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TOWARD PACLITAXEL–[60]FULLERENE IMMUNOCONJUGATES AS A TARGETED PRODRUG AGAINST CANCER

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Two newly synthesized water-soluble conjugates of Paclitaxel with malonodiserinolamide-derivatized [60]fullerene (C_{60}-ser) undergo hydrolysis and release their medical payload under biological conditions. *In vivo* testing of one of these compounds in a murine model showed tumor volume reduction similar to the FDA-approved drug Abraxane, but without the associated weight-loss, indicating better tolerance of this new formulation.

Keywords: Fullerene, Paclitaxel, Cancer, Immunoconjugate.

1. Introduction

Paclitaxel is a clinically used anticancer drug, which acts by binding stoichiometrically and specifically to the β-tubulin subunit in the microtubules in cell nuclei, thus preventing cell division [1]. Paclitaxel dissolved in Cremophor EL, polyethoxylated castor oil, (Taxol®) is widely used to treat various types of cancer, including breast cancer and non-small cell lung cancer (NSCLC) [2]. Abraxane is a water-soluble formulation of Paclitaxel, bonded to albumin as a delivery vehicle; it received US FDA approval in 2005 for medical use, including the treatment of NSCLC [3].

Paclitaxel's clinical use is primarily limited by its high non-specific cytotoxicity and its poor water solubility. Since there are many reports indicating that prolonged administration of higher Paclitaxel doses is poorly tolerated by patients because of myalgia and neurotoxicity [4–6], it is important to choose the right delivery vehicle for Paclitaxel which can mitigate these undesirable properties. [60]Fullerene, due to its antioxidant properties, was recently shown to serve as a possible prevention and treatment of various neurological diseases such as Parkinson's disease, Alzheimer's disease, senility, and schizophrenia, in which the role of free radical production has recently come into focus [7–9]. Thus, combining the water-soluble fullerene with Paclitaxel is a new strategy using the fullerene as a non-toxic transfection agent vector to deliver a chemotherapeutic prodrug to cancer cell nuclei.

Recently, we have shown that a fluorescently-labeled, biocompatible malonodiserinolamide-derivatized [60]fullerene (C_{60}-ser) is internalized within living cancer cells in association with serum proteins through multiple energy-dependent pathways. The labeled C_{60}-ser escapes endocytotic vesicles to eventually localize and accumulate in the nucleus of the cell through

the nuclear pore complex [10, 11]. *In vitro* studies indicate a very low cytotoxicity for C_{60}-ser in cells. Together, these findings suggest that C_{60}-ser can serve as a potential delivery vehicle for therapeutic agents with intranuclear activity (DNA plasmids, drugs such as paclitacel, gemcitabine, camptothecin, cisplatin, siRNA, transcription factors, epigenetic agents, etc) to treat cancer. Targeted delivery of these vehicles can be achieved by their binding with tumor-specific antibodies, due to the known affinity of water-soluble fullerenes for antibodies to form immunoconjugates [12].

As part of our efforts to treat cancer with carbon nanostructure-mediated RF hyperthermia (including [60]fullerene) [13], here we report the syntheses of two new water-soluble conjugates of Paclitaxel with C_{60}-ser, **3** and **7**, which are expected to undergo hydrolysis and release Paclitaxel under controlled conditions. The rationale behind this approach is 1) to facilitate the membrane transfer, both cellular and nuclear, of Paclitaxel due to the properties of the [60]fullerene moiety as a transfection agent [14], 2) to achieve selective delivery of Paclitaxel from a prodrug by esterase hydrolysis inside cancer cells, and 3) to achieve high water solubility of the Paclitaxel prodrug in comparison to Paclitaxel itself.

Cell culture and *in vivo* testing of these [60]fullerene-Paclitaxel conjugates have shown that both **3** and **7** have equal or superior efficacy to current Cremophor EL and Abraxane® Paclitaxel formulations, though they both will require further refinement to become clinically viable.

