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Synthesis and Evaluation of Daunorubicin-Paclitaxel Dimers

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Abstract—Bifunctional, heterodimeric compounds were synthesized to test their ability to create polyvalent arrays between DNA and microtubules in cells. Each dimer was examined for the capacity to bind to microtubules and for cytotoxicity against MES-SA and MES-SA/Dx5 cell lines.

Noncovalent binding of small molecule drugs to biological macromolecules is the initiating event for many anticancer agents. Examples of these interactions include the binding of paclitaxel (PTX) to microtubules (MTs) and the binding of daunorubicin (DNR) to DNA. MTs and DNA are biopolymers that possess multiple ligand binding sites. Whitesides and others have shown that the binding of low affinity, extracellular ligands can be significantly improved by covalently tethering many ligands together to form a polyvalent array.[1–6] We proposed to use a conceptually similar approach for intracellular targets by taking advantage of the fact that certain biopolymers contain multiple binding sites (e.g. MTs and DNA), in conjunction with synthetic bifunctional heterodimers, to create polyvalent arrays in cells. The expectation was that this would enhance binding of the ligands to their respective receptors and thereby improve cytotoxicity.

Design and Synthesis of Dimers

Recent structure–activity relationship studies on PTX and DNR have revealed that certain modifications are well tolerated while others cause significant decreases in biological activity. In general, modifications to PTX in the C7 to ClO region are generally tolerated without loss of biological activity.[7,8] Studies with fluorophore-linked analogues have shown that bulky groups can be attached to the C7 hydroxyl group of PTX without abolishing efficacy.[9] Nicolaou et al. have demonstrated that the C7 hydroxyl group of paclitaxel can be derivatized as an ester that contains a nascent pendant amine.[9] Structure–activity[10] and crystallographic[11] data on the interaction of DNR with DNA indicated that, while the sugar moiety is vital for efficacy, certain alterations are tolerated. Furthermore, a bis-daunorubicin compound consisting of two DNR molecules linked through the sugar amine moieties binds to DNA more tightly than the monomeric species.[12] We expected that the nature of the linking group between the two halves of the dimers could be very important. Therefore a variety of linkers, rigid and flexible, aliphatic and aromatic, were investigated.

The procedure of Nicolaou et al.[9] was used to prepare the amino-functionalized PTX, resulting in 7-β-alanyl-paclitaxel (**2**, Scheme 1). DNR was functionalized with the linking groups by reacting the aminosugar with one end of a dicarboxylic acid or its equivalent to afford DNR acids **4a**–**f** (Scheme 1, step d). Dimer synthesis was achieved in the final step by coupling **2** and **4a**–**f** to afford the target molecules **5a**–**f** in a 40–70% yield after chromatographic purification.[13] The chromatographic mobilities, polarities, and solubilities of the heterodimeric molecules are more similar to paclitaxel than they are to daunorubicin.

Analysis of Dimers

The dimers were initially evaluated to determine whether each half retained the ability to interact with its respective target. DNR absorbs strongly at 480 nm and fluoresces at 557 nm. This fluorescence is quenched upon binding to DNA.[14] Fluorescence quenching experiments were performed on dimers and DNR and the results were compared. Our results indicated that the DNR half of the dimers retain the ability to bind to DNA, with slightly decreased strength relative to DNR alone (data not shown).

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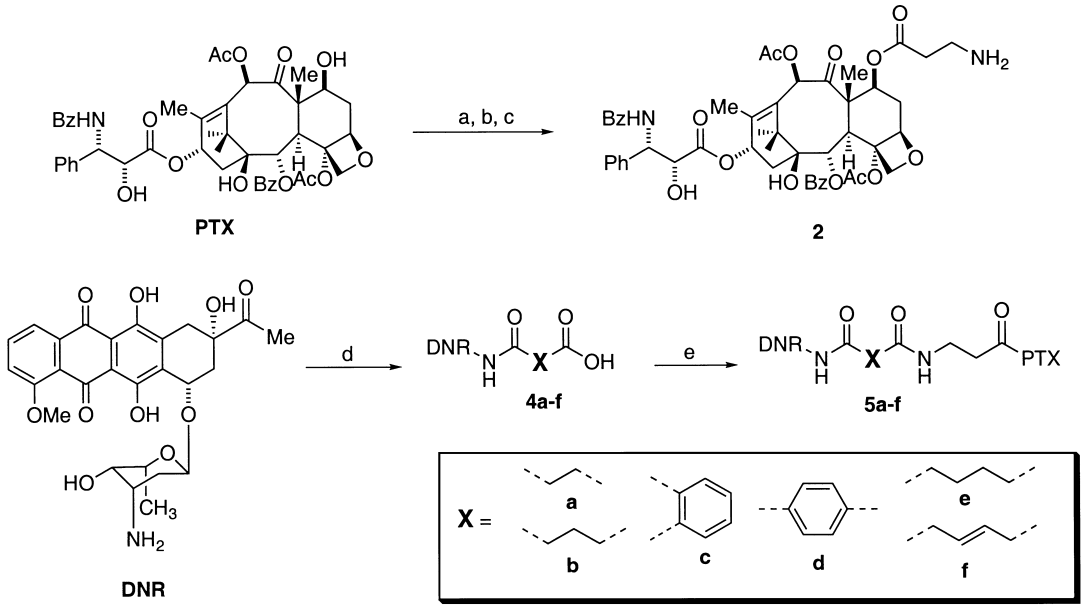

