Communication

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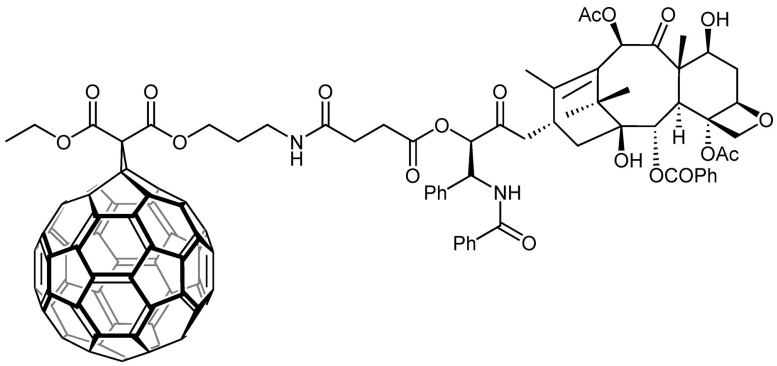

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A Fullerene−Paclitaxel Chemotherapeutic: Synthesis, Characterization, and Study of Biological Activity in Tissue Culture

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Liposome aerosol delivery has been successfully used for lung cancer therapy employing a variety of lipophilic chemotherapeutics.[1] It offers significant advantages over other methods of drug administration, including substantially higher drug concentration in the lungs, lower dosage requirements, reduced systemic toxicity, and noninvasive administration.[2] However, rapid clearance of the drugs from the lungs after cessation of aerosol delivery[3] has prompted us to design a lipophilic slow-release system to enhance therapeutic efficacy. Fullerene (C_{60}) has potential to produce such an ideal lipophilic slow-release system since it is biologically stable and a convenient three-dimensional scaffolding for covalent attachment of multiple drugs to create single-dose "drug cocktails". This drug-delivery strategy adds to the growing list of potential biomedical applications for fullerene derivatives which include neuroprotective agents,[4] HIV-1 protease inhibitors,[5] bone-disorder drugs,[6] and X-ray contrast agents.[7] Paclitaxel (**1**, Figure 1) was selected as a prototype drug for this study since it is regarded as one of the most promising drugs against lung cancer.[8] Furthermore, it has been shown in vitro that the cytotoxicity of paclitaxel is more dependent on exposure time than on increased paclitaxel concentration.[9] In this Communication, we report the synthesis of a C_{60}−paclitaxel conjugate designed as a slow-release drug-delivery system and demonstrate that, as a liposome formulation, it possesses significant anticancer activity in tissue culture.

The extensive reports describing structure−activity relationships for paclitaxel[10] were used to suggest a successful approach for the conjugate design. All modifications of the 2′-hydroxyl group of paclitaxel reported so far have resulted in loss of biological activity of the derivatives, except for the ones which contained groups such as esters or carbonates that can be cleaved by enzymatic or other physicochemical mechanisms.[10,11] Similar potencies and selectivities of the latter prodrugs and paclitaxel itself, as well as isolation of paclitaxel from aqueous solutions of these prodrugs under conditions appropriate for cell culture experiments, are consistent with a mechanism of action dependent on paclitaxel release.[11] Since it has also been established that the 2′-hydroxyl group is more reactive than the 1- and 7-OH groups,[10] we selected this position for modification to an ester with further coupling to a fullerene amino derivative (**5**, Scheme 1) through a spacer containing a free carboxyl group (**2**, Figure 1). Succinate was used as a linker because derivatization of paclitaxel with succinic anhydride has been shown to proceed in high yield.[13]

Synthesis of the C_{60}−paclitaxel conjugate was initiated with the asymmetrical malonate (**4**) available by treatment of *tert*-butyl *N*-(3-hydroxypropyl)carbamate (**3**) with ethylmalonyl chloride (Scheme 1). Bingel−Hirsh addition to C_{60}, followed by deprotection of the amino group, gave **5**. Paclitaxel-2′-succinate (**2**) was prepared according to the published procedure[12] and coupled to **5** using EEDQ to yield **6**.

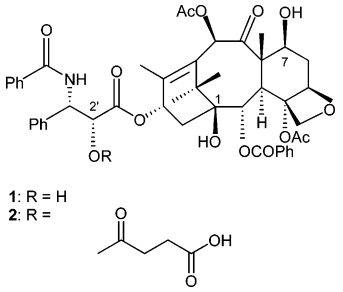
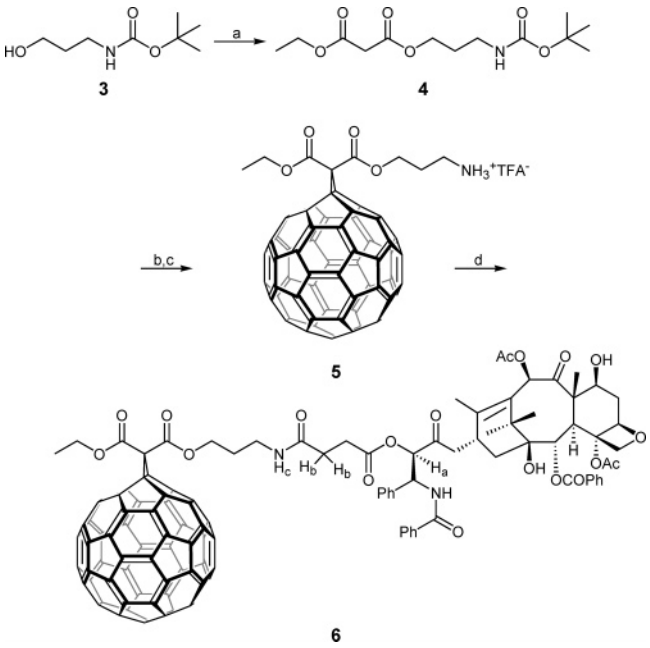


Figure 1. Paclitaxel (**1**) and paclitaxel-2′-succinate (**2**).