2. Results and Discussion

2.1. Synthesis and characterization of C_{60}-Paclitaxel conjugates

C_{60}-Paclitaxel conjugates **3** and **7** have been synthesized and purified according to the procedure briefly described below. The reaction scheme is shown in Fig. 1.

Proton nuclear magnetic resonance spectra were measured with a *Bruker 400 MHz NMR spectrometer* with tetramethylsilane as an internal standard. MS spectra for water-insoluble compounds were collected using a *MS Reflex IV MALDI-TOF mass spectrometer* and for water-soluble fullerene derivatives, by *MS electrospray ionization time-of-flight (ESI-TOF) mass spectrometer*, both instruments from Bruker Daltonics Inc (Fremont, CA).

1 (Paclitaxel-2'-poly(ethylene glycol) ester) was synthesized generally following the scheme outlined in [15]. In a typical experiment, 1 g (1.171 mmol) of vacuum-dried Paclitaxel was put into a 100 ml flask with 50 ml of dry triclene (1,1,2-trichloroethene) with 0.54 g ($8.955 \cdot 10^{-4}$ mol) of PEG600 carboxylic acid and 4-dimethylaminopyridine (DMAP, 0.3 g, 2.4 mmol) dissolved. The stirred solution was cooled with ice, and a solution of diisopropylcarbodiimide (*i*PrN=C=N*i*Pr, 0.15 g, 1.171 mmol) in 1 ml of triclene was added. Reaction progress at RT was monitored by the dissolving of Paclitaxel. Flash purification on silica using a CH_2Cl_2–MeOH eluent system yielded 0.58 g of **1** (white solid, .041 mmol, 46%). MS Autoflex MALDI-TOF (positive ion mode): calculated masses = 1321.55 (n=8), 1365.58 (n=9), 1409.60 (n=10), 1453.63 (n=11), 1497.66 (n=12), 1541.68 (n=13), 1585.71 (n=14) Da; observed masses = 1322.736, 1366.754, 1410.789, 1454.808, 1498.844, 1542.902 Da [$M+H^+$], 1388.827, 1432.827, 1476.859, 1520.893, 1564.968, 1608.990 Da [$M+Na^+$]. NMR (CDCl3, TMS reference): ^1H δ(ppm) = 1.109 (d), 1.177 (s), 1.223 (d), 1.643 (s), 1.671 (d), 1.808 (t), 1.931 (d), 2.043 (dd), 2.213 (d), 2.333 (s), 2.471 (t), 3.489-3.697 (m), 3.721 (dd), 3.746 (s), 4.013 (bs), 4.162 (dd), 4.262 (t), 4.244(s), 4.262 (t), 4.272–4.333 (m), 4.367 (dd), 4.433 (dd), 4.904 (d), 4.934 (d), 5.542 (q), 5.596 (s), 5.610 (s), 5.625 (s), 5.954 (d), 6.085 (t), 6.230 (s), 6.291 (s), 7.155 (t), 7.301–7.556 (m), 7.631 (q), 7.754 (d), 7.903 (d), 8.071 (d), 8.137 (d).

