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Tissue Sites of Uptake of ^{14}C-Labeled C_{60}

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This paper describes the *in vivo* behavior and potential metabolism of C_{60} and a more water-soluble quaternary ammonium salt-derivatized C_{60}. In both cases, a ^{14}C-labeled fullerene core was utilized for the target molecules that were intravenously injected into female Sprague–Dawley rats. The ^{14}C-labeled C_{60} (*C_{60}) was rapidly (within 1 min) cleared from the circulation and the majority of the *C_{60} accumulated in the liver (90–95%). *C_{60} was not eliminated from the liver over the 120-h period of this study. Our results also suggest that C_{60} is not metabolized by the typical oxidative patterns characteristic of other polycyclic aromatics. Therefore, although not acutely toxic, use of C_{60}, or its derivatives that could be cleaved back to the parent C_{60} *in vivo*, would likely lead to long-term fullerene accumulation in the liver. The uptake of *C_{60} and ^{14}C-labeled ammonium salt-derivatized C_{60} **(1)** by human keratinocytes *in vitro* showed that while both *C_{60} and **1** are readily taken up by cells, **1** accumulates more slowly. Additionally, while C_{60}, at rather high concentrations (2.0 μM) and over extended periods of time (8 days), is able to inhibit the growth of human keratinocytes by about 50%, this effect showed little, if any, photoinducability. © 1996 Academic Press, Inc.

INTRODUCTION

There have been numerous proposals regarding the potential *in vivo* use of fullerene derivatives (*1–3*). Concurrently, several potential health risks, including possible carcinogenic properties, have been suggested for C_{60} due to both its polycyclic aromatic nature and its ability to convert oxygen to the singlet state (*3–6*). To date, none of these risks have been substantiated. In fact, topically applied fullerenes (unfunctionalized C_{60} and C_{70}) did not exhibit tumor-promoting activity in the classic 7,12-dimethylbenzanthracene-initiated mouse skin model, or cause acute toxicity in mouse skin as measured by epidermal DNA synthesis and the induction of ornithine decarboxylase activity (*7*). Most biological studies of fullerenes have focused on water-soluble C_{60} derivatives, and several potentially interesting biological properties have been reported. For example, the water-soluble bis(phenethylamino-succinate)-C_{60} derivative has shown activity against human immunodeficiency virus type 1 (HIV-1) in acutely and chronically infected human lymphocytes (EC_{50}

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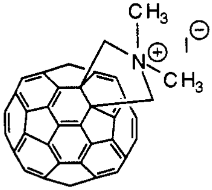

SCHEME 1. ^{14}C-Labeled on the fullerene core **1.**

about 6 μM) and it inhibits HIV-1 protease in cell-free systems (K_i of 5.3 μM) (*8–10*). In addition, a fullerene carboxylic acid derivative inhibited the growth of the HeLa S3 cell line (IC_{50} about 6 μM) when the cells were exposed to light, but not in the dark (*11*). The C_{60}-carboxylic acid also exhibited photo-induced DNA strand scission properties, with cleavage selectively at guanine bases, suggesting that the single-strand breaks were generated via singlet oxygen (*11*). Furthermore, an organofunctional C_{60} bearing a 14-mer oligodeoxynucleotide side chain exhibited photo-induced sequence-specific DNA cleavage, raising the possibility of a use of fullerene-oligonucleotide conjugates as photoprobes in molecular biology studies (*12*). A water-soluble bioactive fullerene peptide has also been synthesized (*13*).

