conjugated with cyanonilutamide linked alcohols by reaction with DMAP and EDCI in DCM at room temperature. Deprotection of both silyl protecting groups gave docetaxel conjugated at the 10-position with cyanonilutamide via linkers of varying lengths (**5**−**10**).

The synthesis of a cyanonilutamide conjugate at the 3′-position also began with 2′-TBS-7-TES-docetaxel. This was treated with trifluoroacetic acid in DCM at 0 °C to remove the *tert*-butyloxycarbonyl (BOC) group at the 3′-position. Subsequent N-acylation by a cyanonilutamide-linked carboxylic acid and removal of the silyl protecting groups afforded the 3′N-acyl conjugate **11**.

Tubulin Binding and Tubulin Polymerization Studies. *Affinity of Taxanes for the Paclitaxel Site on GMPCPP Microtubules.* The relative affinities of paclitaxel, docetaxel and compounds **1**−**11** for GMPCPP microtubules were assessed by competition experiments. Briefly, GMPCPP microtubules (1 μM) in PME (100 mM PIPES, 1 mM $MgSO_4$, and 2 mM EGTA) were incubated with the fluorescent paclitaxel *N*-debenzoyl-*N*-(*m*-aminobenzoyl)paclitaxel (N-AB-PT, 1 μM) and varying concentrations of the nonfluorescent taxane for 30 min at 37 °C. Owing to the low solubility of some of the synthetic taxanes, all samples