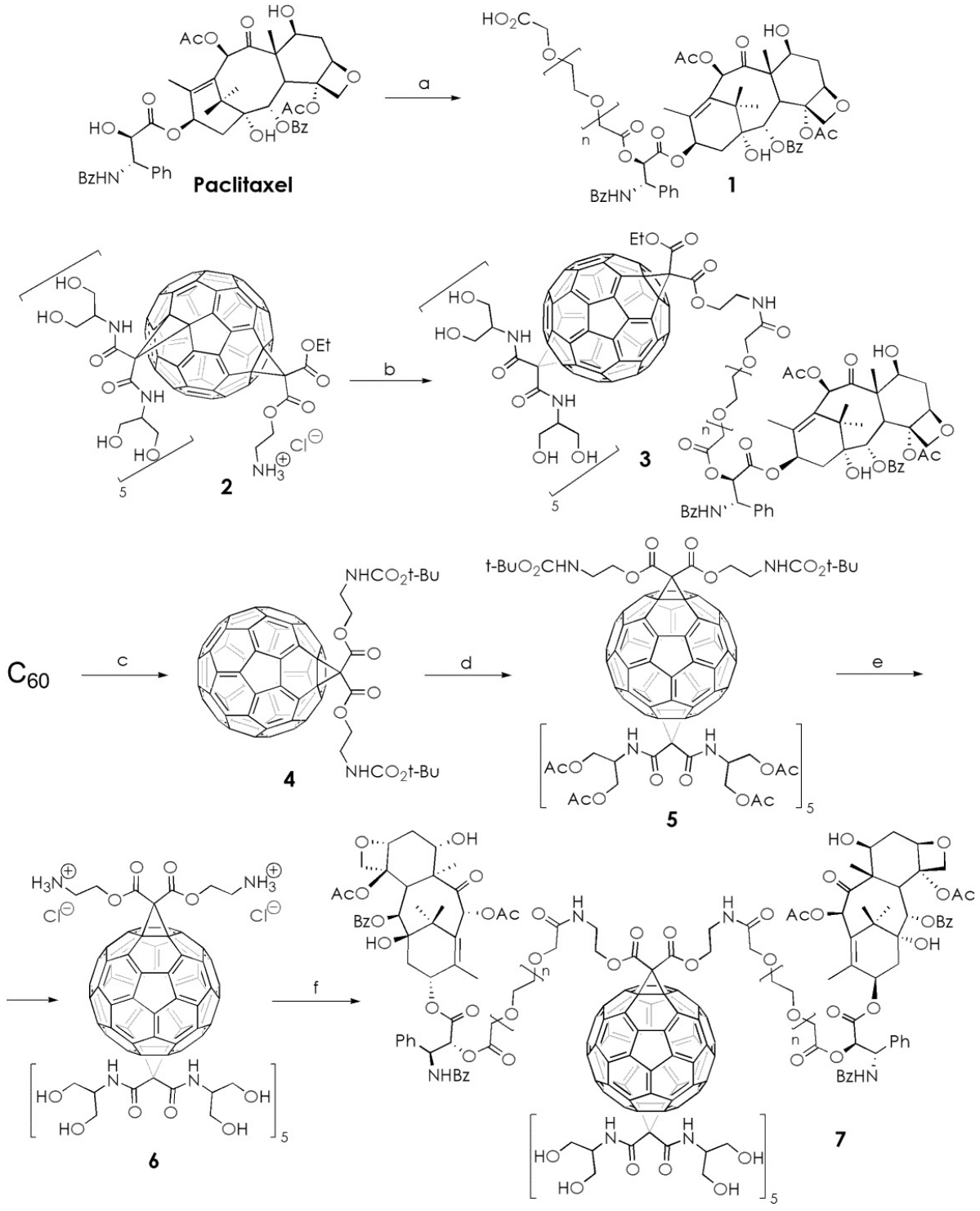

FIG. 1. The synthesis of C_{60}–Paclitaxel conjugates 3 and 7 ($n \approx 10$). Reagents and conditions: (a) PEG-600 dicarboxylic acid, in $CHCl{=}CCl_2$, iPrN=C=NiPr, 4-dimethylaminopyridine, 36 h; (b) 1, EDC, MES hemisodium salt buffer (pH = 6.5), 15–30 min; (c) bis-(β-*tert*-butoxycarbonylaminoethyl) ester of malonic acid, CBr_4, DBU, 3 h; (d) N,N'-bis[2-(acetyloxy)-1-[(acetyloxy)methyl]ethyl]-malonamide, CBr_4, DBU, overnight; (e) 1 M HCl in dioxane: H_2O (99:1), 24 °C, 48 h; (f) 1, EDC, MES buffer (pH = 6.5), 4 h