Relatively little is known concerning the biological properties of parent C_{60}, and studies in this area have been hampered by the poor solubility of fullerenes in aqueous solutions. However, some progress has been made in this area with the finding that a water soluble complex of C_{60} is formed on heating a solution of γ-cyclodextrin with solid C_{60} (*14*). Additionally, a procedure for the dissolution of fullerenes into water with polyvinylpyrrolidone (PVP) has been reported (*15*). PVP-solubilized C_{60} or C_{70} (200 μg/ml) had no hemolytic effect on sheep red blood cells (*15*). However, PVP-solubilized C_{60} did promote chondrogensis of rat embryonic limb bud cells *in vitro* (*16*). We recently described the synthesis of ^{14}C-labeled C_{60} ($^*C_{60}$) where the label is part of the C_{60} cage, and a method to make a fine (0.3 μm) aqueous suspension of the $^*C_{60}$ which is suitable for biological studies (*17*). We found that approximately 50% of $^*C_{60}$ given to human keratinocytes in culture became cell-associated within 6 h and remained cell-associated during a subsequent 11-h chase period. In addition, aqueous suspensions of C_{60} (up to 2 μM) did not affect [^{3}H]thymidine incorporation into DNA in cultured human keratinocytes and human fibroblasts, indicating that the accumulation of C_{60} in human cells does not result in acute toxicity (*17*). Others recently conducted *in vivo* studies of a water-soluble side-chain-labeled C_{60} derivative (*18*). In an effort to further assess the *in vivo* behavior and potential metabolism of the parent, underivatized C_{60}, we describe here our results on the plasma clearance and tissue distribution of aqueous $^*C_{60}$ suspensions injected intravenously into rats. We also compare the data to a more water soluble quaternary ammomium salt-derivatized ^{14}C-labeled C_{60} **(1)** that was similarly injected. The $^*C_{60}$ was rapidly (within 1 min) cleared from the circulation and the majority of the $^*C_{60}$ accumulated in the liver (90–95%). Unlike the water-soluble C_{60} derivative described in previous *in vivo* studies (*18*), the parent fullerene was not eliminated from the liver over the 120-h period of this study. Our results also suggest that the underivatized C_{60} is not metabolized by the typical oxidative patterns characteristic of other polycyclic aromatics, possibly due to its insolubility and/or large size (~10-Å diameter) and thus its inability to fit into the active site of most mammalian cytochrome P450s (*6*, *19*). The lack of metabolites also argues against the attack of exogenous radicals that may initiate metabolic decomposition of the fullerene core. Therefore, although not acutely toxic, use of C_{60}, or its derivatives that could be cleaved back to the parent C_{60} *in vivo*, would likely lead to long-term fullerene accumulation in the liver.

METHODS

^{14}C-Enriched C_{60} (C_{60}).* *C_{60} used in this study, which had a specific activity of 11.2 mCi/mmol (approximate $^{12}C:^{14}C = 200:1$), was prepared and suspended in water (2.1 μM, average diameter of particles 0.3 μm) as we have described in detail previously (*17*).

^{14}C-Enriched C_{60}-N-dimethylpyrrolidine ammonium iodide derivative **(*1*).** Described is the procedure for the preparation of the nonradioactive material which was then duplicated on a smaller scale in the case of the radioactive material. C_{60}-*N*-methylpyrrolidine was prepared from C_{60} according to a reported procedure (*20*). C_{60}-*N*-methylpyrrolidine (40 mg. 0.05 mmol) was then suspended in iodomethane (3 ml) in a screw-cap tube. The suspension was stirred overnight at room temperature and a precipitate was formed. (Note that this precipitated material, possibly due to an initially unusual micellar form, was not soluble in any single common solvent that we investigated. The mixture of residual iodomethane and DMSO was a suitable solvent, while DMSO alone was not.) Most of the iodomethane was removed under a nitrogen stream to give a wet copper-colored crude product that was then dissolved in dimethylsulfoxide to give a homogeneous solution. Removal of the remaining iodomethane under a nitrogen stream, and removal of the dimethylsulfoxide by heating at 90°C under a nitrogen stream and further in a vacuum oven (45°C, 1 mm Hg), afforded 0.046 g (100%) of the title product as brown solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 5.74 (s, 4 H), 4.09 (s, 6 H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 152.91, 147.76, 146.78, 146.53, 146.04, 145.99, 145.94, 145.72, 144.86, 143.59, 143.16, 142.56, 142.41, 141.90, 140.27, 136.79, 73.67, 69.81, 53.31. Positive ion electrospray MS calculated for $C_{64}H_{10}N$: 792. Found: 792. No decomposition of the ammonium iodide was observed after weeks in aqueous environments. The radioactive material was analogously prepared starting from *C_{60} (1.5 mg, 2.1 μmol, 22.4 μCi). The ^{14}C-labeled C_{60}-*N*-methylpyrrolidine was carried through directly to afford **1** (2 μCi, 9% for two steps).