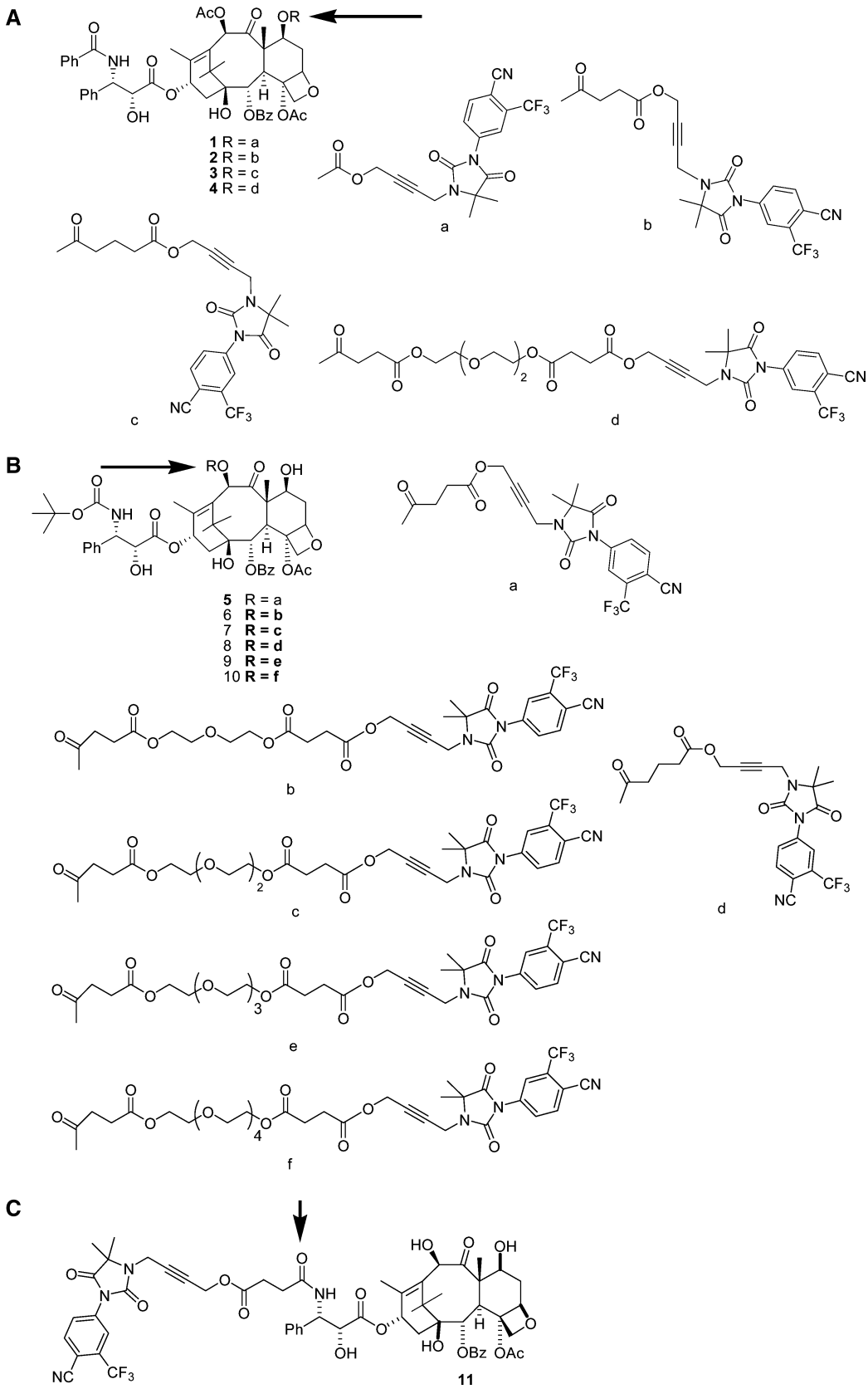

Figure 1. Taxanes investigated. (A) C-7 substituted paclitaxel derivatives **1**−**4**. (B) C-10 substituted docetaxel derivatives **5**−**10**. (C) N-3′ substituted docetaxel derivative **11**.

contained 15% DMSO. We have previously validated the competition binding assay using this concentration of DMSO.[16] The fluorescence intensity of the N-AB-PT containing microtubules was measured in the absence of

competitor (F_0) and in the presence of varying concentrations of taxane (F). Curves were fit to a sigmoidal dose−response curve using SigmaPlot 10.0.

Tubulin Assembly Activity. Tubulin polymerization was monitored by apparent light scattering using a Hewlett-Packard 8453 absorption spectrophotometer with a thermostatted multicell holder that was maintained at 37 °C with a circulating water bath. Tubulin (5 μM) in PME buffer containing 0.1 mM GTP was equilibrated in the sample cell and a baseline was recorded. The selected taxane was added to a final concentration of 2 μM and 4% DMSO (v/v). Tubulin assembly was followed by monitoring the increase in apparent absorption at 350 nm as a function of time.

Cell Culture and Gene Expression Studies. *Cell Culture.* The LAPC4 prostate cancer cell line was obtained courtesy of Charles Sawyers (Memorial Sloan Kettering Cancer Center), grown in Iscove's modified Dulbecco medium with 10% fetal bovine serum. The PC3 prostate cancer cell line was obtained from ATCC (Manassas, VA) and grown in F-12K medium with 10% fetal bovine serum. Before exposure to drug, cells were put on the same medium with 10% charcoal-stripped serum, along with 100 nM paclitaxel, and the conjugated taxanes, or with DMSO alone as a control. Cells were exposed to drug for 24 h, and total RNA was isolated using TRIzol (Invitrogen).

Microarray Analysis. RNA was used in a reverse transcription reaction containing either Cy3-dUTP or Cy5-dUTP, oligo(dT) primer, and SuperScript III. Following reverse transcription, RNA was hydrolyzed using NaOH, the Cy3 and Cy5 reactions were mixed, and the probe was cleaned up using a Microcon YM-30 column (Millipore). The probe was hybridized to the U133 plus array (Affymetrix) and analyzed as described previously.[17]

Analysis of Gene Expression by Quantitative-PCR. Up to 1 μg of total RNA from each sample was reversed transcribed into cDNA using the Single-Strand cDNA Synthesis Kit (Stratagene, Inc.) according to the manufacturer's directions. Quantitative-PCR (qPCR) analysis was performed using TaqMan probes (Applied Biosystems) according to the manufacturer's instructions, in a 10 μL final reaction volume, in 384-well microtiter plates (Applied Biosystems). Thermocycling conditions using an Applied Biosystems ABI-7900 SDS were as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Specific primers for qPCR of GAPDH and survivin were purchased as Assays-on-Demand from Applied Biosystems, making their sequences proprietary in nature. Accurate quantification of each mRNA was achieved by normalizing the sample values to GAPDH and to vehicle treated cells.