Scheme 1. (a) Benzylchloroformate, pyr, CH_2Cl_2, 65%; (b) *N*-Cbz-β-alanine, DCC, DMAP, CH_2Cl_2, 95%; (c) H_2, Pd/C, MeOH, 95%; (d) Condition 1: succinic, glutaric or phthalic anhydride (5 equiv), 2,6-lutidine (2 equiv), MeOH, rt, overnight, yields: 56–90%. Condition 2: terephthalic acid or *trans*-β-hydromuconic acid (2 equiv), PyBroP (1.1 equiv), DIPEA (2 equiv), DMF, rt, 30 min, yields: 35–50%. Condition 3: adipic acid mono ethyl ester (2 equiv), PyBroP (1.1 equiv), DIPEA (2 equiv), DMF, rt, 30 min, then LiOH (3 equiv), THF:H_2O (3:1 v/v), 40%; (e) **2** (0.9 equiv), PyBroP (1.1 equiv), DIPEA (2 equiv), THF, rt, 30 min, yields: 40–70%.

Two experiments were performed to test the interaction between our synthetic bifunctional molecules and tubulin/MTs. Transmission electron microscopy showed that dimers **5a** and **5d**, like PTX, cause tubulin to polymerize into stable MTs (Fig. 1); however, fewer MTs were formed overall. In addition to MTs, aggregated tubulin protein was observed in the dimer treated samples and was not observed in the taxol treated samples. Neither MTs nor aggregated tubulin were observed when tubulin was treated with DNR.

In order to quantitate the interaction between the dimers and MTs, a competition binding assay was performed using [^{3}H]-paclitaxel (Fig. 2).[15] EC_{50} concentrations for PTX and intermediate **2** were 9 and 35 μM respectively. Dimers **5a**–**f** showed no competition up to 100 μM. This shows that derivitization of PTX with β-alanine results in only a moderate decrease in binding affinity (ca. 4-fold) as expected, whereas attachment of the large DNR moiety causes a more significant decrease (>10-fold). DNA was added to the samples to determine if the affinity could be improved by forming self-assembled polyvalent arrays. In the presence of DNA, the synthetic bifunctional molecules did not improve as competitors of [^{3}H]-PTX bound to MTs.

The microscopy experiments demonstrate that the PTX–DNR heterodimers induce weak polymerization of tubulin. However, the results of the competion binding assay show no detectable binding of dimer to the PTX binding site. We explored the possibility that the binding of the DNR portion of the dimers to tubulin was responsible for the formation of polymerized tubulin through a simple MT sedimentation assay. Tubulin protein was incubated with DNR in either the presence or absence of PTX and then pelleted by centrifugation. The amount of DNR remaining in the supernatant was then measured spectroscopically.[16] Incubation of DNR with MTs or tubulin resulted in no appreciable loss of signal compared to the absorbance of DNR in the absence of protein, indicating that DNR is not bound to sedimented MTs (data not shown).

The dimers were next tested for their ability to kill mammalian cells in culture. MTT cytotoxicity assays were performed as described.[17,18] Two cell lines examined were the MES-SA uterine tumor line and MES-SA/Dx5, a multi-drug resistant strain due to P-glycoprotein (P-gp) overexpression. The results in Table 1 show that the dimers are 100- to 4000-fold less cytotoxic than PTX and 5- to 100-fold less potent than DNR in the MES-SA line. Interesting differences between the dimers were observed in the Dx5 line. Treatment of Dx5 cells with the aliphatic-linked dimers resulted in no cytotoxicity, whereas the aromatic-linked dimers (**5c** and **5d**) retained weak activity and are five times less potent than the PTX or DNR monomers. As expected, co-treatment of the dimers with the P-gp inhibitor PSC-833 restored the cytotoxicity of the dimers in the Dx5 line and had no effect on the MES-SA line.[19]