*Plasma Clearance in Rats of *C_{60} or **1**.* All animal procedures were approved by the University of South Carolina's Institutional Animal Care and Use Committee. The fullerenes to be injected, both *C_{60} and **1,** were concentrated 10- to 100-fold prior to injection by gentle heating under a nitrogen stream. Dulbecco's phosphate-buffered saline (PBS) containing *C_{60} or ethanol containing **1** was injected (0.2 to 1.6 μCi/rat in 0.05–0.5 ml) into jugular cannulas of 120 to 200 g female Sprague–Dawley rats. The surgery was performed by the vendor. The cannulas were then rinsed with 50 μl of PBS or ethanol. Beginning at 60 s, 50 μl of blood was collected into heparinized hematocrits through tail vein nicks. Sampling continued at intervals up to 2 h postinjection. Plasma was collected by centrifugation and radioactivity present in the plasma was determined. The cellular fraction of the blood was dissolved by incubation overnight at 65°C with 1 ml of hyamine hydroxide. The samples were then neutralized with 0.2 ml of glacial acetic acid and radioactivity was determined.

Tissue distribution of $^*C_{60}$ *or* ***1.*** At 2 and 120 h postinjection for rats administered $^*C_{60}$ and at 2 h postinjection for rats given **1,** animals were exsanguinated via cardiac perfusion with 60 ml of PBS. Tissues were harvested and stored at −20°C until processing. Duplicate samples were made with small aliquots of large tissues, or by dividing whole tissues. Samples were weighed, minced, dissolved with 1 ml of hyamine hydroxide (overnight at 65°C), and neutralized with 0.2 ml of glacial acetic acid and radioactivity was determined. Weights of skin, muscle, and fat as well as volume of blood was estimated using published values. In the rats in which the tissue distribution of $^*C_{60}$ was determined 120 h postinjection, the animals were housed after injection in metabolic cages. Urine and feces were collected at 24-h intervals and radioactivity was determined.

Extraction of $^*C_{60}$ *from rat liver.* Aqueous $^*C_{60}$ suspensions and liver samples obtained from rats 120 h after injection of $^*C_{60}$ were extracted in a Dean–Stark apparatus using benzene. As a control, aqueous $^*C_{60}$ suspensions (100–500 ml) were used directly and were extracted until the distillate had become clear (~1 h). Partially frozen rat liver samples (~5 g) that contained $^*C_{60}$ were chopped finely with a razor blade prior to extraction. The liver samples were also extracted until the distillate was clear (~6 h). The benzene extracts were reduced to 1-ml volumes under a nitrogen stream and then filtered through a 0.45-μm HPLC syringe filter. The final extract volume was adjusted with benzene so that 10 μl contained 2000–8000 cpm. The liver extracts contained substantial amounts of phospholipids which made the solutions viscous. These materials precluded the liver extracts from being reduced below about 1 ml total volume in benzene and caused some difficulties in later HPLC analyses. Separation of the liver-derived samples from the troublesome phospholipids was attempted; however, we could not ensure the possible segregation of potential $^*C_{60}$ metabolites. Therefore, the phospholipid-containing extracts were used in the HPLC analyses.