3 (C_{60}-ser-Paclitaxel-2'-poly(ethylene glycol) ester) was synthesized by dropwise addition of 10.8 mg (0.005 mmol) of **2** in 400 ml of water (prepared and purified according to procedure given in [10]) to 7.9 mg (0.0055 mmol) of **1**, with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 1 mg, 0.006 mmol), and hydroxybenzotriazole (HOBt, 0.5 mg, 0.004 mmol, catalytic amount) in 10 ml of 1M MES hemisodium salt buffer (pH = 6.5), stirring for 30 min at RT. Then the product was extracted with 3×10 ml of chloroform, dried *in vacuo*, purified by HPLC (MeOH – water, Atlantis T3 column, 4 ml/min flow rate, Waters LC Module 1Plus) to afford 5.1 mg of product (29% yield). MicroTOF ESI (positive-ion mode): calculated masses = 3437.11 (n=8), 3481.14 (n=9), 3525.17 (n=10), 3569.19 (n=11), 3613.22 Da (n=12); observed mass = 3440.0, 3483.4, 3528.1, 3572.1 Da [M+], 3461.6, 3505.5, 3550.3 Da [M+Na^+], 3566.9 Da [M+K^+].

4 (1',1'-dicarboxylic acid di-(2-*tert*-butoxycarbonylamino-ethyl ester) 1,2-methano[60]fullerene) was synthesized in accordance with a known literature procedure [16]. 727 mg of C_{60} (1 mmol) was dissolved in dry toluene (500 ml) with 500 mg of CBr_4 (1.5 mmol), with 585 mg (1.5 mmol) of malonic acid bis-(b-*tert*-butoxycarbonylaminoethyl) ester, prepared as described in [17]. The solution of 230 mg of DBU (1.5 mmol) in toluene was added within 30 min at RT, after which the reaction proceeded for 3 hours. The product was isolated via flash column chromatography using a toluene – ethyl acetate (9:1) eluent system. Yield was 455 mg (46%). MALDI-TOF MS (pos. ion mode): calculated mass = 1109.1, observed mass = [M+H^+] 1110.0. ^{1}H NMR ($CDCl_3$, TMS ref.) ^{1}H δ(ppm) = 1.58 (s, 18H, C(CH_3)$_3$), 3.61 (t, 4H, CH_2NH), 4.59 (t, 4H, CH_2O), 5.11 (s, 2H, N*H*).

5 (3',3'-di-((2-((*tert*-butoxycarbonyl)amino)ethoxy)carbonyl)- 3", 3", 3"', 3"', 3"", 3"", 3""', 3""', 3""", 3"""-deca-(2-acetoxy-1-acetoxymethylethylcarbamoyl)-3'H, 3"H, 3"'H, 3""H, 3"""H, 3"""H-hexacyclopropa [1, 9 : 16, 17 : 21, 40 : 30, 31 : 44, 45 : 52, 60] (C_{60}–I_h) [5,6] fullerene). 111 mg (0.1 mmol) of **4** dissolved in 300 ml of toluene, with 332 mg of CBr_4 (1 mmol), and 837 mg (2 mmol) of acetic acid 3-acetoxy-2-[2-(2-acetoxy-1-acetoxymethyl-ethylcarbamoyl)-acetylamino]-propyl ester (2 mmol) ("serinol malonate", prepared according to [18], to which a solution of 141 mg of *tert*-butylimino-tris(dimethylamino)phosphorane (0.6 mmol) in 100 ml of dry methylene chloride was added in 3 hours. After 18 hours, the product was isolated via flash column chromatography with a chloroform - ethanol eluent (19:1). Yield 153 mg (48%). MALDI-TOF MS (pos. ion mode): calculated mass = 3190.95, observed mass = [M+H^+] 3191.91. 1H NMR (400 MHz, $CDCl_3$, TMS reference) d (ppm) = 1.48 (s, 18H, C(CH_3)$_3$), 2.08 (s, 60H, acetate CH_3), 3.55 (t, 2H, CH_2NH), 4.38 (t, CH_2O), 4.58 (m, 10H, in serinol), 4.27 (m, 40H, in serinol), 4.9 (s, 12H, N*H*).