Cells that are treated with PTX undergo apoptosis, and one of the hallmarks of this process is the phosphorylation of the Bcl-2 protein, whereas DNR mediated cytotoxicity does not affect Bcl-2.[20,21] As shown by Western blot in Figure 3, cells treated with PTX display phosphorylated Bcl-2 while untreated (CTRL) and DNR-treated cells do not phosphorylate Bcl-2. A mixture of PTX and DNR (Fig. 3, both) also causes Bcl-2 phosphorylation. Incubation and Western blot analysis of dimers **5a** and **5d** show phosphorylation and, therefore, a PTX-like efficacy, although this does not supplant the

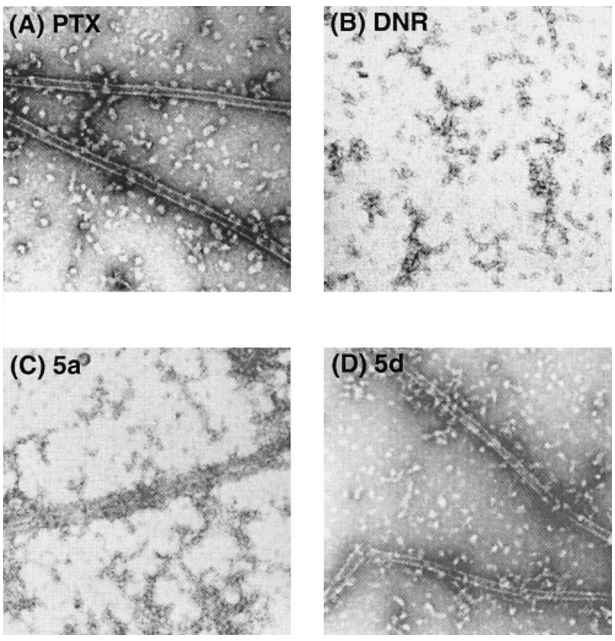

Figure 1. Electron micrographs of tubulin incubated for 1 h at 37 °C with (A) PTX, (B) DNR, (C) **5a**, or (D) **5d**. Protein was adsorbed onto carbon-coated grids and negatively stained for TEM.

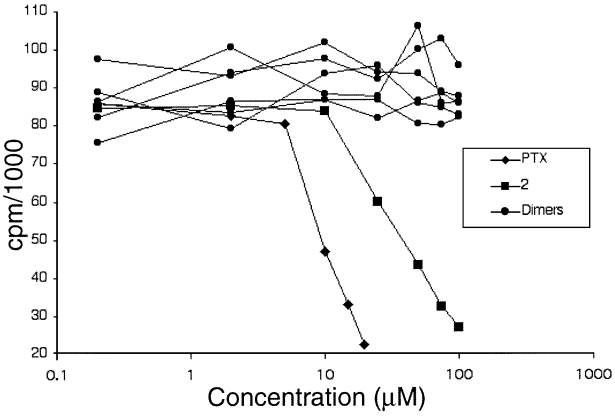

Figure 2. Competition binding assay. Steady-state MTs (1 mg/mL tubulin, 2 mM GMPCPP, BRB 80, 37 °C, 45 min) were treated with 1 μM [^{3}H]-PTX and a competing drug (0–100 μM) for 45 min and pelleted. [^{3}H]-PTX activity in pellet was plotted versus competitor concentration.

possibility that the cytotoxicity of the dimers is due, in some part, to DNR-mediated events.

Discussion

PTX causes cell-cycle arrest at the metaphase-anaphase transition of mitosis, at which point the nuclear envelope is no longer present in human cells. This makes it possible for bifunctional heterodimers to interact with both MTs and DNA in a polyvalent fashion, potentially resulting in greater efficacy than that of the parent monomers. However, the cytotoxicity assays showed that the dimers were less cytotoxic than the parent monomers. The decreased cytotoxicity could be due to the large size of the dimers, which makes it more difficult for them to enter cells. Alternatively, the larger dimers could be better substrates for the P-gp efflux pump. The fact that some of the dimers (**5c** and **5d**) are cytotoxic to the resistant Dx5 line and that the P-gp inhibitor had no effect on the MES-SA line argues against the latter possibility.

Table 1. Results from MTT assays[a]

Compound	MESSA	Dx5	Dx5 w/PSC
PTX	0.0006	2.7	0.0003
DNR	0.021	2.0	0.014
PTX + DNR	0.011	1.0	
5a	0.7	>50.0	
5b	0.95	>50.0	1.1
5c	0.099	16.0	0.036
5d	1.0	10.0	1.0
5e	2.3	>50.0	
6	0.28	>50.0	0.3

[a]Dx5 is a MESSA/MDR cell line. PSC = P-gp inhibitor PSC-833. Each result is an average of 8-16 determinations. IC_{50} reported in μM.