Cell Cycle Analysis, Cell Toxicity and PARP Cleavage Studies. *Cell Cycle Analysis.* Cells were treated with taxanes in exactly the same manner as with the gene expression studies above, trypsinized, washed and resuspended in 250 μL of stain solution (0.1 mg/mL propidium iodide, 0.6% Triton-X100). Subsequently, 250 μL of RNase solution (200 U/mL in PBS) was added and the cells were incubated for 30 min in the dark at room temperature. Cells were analyzed using a FACSort flow cytometer (Becton Dickinson, San José, CA) equipped with a 488 nm argon laser. The program ModFitLT v.3.0 (Verity Software, Topsham, ME) was used to determine the percentage of cells in each phase of the cell cycle.

Drug Sensitivity Studies. 20,000 LAPC4 and 3,000 PC3 cells per well were plated on 96-well plates and treated with the indicated concentration of taxane for 72−120 h. The CellTiter-Blue Viability Assay (Promega) was performed as per the manufacturer's instructions.

PARP Cleavage. LAPC4 and PC3 cells were treated with the indicated concentration of taxane for 72 h. Total protein was isolated using lysis buffer, and protein was loaded and run on polyacrylamide gels. Protein was transferred to nitrocellulose membranes, blocked with 5% milk in TBST, incubated with primary and secondary antibodies and washed using standard methods. Mouse anti-PARP (Santa Cruz, Santa Cruz, CA) and mouse anti-actin (Sigma-Aldrich, St. Louis, MO) were used as primary antibodies. Goat anti-mouse (Li-cor Biosciences, Lincoln NE) was used as the secondary antibody, and the Li-cor Odyssey Infrared Imager was used to detect and quantitate signal.

Results

Tubulin Binding and Tubulin Polymerization. The activity of taxanes that is critical to its therapeutic effect is mediated by binding to tubulin and maintaining tubulin in the polymerized state.[18,19] Therefore, we tested the tubulin binding affinity of these novel taxanes, along with their tubulin polymerization activity. Tubulin affinity experiments were carried out under conditions that promote maximum solubility of the drugs. Figure 2A shows 7-position taxane conjugates, all of which have affinities for tubulin that are lower than paclitaxel. This may be attributable to their poor solubility in addition to their supposedly poor affinity to GMPCPP microtubules. Figure 2B displays 10-position taxane conjugates, as well as **11**, which is a side chain conjugate. For **5**, **8**, **9**, and **10**, in the region of lower concentrations (left side of the binding curve), the drop in F/F_0 values is comparable to or steeper than that for paclitaxel at identical concentrations, which indicates that they could have a higher binding affinity for tubulin than paclitaxel. However, the binding curve for **11** is shifted to