6 (3',3'-di-((2-aminiumethoxy)carbonyl)- 3", 3", 3"', 3"', 3"", 3"", 3""', 3""', 3""", 3"""-deca-(2-hydroxy-1-(hydroxymethyl)ethylcarbamoyl)-3'H, 3"H, 3"'H, 3""H, 3"""H, 3"""H-hexacyclopropa [1, 9 : 16, 17 : 21, 40 : 30, 31 : 44, 45 : 52, 60] (C_{60}–I_h)[5,6]fullerene dihydro-chloride) was obtained by hydrolysis of **5** (153 mg, 0.05 mmol), dissolved in 10 ml of dioxane-water (99:1) and saturated with HCl gas at RT up to a 1M concentration. The mixture was kept closed for 48 hours at RT, then the solvents was removed *in vacuo*, and the product was purified using a cellulose ester dialysis membrane (Thermo Scientific, 2000 MWCO) for 7 days using DI water with pH=4.0, adjusted by $HCl_{(aq.)}$. Yield 107 mg (98%). ESI-TOF MS (pos. ion mode): calculated mass (cation) = 2152.0, observed masses = [M^++2H_2O] 2188.1. ^{1}H NMR (400 MHz, DMF-D_7, 2.75 peak used for ref.) d (ppm) = 2.96 (t, 4H, $CH_2NH_3^+$), 3.84 (t, 4H), 3.96 (m, 40H, CH_2OH), 3.55 (m, 10H, CH_2NH).

7 (C_{60}-ser-*bis*-Paclitaxel-2'-poly(ethylene glycol) ester) was synthesized by dropwise addition of 5.5 mg (0.0025 mmol) of **6** in 400 μl of water to a solution of 7.9 mg (0.0055 mmol) of **1**, 1 mg (0.006 mmol) of EDC, 1 mg (0.008 mmol) of HOBt in 10 ml of 1M MES

TABLE 1. Hydrolysis half-lives in hours of 1, 3 and 7 at 23.5 °C

	1	**3**	**7**
0.1% $CF_3CO_2H_{(aq.)}$ (pH $\approx$ 2)	1.68 $\pm$ 0.30	1.22 $\pm$ 0.21	n/a
1M MES buffer (pH = 5.6)	75.6 $\pm$ 13.0	64.5 $\pm$ 12.0	49.5 $\pm$ 12.0
1x PBS buffer (pH = 7.4)	47.4 $\pm$ 12.0	38.5 $\pm$ 11.0	35.5 $\pm$ 11.0

buffer (pH = 6.5), stirred for 4 h at RT. The product was extracted with 3$\times$10 ml of chloroform, dried *in vacuo*, purified by HPLC (MeOH – water, 5PYE column, 4 ml/min flow rate, Waters LC Module 1Plus) to afford 5.0 mg of product (40% yield). MicroTOF ESI (positive-ion mode): calculated masses = 4890.91 (n+n=19), 4934.97 (n+n=20), 4979.02 (n+n=21) Da; observed mass = 4891.3, 4935.4 [M+H^+], 5002.6, 4958.3 [M+Na^+].

2.2. Aqueous solutions of the C_{60}-Paclitaxel conjugates

Dissolution of **1**, **3** and **7** in acidic or buffered solutions at pH=2.0 through physiological pH=7.4 resulted in the release of Paclitaxel, as expected. Half-lives, $t_{1/2}$, were calculated assuming that hydrolysis reactions follow Michaelis–Menten kinetics, are shown in Table 1.

Solubilities in water were estimated using standard filtration at RT and the freeze-drying method: 15$\pm$3 mg/ml (**1**); 380$\pm$80 mg/ml (**3**); $\geq$250 mg/ml (**7**).

Water-soluble fullerenes tend to form aggregates in aqueous solution [19, 20]. Fig. 2 shows dynamic light scattering (DLS) data of a solution of **3** at 100 μg/mL, using a Malvern Zetasizer, Model Zen 3600 (He-Ne laser, 4.0 mW, 632.8 nm; Malvern Instruments Ltd, Malvern, Worcestershire, United Kingdom). As shown in the Figure, under these conditions the average hydrodynamic diameter of aggregated Compound **3** is ca. 240 nm.