Scheme 1. Synthesis of Paclitaxel−Fullerene Conjugate[a]



[a] Reagents and conditions: (a) ethylmalonyl chloride, pyridine, CH_2Cl_2, 60%; (b) C_{60}, I_2, DBU, toluene, 54%; (c) TFA, CH_2Cl_2, quantitative; (d) **2**, TEA, EEDQ, CH_2Cl_2, 47%.

Spectroscopic and MALDI-TOF MS data for **6** are consistent with the assigned structure. The presence of the conjugate was verified by MALDI-TOF MS with the molecular ion peak at m/z = 1844. The ^{1}H NMR of **6** in $CDCl_3$ displayed resonances at δ 5.47 ppm (H_a in Scheme 1), which confirmed the presence of a

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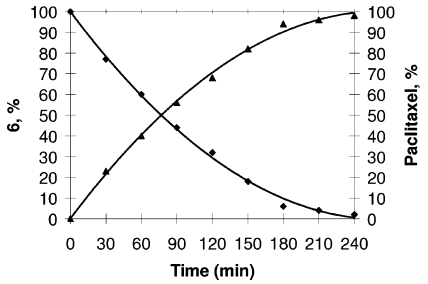

Figure 2. Kinetics of hydrolysis of **6** in bovine plasma at 37 °C. Conjugate **6** was incubated in the presence of bovine plasma, and the concentrations of **6** (■) and paclitaxel (▲) were determined at the indicated time points using reverse-phase HPLC. The curved fits are only to guide the eyes.

2′-ester group, and at δ 2.79 (H_b in Scheme 1) and 6.01 ppm (H_c in Scheme 1), which proved amide formation upon EEDQ coupling.

It has been suggested previously that the biodistribution and biological activity of fullerene derivatives depend on their derivatization and aggregation state.[13] Thus, the aggregation properties of **6** and paclitaxel were studied as a function of concentration in aqueous solution (10% DMSO) using a Brookhaven 90Plus submicrometer particle-size analyzer. For **6**, the average hydrodynamic diameter (D_h) varied from 120 to 145 nm for the concentration range 0.004−0.05 μg/mL and was found to be essentially invariant with concentration. The aggregate sizes were broadly distributed for all concentrations, with a polydispersity index (PDI) between 0.35 and 0.45 nm; the data clearly displayed a bimodal distribution. The intensity of light scattering decreased with decreasing concentration, which can be attributed to a decrease in the concentration of the aggregates without affecting the particle size. These data are in striking contrast to the aggregation behavior of paclitaxel itself, for which 4 and 0.04 μg/mL solutions did not show any scattering. Thus, the C_{60} component of **6** greatly increases the tendency of paclitaxel to aggregate in aqueous (10% DMSO) solution.

The C_{60}−paclitaxel conjugate (**6**) is stable in the solid state and in aprotic organic solvents as well as in aqueous media (10% DMSO) at physiological pH. However, incubation of **6** with bovine plasma at 37 °C resulted in the release of paclitaxel, with the half-life of hydrolysis around 80 min (Figure 2). Assuming a similar half-life for **6** in vivo and the ability of **6** to remain in lungs, a several-fold increase in the exposure time of cancer cells to the drug should be achieved by **6** since the half-life of paclitaxel itself in the lungs has been reported to be only 20 min after delivery by aerosol.[3]

Finally, **6** was examined for its ability to form stable dilauroylphosphatidylcholine (DLPC) liposome formulations and its antitumor activity against human epithelial lung carcinoma A549 cells as a liposome suspension. DLPC is desirable for aerosol delivery of lipophilic agents because it has a low transition temperature (about 0−5 °C), similar to the fluidity of phosphatidylcholine in mammalian cell membranes.[14] To prepare the **6**−DLPC liposomes, 1 mg of **6** was dissolved in 10−20 μL of DMSO and mixed with 1 mL of *tert*-butyl alcohol containing 10 mg of DLPC. The drug−phospholipid mixture was then frozen at −80 °C and lyophilized to a dry powder. Before use, it was resuspended in sterile water and vortexed to form a homogeneous liposomal suspension which was examined by microscopy under polarized light. The suspension was found to be stable with no evidence for drug precipitation. The mean diameter of **6**−DLPC liposomes was found to be 2.77 μm, as measured by light scattering (NICOMP). The IC_{50} value for **6**−DLPC was determined through an experiment designed to compare this value with IC_{50} values for paclitaxel−DLPC, **5**−DLPC, and DLPC-only liposomes, prepared as described for **6**−DLPC. Cells were exposed to different concentrations of these formulations for 1 h, medium was replaced with drug-free medium, and growth was compared to that of untreated control cells after 2 days of additional incubation. **5**−DLPC and DLPC-only did not show any cytotoxicity in the studied concentration range. The mean IC_{50} values for **6**−DLPC and paclitaxel−DLPC were 410 and 253 nM, respectively, revealing similar potencies of these formulations. Thus, it seems reasonable to expect sufficient concentrations of paclitaxel, delivered by **6**−DLPC to lungs by small-particle aerosol, to be therapeutic against lung cancer.

In summary, we have designed and synthesized the first C_{60}-based slow-release system of paclitaxel for liposome aerosol delivery to the lungs. With both clinically relevant kinetics of hydrolysis and significant anticancer activity in tissue culture, the conjugate holds promises for enhanced efficacy of paclitaxel in vivo. Pharmacokinetics and antitumor activity studies of **6** in animal models are in progress.

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Supporting Information Available: Spectral data for compounds **4**−**6**, experimental procedures, and DLS data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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