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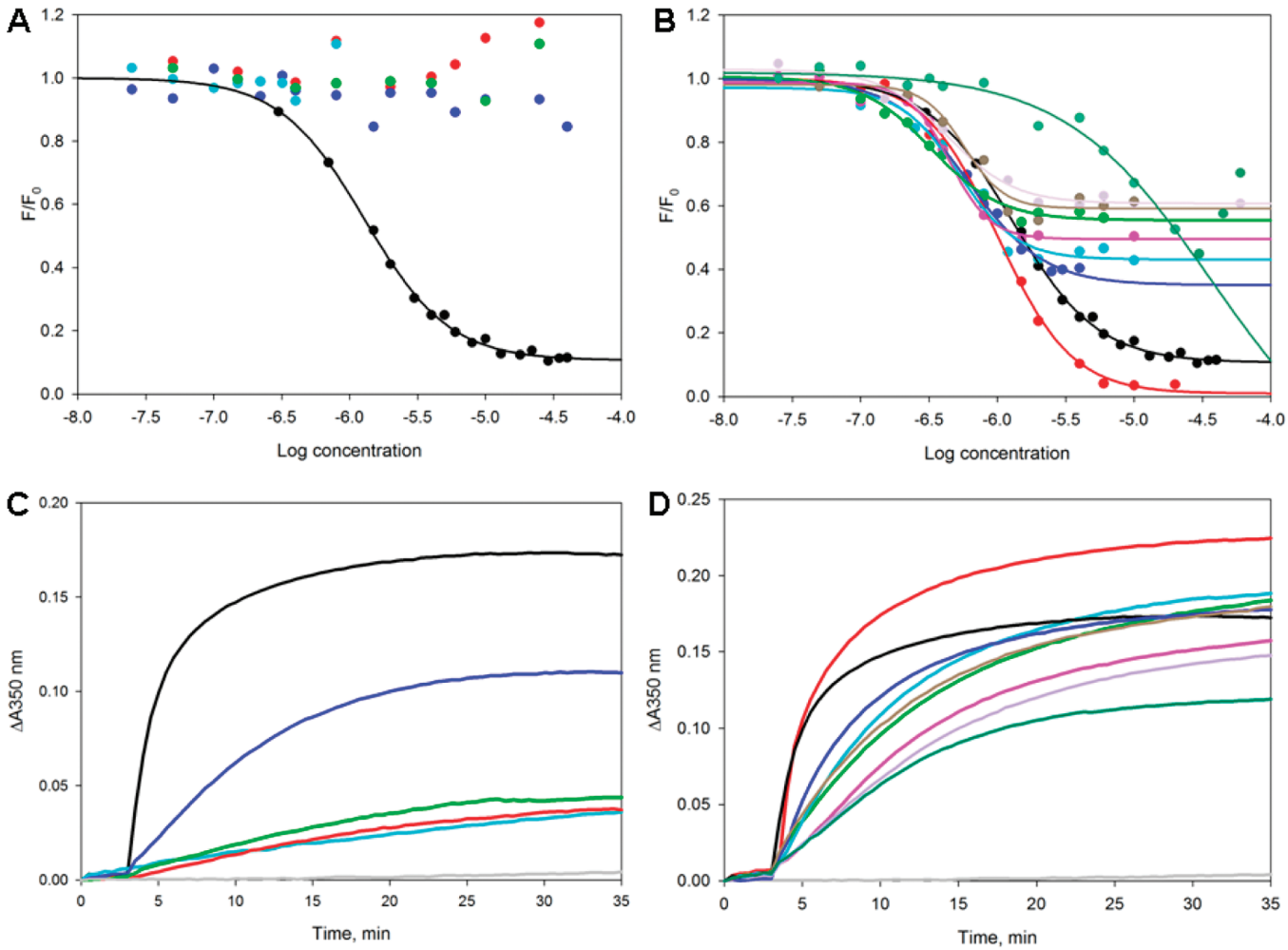

Figure 2. Competition binding and tubulin assembly studies. Panels A and B: Competition of paclitaxel, docetaxel and analogues with fluorescent paclitaxel N-AB-PT for binding to GMPCPP microtubules at 37 °C. The concentrations of GMPCPP-microtubules and N-AB-PT were 1 μM each, and DMSO was 15% (v/v). Panel A: paclitaxel (black), **1** (dark blue), **2** (red), **3** (bright green) and **4** (light blue). Panel B: paclitaxel (black), docetaxel (red), **5** (dark blue), **6** (gray), **7** (tan), **8** (bright green), **9** (light blue), **10** (pink) and **11** (blue green). Note that compounds **5**, **6**, and **8**−**11** were insufficiently soluble under these experimental conditions to yield a complete dose/response curve. Panels C and D: Assembly of tubulin (5 μM) induced by paclitaxel, docetaxel and analogues at 37 °C. The concentration of all ligands was 2 μM, and the concentration of DMSO was 4% (v/v). Panel C: paclitaxel (black), **1** (dark blue), **2** (red), **3** (bright green) and **4** (light blue). No assembly was observed in the protein sample without added taxane (gray). Panel D: paclitaxel (black), docetaxel (red), **5** (dark blue), **6** (gray), **7** (tan), **8** (bright green), **9** (light blue), **10** (pink) and **11** (blue green).

the right, indicating a lower binding affinity than paclitaxel. Next, these compounds were tested for their tubulin assembly activity. Figure 2C shows the activities of the 7-position conjugates compared to paclitaxel. Only compound **1** shows moderate activity; compounds **2**−**4** are only weakly active, and Figure 2D shows the activities of 10-position taxane conjugates and **11** compared to paclitaxel and docetaxel. The activities of most of these compounds, **11** excluded, are comparable to or slightly lower than that of paclitaxel. As a group, the 10-position conjugates have a higher activity than the 7-position conjugates.