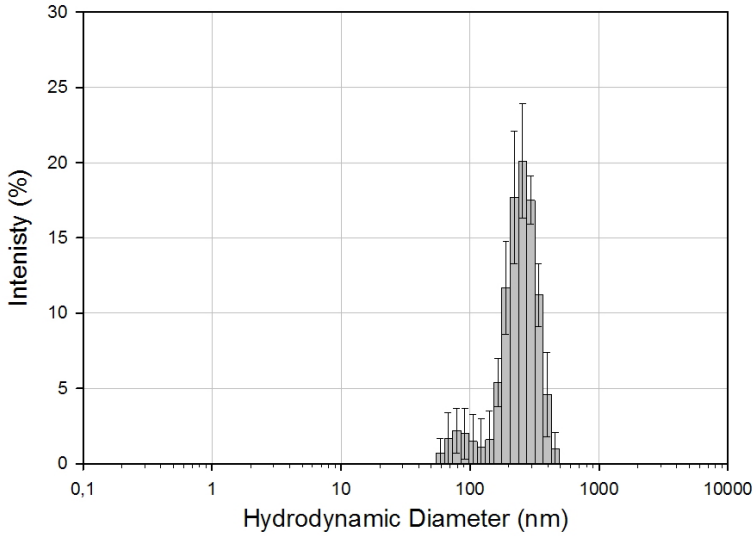

FIG. 2. Aggregate size distribution in solution of 3 (Concentration: 100 μg/mL, average $D_h = 244.5$ nm, solution filtered using 0.45 μM PES membrane)

2.3. Materials, cell cultures and cell lines

Normal human pancreatic ductal epithelial (HPDE) cells and pancreatic adenocarcinoma cells Panc-1 and AsPC-1 were seeded in 96-well plate. After overnight incubation, the cells were treated for 72 h with 1 mM Paclitaxel alone or **7**. C_{60}-ser, a similar unlabeled compound known

TABLE 2. In vitro timed pulse cytotoxicity assay of Abraxane, Paclitaxel, and Compound 3 in Hep3B cells

	IC_{50} Values of Paclitaxel and its Derivatives	
	24 h. exposure timepoint	72 h. exposure timepoint
Paclitaxel	7.94	10.31
Abraxane	21.5	5.40
3	3.58	1.02

to be a transfection agent [14], and untreated cells were used as controls. The cytostatic effect of treatment was determined by adding MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) salt solution in PBS, which is reduced to purple formazan crystals in living cells.

Results support the assumption that C_{60}-ser showed no cytotoxic effect on normal and cancer cells and can therefore be used as a delivery vehicle for Paclitaxel. A dose of 1 μM of Paclitaxel was toxic to all cells. Conjugate **7** is less cytotoxic toward cancer cells than Paclitaxel itself, as shown in Fig. 3. However, functionalization of C_{60}-ser with Paclitaxel to form **7** produced a Paclitaxel derivative that was more cytotoxic for normal HPDE cells than free Paclitaxel.

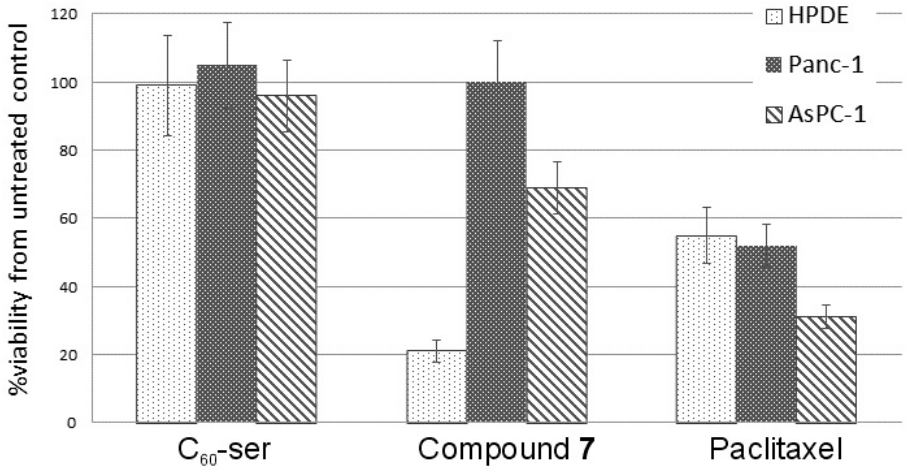

FIG. 3. Cytotoxic effect of Paclitaxel and Compound 7 for normal human ductal epithelial cells (HPDE) and malignant pancreatic cells (Panc-1 and AsPC-1). All cells received PTX at 0.2 M for 72 h. Cell viability was determined as a ratio to the untreated cells that were used as control