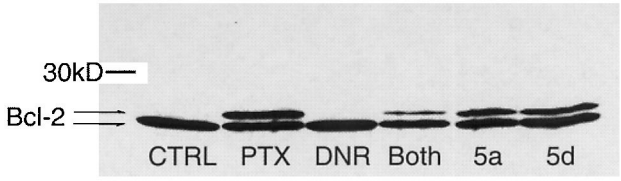

Figure 3. Bcl-2 phosphorylation gel-shift. MES-SA cells were treated with a drug for 12 h. Protein was detected by Western blot analysis. The bottom arrow is Bcl-2. The top arrow is phophorylated Bel-2.

Linking DNR to PTX may disrupt or diminish PTX's ability to interact with MTs. The EM and competition binding assays suggest that a decreased affinity for MTs may be affecting the cytotoxicity. Even though the C7 hydroxyl group of PTX is amenable to many alterations, it may be that the addition of something as large as DNR significantly attenuates binding to MTs at even an 'insensitive' position. Alternatively, the increased bulk due to the tethered DNR may prevent the bifunctional molecule from passing to the interior of MTs, which is the recently proposed location of the PTX binding-site.[22]

Conclusion

We have shown that daunorubicin and paclitaxel can be covalently linked, and that each half of the resulting dimer retains the ability to interact with its respective target. The dimers are, however, less cytotoxic than the parent monomers. This is probably due to decreased affinity for targets because of the increased bulk of the dimers. The decreased affinity makes it difficult to determine whether it is possible for the dimers to access both targets simultaneously. Additional experiments are currently underway to explore this possibility.

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13. The ^{1}H NMR spectra of each intermediate or final product was compared to spectra of daunorubicin and paclitaxel. All spectra were in accord with the expected structures. To further confirm the structures, ES-MS of the dimers were obtained which verified their respective molecular weights. ES-MS: **5a**: calcd 1534, found 1557 (M + Na); **5b**: calcd 1548, found 1550 (MH + + ^{13}C); **5c**: calcd 1582, found 1605 (M + Na); **5d**: calcd 1582, found 1605 (M + Na); **5e**: calcd 1562, found 1563 (MH +); **5f**: calcd 1560, found 1583 (M + Na).
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15. Briefly, PC-tubulin (1 mg/mL) was incubated under assembly conditions (BRB80 buffer, pH 6.8, 2 mM GMPCPP, 37 °C) until MT assembly reached steady state (45 min). Preliminary experiments had defined these conditions as inducing essentially maximal MT polymerization. Next, 1 μM [^{3}H]PTX (5 μgCi/μmol, Moravek Biochemical) and other competing drugs were added to the solution containing the assembled MTs and incubated for an additional 45 min at 37 °C. The MTs were then pelleted by centrifugation at 14,000 rpm in an Eppendorf microfuge for 30 min. The supernatants were carefully removed and the pellets were resuspended in 0.1 M NaOH solution then neutralized with 0.1 M HCl and transferred to a scintillation vial. Approximately 5 mL of Cytoscint liquid scintillation cocktail was added to each, and the samples were counted on a Beckman LS 3801 Liquid Scintillation Counter.
16. UV–visible absorption spectra were measured for solutions of DNR (0–50 μM). Next, tubulin (1.0 mg/mL) was treated with varying amounts of DNR (0–50 μM) in either the presence or absence of PTX (10 μM). All protein containing samples were pelleted by centrifugation, and the supernatants were analyzed spectrophotometrically. The absorption at 480 nm for all solutions was plotted as a function of DNR concentration.
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18. MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Assay: MES-SA (uterine tumor) or MES-SA/Dx5 (MDR overexpression) cells were plated in 96-well microtiter plates at 80,000 cells/mL. After 24 h at 37 °C in a humidified atmosphere containing 5% CO_2, the cells were exposed to drugs at the appropriate dilutions and incubated for 72 h. MTT reagent (20 μL of 5 mg/mL in PBS buffer) was added to each well. After 3 h, the medium was aspirated and 0.1 N HCl-isopropanol solution was added to solubilize the formazan salts and thoroughly mixed. Absorbances were measured on a multiwell spectrophotometer (Molecular Devices, Menlo Park, CA) at 570 nm. The data were plotted in Excel to determine the IC_{50}. Values reported for PTX + DNR combination are the concentrations of each compound for a total drug concentration twice the reported value.
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