Gene Expression. In order to determine how these novel taxanes change the transcriptional program of cancer cells compared to paclitaxel, gene expression was assessed using cDNA microarrays. A group comparison was used to determine gene expression differences among the treatment groups ($P < 0.01$). Experiments were conducted in biological triplicate. Figure 3A shows a gene expression signature that distinguishes the response to paclitaxel and **8** from the response to four other 10-position conjugates. Among the genes induced selectively by paclitaxel and **8** are microtubule and cytoskeleton associated genes, including centromeric proteins, trophinin-associated protein (TROAP), TPX2 and two forms of kinesin. Also induced with this gene expression signature is survivin. Expression of survivin was further confirmed by qPCR (Figure 3B).

Cell Cycle, Cytotoxicity and Apoptosis. Although survivin is induced by paclitaxel in a cell cycle independent manner, survivin expression is also associated with the cell cycle.[8] In order to investigate if the difference between

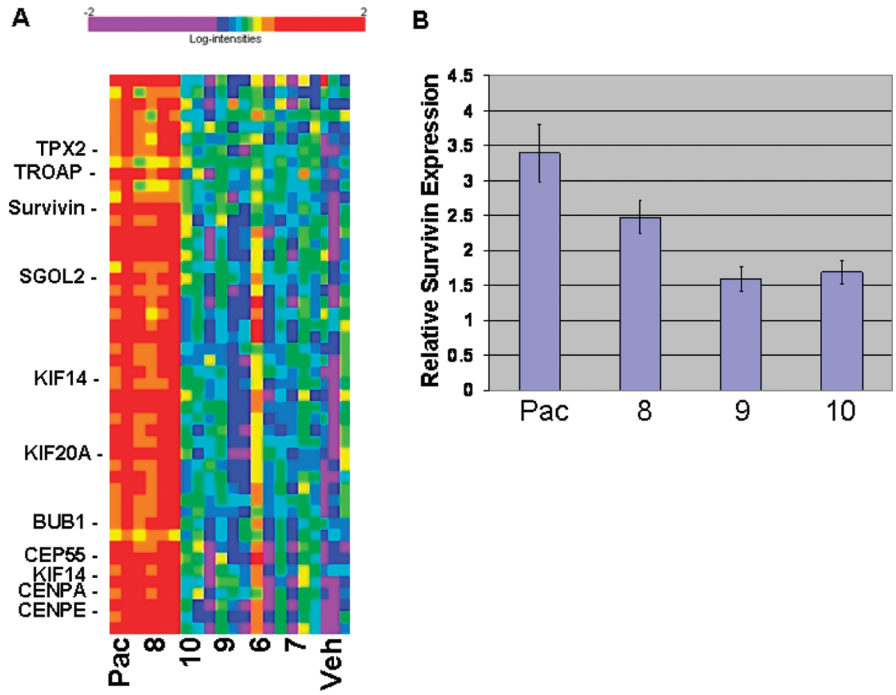

Figure 3. Novel taxanes do not induce survivin expression. A: Paclitaxel and compound **8** induce a DNA microarray gene expression signature after treatment of LAPC4 prostate cancer cells that are not induced by the taxanes **6**, **7**, **9**, and **10**. Results from three biological replicates are shown. B: Confirmation of survivin expression after treatment with selected taxanes with qPCR. Survivin expression normalized to GAPDH is shown. Values are normalized to vehicle treated LAPC4. Error bars indicate the standard error.

taxanes which induce or lack this gene expression signature is due to a difference in cell cycle regulation, cell cycle analysis was done after treatment (Figure 4A). There is no difference in G2/M arrest induced by **8** and the other 10-position conjugates (**6**, **7**, **9** and **10**), which do not induce survivin expression.

LAPC4 and PC3 prostate cancer cells were tested for sensitivity against these taxanes (Figure 4B and 4C). None of these compounds were more potent than paclitaxel. However, despite comparable tubulin binding and tubulin polymerization activity, **8**, which induces survivin, was significantly less active than the other taxanes which do not induce survivin.

Finally, in order to determine if the relative cytotoxicity of these compounds is associated with apoptosis, PARP cleavage was assessed (Figure 4D). The quantity of cleaved PARP that results from treatment with compounds **8**, **9** and **10** followed the pattern of cytotoxicity.