Cytotoxicity studies of **3** performed on hepatocellular carcinoma cell line HEP 3B showed that efficacy increased significantly when compared with Palitaxel alone or when the drug was conjugated with albumin (Abraxane) (Table 2).

2.4. *In vivo* cytoxic activity of 3

Cells from the liver cancer cell line Hep3B which had been transfected with luciferase plasmid and green fluorescent protein were cultured according to the American Type Culture Collection (ATCC) guidelines for this cell line. Approximately 1.6 million cells in 10 μL of PBS were injected into mice orthotopically under the liver capsule for 29 female C.B-17 SCID mice (Taconic, Hudson, NY). After four weeks, tumor development was verified using luminescence imaging. The mice were injected with 25 mg/kg firefly D-luciferin in 100 μL saline and then

subjected to non-invasive bioluminescence imaging. After bioluminescence imaging confirmed tumor progression of hepatocellular carcinoma in all mice, animals were randomized in five groups of mice (6 mice/group): the first group received Paclitaxel (in Cremaphor EL), the second group received Abraxane, and the third group received Paclitaxel as Compound **3.** The other two groups received either C_{60}-ser alone or injections of saline and were used as controls. The C_{60}-ser group had five mice instead of six.

All groups were weighed daily and given an IP injection of the appropriate dose of drug in a 100 μL sterile PBS dilution for five consecutive days. The Taxol group received 12.5 mg Paclitaxel/kg/day. The Abraxane and the Compound **3** groups received 30 mg Paclitaxel(equivalent)/kg/day. The dose administered for the Compound **3** group was calculated using the approximation that the prepared conjugate was 90% pure. The reason the Abraxane and Taxol groups received different doses of Paclitaxel was to achieve equal toxicity rather than equal molar doses, as Paclitaxel is more potent per quantity [21]. Based on the behavior of Abraxane, we assumed that the water-soluble conjugate would have a similar toxicity profile. The C_{60}-ser group received 78 mg/kg/day. This dosage represents the molar equivalent of C_{60}-ser for the Compound **3** group. Solutions of Taxol and Abraxane were prepared fresh daily. Compound **3** is susceptible to hydrolysis of the ester linkage so the solutions were prepared in advance, flash frozen, and thawed as necessary immediately prior to use.

At the end of the experiment, animals were euthanized and tumors were resected, weighed and preserved in formalin along with vital organs (kidney, liver and intestine). Results from this experiment are shown in Fig. 4. Compound **3** was equipotent to Abraxane. We noticed that the animals in the group receiving Abraxane tolerated the treatment less well than those receiving Compound **3**. The average body weight of mice in this group decreased $>10\%$ (Fig. 5). Two mice from this group had to be euthanized due to morbidity signs. In this way, the length of the study could be maximized, while still allowing for statistical analysis. In contrast, mice that received Compound **3** showed no evidence of toxicity and did not lose body weight until the end of the study.

3. Conclusions

Currently, the development of the Paclitaxel-C_{60} conjugates included in this work (Compounds **3** and **7**) serve as a contribution to the growing library of research utilizing fullerenes as therapeutic modalities. Compared to Paclitaxel and FDA-approved Abraxane, *in vitro* testing of Compound **3** yielded excellent results, showing $10\times$ and $5\times$ greater cytotoxicity than Paclitaxel and Abraxane, respectively. *In vivo* testing of Compound **3** in a murine model with orthotopic hepatocellular carcinoma showed tumor volume reduction similar to FDA-approved Abraxane, but without the weight-loss associated with present clinically approved formulations of Paclitaxel. This indicates the need for further studies of Compound **3** as a stand alone, water-soluble agent for the delivery of hydrophobic Paclitaxel. Good tolerance of Compound **3** in our studies implies that higher doses of it may be better tolerated than current clinical agents.