HPLC analysis of rat liver extracts. HPLC analyses were conducted using two Waters 6000A HPLC pumps, a Waters 660 gradient controller, and a Waters U6K injector. The column was a Waters 4.5 mm × 10 cm C8 reverse-phase column with 5 μm packing. Benzene samples of 10 μl (containing 2000–8000 cpm of $^*C_{60}$) were injected onto the column and eluted at 1 ml/min under a linear gradient which ran from 100% methanol to 50% methanol/50% toluene over 10 min with the 50% methanol/50% toluene solvent mixture being maintained for an additional 10 min. Fractions were collected every 15 s and radioactivity present in each fraction was determined. Analysis of the control $^*C_{60}$ aqueous suspension showed a single radioactive peak with a retention time of 9.25 min (peak front) and 10.50 min (peak maximum). Analysis of the liver extract was complicated by the substantial amount of phospholipid in the sample; the phospholipids caused significant tailing of the

$^*C_{60}$ peak. Nevertheless, the liver extract contained a single, nonpolar peak with a retention time of 9.25 min (peak front) and 11.50 min (peak maximum). Potential polar metabolites of $^*C_{60}$ would be expected to elute in this system prior to control C_{60}, a region not obscured by the tailing. However, no radioactive material was present in the liver extracts that eluted with a retention time earlier than control $^*C_{60}$, indicating that the liver did not contain substantial levels of polar $^*C_{60}$ metabolites.

*Uptake of $^*C_{60}$ and **1** by immortalized human keratinocytes.* Human keratinocytes immortalized by human papillomavirus type 16 DNA were cultured in 35-mm dishes in serum-free complete MCDB153-LB medium. The establishment of the cell line and the culture conditions were described in detail in previous publications (*21–24*). Briefly, the serum-free MCDB153-LB basal medium was supplemented with hydrocortisone (0.2 μM), insulin (5 μg/ml), transferrin (10 μg/ml), triiodothyronine (10 nM), $CaCl_2$ (0.1 mM), epidermal growth factor (5 ng/ml), and bovine pituitary extract (35–50 μg protein/ml) with medium changes every 48 h. When cells reached 75% confluence, the uptake by the keratinocytes of $^*C_{60}$ and **1** (30,000 dpm/dish) was determined in duplicate dishes using a previously detailed protocol (*17*). In brief, complete MCDB153-LB medium containing the fullerence was added and incubation proceeded at 37°C in the absence of light. At the times indicated, medium was removed by aspiration and the cells were washed three times with Dulbecco's phosphate-buffered saline. Distilled waster (1 ml/dish) was added, the cells were lysed by freeze–thaw, and the radioactivity was measured in aliquots of cell lysates.

Effect of C_{60} on the mass culture growth of immortalized human keratinocytes. Immortalized human keratinocytes were plated at a density of 50,000 cells/35-mm culture dish and incubated at 37°C in the dark. At 24 h after plating, and every 48 h thereafter, the cells were refed with media alone or media containing an aqueous suspension of unlabeled C_{60} (either 0.2 or 2.0 μM). Cells were then incubated at 37°C either in the dark or with periodic light irradiation at 6 W for 1 h every 12 h. Cell number was determined in triplicate dishes per condition every 48 h for a total of 8 days, by trypsinizing and counting cells in a hemocytometer.

RESULTS AND DISCUSSION

Aqueous suspensions of $^*C_{60}$, or ethanol-containing solutions of **1,** were injected into jugular cannulas of female Sprague–Dawley rats. To assess how quickly the labeled fullerenes were cleared from the circulation, blood was collected from the tail vein at various times after the injection (1 to 120 min) and radioactivity determined. Clearance of $^*C_{60}$ from the circulation was extremely rapid, with less than 1% of the injected dose remaining 1 min after the injection (Fig. 1); **1** was also quickly cleared from the circulation, but with kinetics that were slower than that observed for the $^*C_{60}$. About 9% of the injected dose of **1** remained after 1 min, 3% after 60 min, and only 1.5% remained after 120 min (Fig. 1). The rapid clearance of the fullerenes is not surprising given their extreme hydrophobic character, and the slower clearance kinetics of the **1** is likely due to its more hydrophilic character compared to parent $^*C_{60}$.