Discussion

Taxanes are a critical and integral part of chemotherapy for many solid tumors, which include lung, prostate, ovarian and breast cancer.[1−5] Although some tumors respond to taxane chemotherapy, many tumors exhibit primary resistance, and metastatic tumors which do respond initially almost always eventually acquire resistance to this class of compounds. Novel taxanes which have equivalent tubulin assembly activity, yet which are not susceptible to the same mechanisms of resistance may yield better and more sustained responses. Chemical modifications on the taxane backbone, such as the C-10 position, allows preservation or an increase in potency but with other drug properties that are unique.[20] Novel taxanes that dampen the induction of survivin expression, which is induced by chemotherapy with taxanes[8,11] and which mediates resistance to taxane therapy,[12,21] would be a valuable tool for new therapeutic strategies in oncology. Here, we describe novel taxanes that have potent tubulin-binding and tubulin polymerization activity. Importantly, survivin induction does not occur upon treatment with a subset of these taxanes. Moreover, the novel taxanes with 10-position conjugates that do not induce survivin (**6**, **7**, **9**, **10**) have more potency compared to the taxane with a 10-position conjugate that does induce survivin (**8**). However, none of the novel taxanes are more effective than paclitaxel in cell toxicity and PARP cleavage. We suspect that this may be due to the chemistry of these novel derivatives that may make these compounds more susceptible to becoming substrates for cellular metabolism or cellular efflux. Nonetheless, of these novel taxanes, those that do not induce survivin expression are significantly more toxic compared to those that induce survivin, in a manner similar to paclitaxel.

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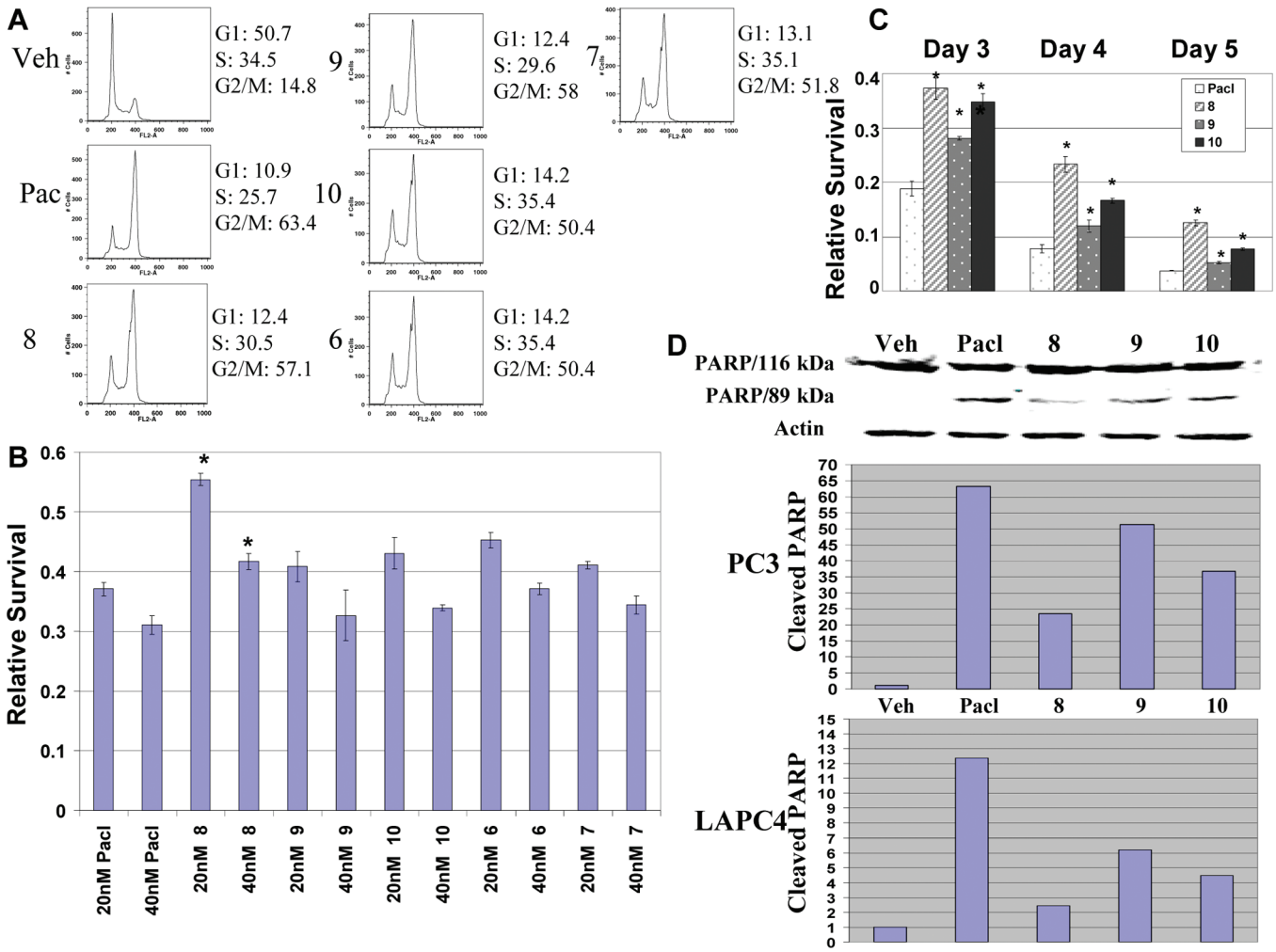