For Compound **7**, with twice the amount of Paclitaxel, *in vitro* testing demonstrated promising cytotoxicity values, but lack of specificity prevented it from being tested *in vivo*.

Our results support previous findings that C_{60}-ser is not toxic to cells and animals and is capable of altering the cytotoxic properties of a chemotherapeutic agent. We plan to investigate C_{60}-ser conjugated to other anticancer agents to find out which class of prodrugs is most suitable for delivery via the C_{60}-ser carrier vector.

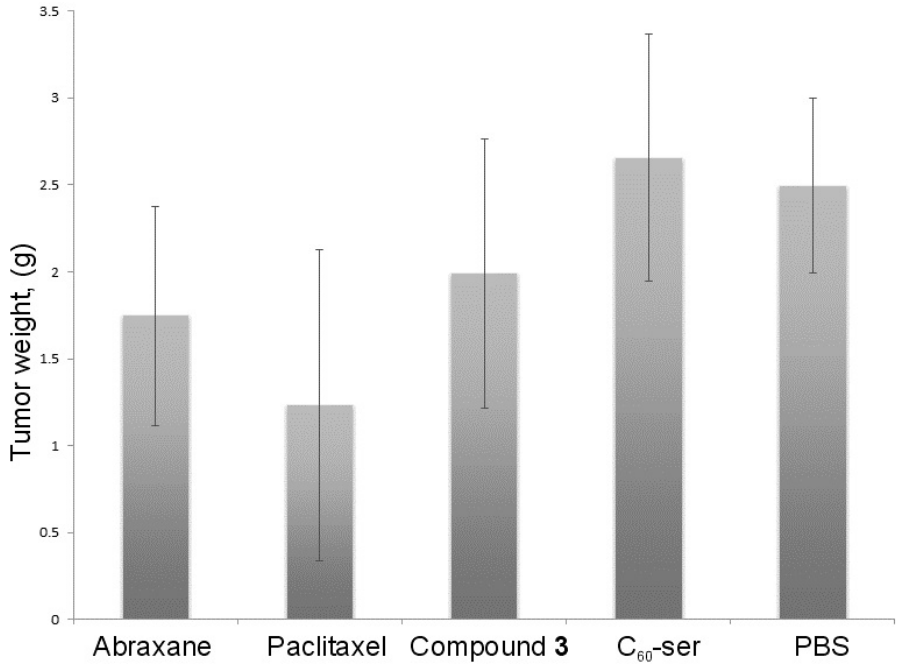

FIG. 4. Average tumor weights in murine subjects after treatment (average with standard dev.)

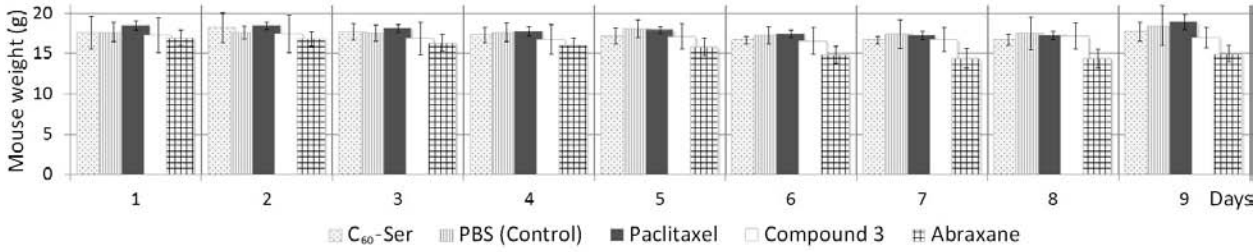

FIG. 5. The change in average body weight of mice during treatment (average with standard dev.)

Acknowledgments

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