Figure 4. Cell cycle analysis, PARP and cell sensitivity studies. A: Taxanes with 10-position conjugates induce similar degrees of G2/M arrest. B: Compound **8**, which induces survivin, has lower potency in LAPC4 compared with the taxanes that do not induce survivin. *, $P < 0.05$. C: Compound **8** is significantly less cytotoxic to PC3 than compounds **9** and **10**, which do not induce survivin. Cells were treated with 100 nM of each drug. *, $P < 0.05$. D: Treatment of PC3 and LAPC4 cells with compound **8** induces less PARP cleavage than compounds **9** and **10**. Western blot of whole cell lysate from PC3 is shown (top). Quantitation of cleaved PARP relative to vehicle treated cells and normalized to actin is shown in PC3 cells (middle) and LAPC4 (bottom).

One of the intents of this study was to dampen androgen receptor activity with the cyanonilutamide moiety of these compounds, which has intrinsic androgen receptor antagonist activity.[22] However, the gene expression profile did not suggest an effect of these compounds on androgen-responsive genes (not shown), and it is therefore unlikely that lack of survivin induction is in any way related to androgen receptor activity.

The exact mechanism of survivin induction with taxanes and, therefore, the reason for the lack of induction with **6**, **7**, **9**, and **10** are unclear. Although survivin is regulated by the cell cycle, survivin expression in cancer cells is independent of the cell cycle,[23] as it is expressed in tumors with a low mitotic index,[6] and our work suggests that induction of G2/M arrest does not account for survivin expression upon exposure to these taxanes. Survivin is intimately associated with polymerized tubulin, kinetochores and centromere antigens.[7,23] Notably, the taxane conjugates with the longer alkyne-based linkers (**6**, **7**, **9**, **10**) are the ones that do not induce survivin expression, compared with the taxane with an identical cyanonilutamide conjugate but with a shorter linker (**8**). This suggests the possibility that differences in survivin regulation with these tubulin-binding compounds may be related to the linker length and a signal that is perturbed or repressed by taxanes bound to polymerized tubulin. Altering the linker length may influence the assembly

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of proteins around polymerized tubulin and downstream signaling that is dependent on these processes. Although more work remains to be done to elucidate what mechanisms mediate survivin induction with paclitaxel and, conversely, what inhibits survivin expression with compounds **6**, **7**, **9** and **10**, the novel taxanes and their properties described here serve as an important starting point in elucidating these processes.

In conclusion, we describe the synthesis and properties of novel taxanes. A subset of these taxanes have potent tubulin-binding and tubulin polymerization activity, yet lack the capability to induce survivin expression: an important mechanism of drug resistance that occurs with paclitaxel therapy. These findings suggest that treatment with novel taxanes that are not susceptible to canonical drug resistance mechanisms may be achievable in the future.

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Supporting Information Available: Detailed synthetic procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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