In vivo biological behavior of a water-miscible fullerene: ^{14}C labeling, absorption, distribution, excretion and acute toxicity

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Background: Water-soluble fullerenes have recently been shown to exhibit considerable *in vitro* biological activity including cytotoxicity, site-selective DNA cleavage and inhibition of HIV protease. To assess the potential of these compounds as drugs, studies on the *in vivo* behavior of fullerenes are needed. We therefore set out to synthesize a radiolabeled, water-soluble fullerene, in order to obtain data on the oral absorption, distribution and excretion of this class of compounds.

Results: We synthesized a ^{14}C-labeled water-soluble [60]fullerene using dipolar trimethylenemethane, which undergoes cycloaddition to [60]fullerene. When administered orally to rats, this compound was not efficiently absorbed and was excreted primarily in the feces. When injected intravenously, however, it was distributed rapidly to various tissues, and most of the material was retained in the body after one week. The compound was also able to penetrate the blood–brain barrier. Acute toxicity of the water-miscible fullerene was found to be quite low.

Conclusions: Although the water-soluble fullerenes (and possibly their simple metabolites) are not acutely toxic, they are retained in the body for long periods, raising concerns about chronic toxic effects. The fact that fullerenes distribute rapidly to many tissues suggests that they may eventually be useful to deliver highly polar drugs through membranes to a target tissue, however, and they may even have applications in the delivery of drugs to the brain. Recent advances in fullerene synthetic chemistry may also make it possible to control fullerene absorption/excretion profiles in the future.

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Introduction

Despite intense interest in the fullerene molecule ever since its discovery, fullerene biology has only become a realistic target of scientific research since the recent demonstration that water-soluble fullerenes (for example, compounds **1** and **2** in Fig. 1) show considerable *in vitro* biological activity, including cytotoxicity, DNA cleavage [1] and anti-viral activity [2,3]. We recently showed that a C_{60}–DNA conjugate could be used to cleave single- and double-stranded DNA at specific sites [4], further demonstrating that tailor-made fullerenes could be useful in biochemical and biological studies. Current studies on the *in vivo* behavior of fullerenes are expected to provide fundamental information for the further development of fullerene biology, and eventually to bring forth new drug delivery systems for diagnosis and therapy. Here, we report the first data, to our knowledge, on the oral absorption, distribution and excretion of a water-miscible fullerene. These studies required the synthesis of the radioactive compound **1a** (see Fig. 1).

The fullerene **1a** was not effectively absorbed when administered orally (p.o.). Nonetheless, a small amount was absorbed, metabolized and excreted into the urine. When injected intravenously (i.v.), most of the radiolabeled compound moved rapidly to the liver and was then distributed to various other tissues. Thus it seems that, despite its chemical reactivity [5], the fullerene does not become quickly bound to reactive substances, such as the amine residues of proteins (for example, lysine and ornithine). Excretion was slow, however, and over 90 % was still retained in the body after one week. Thus, the chronic toxicity of water-miscible fullerenes will be an important focus of future work.

Results and discussion

We first investigated the acute toxicity of the water-soluble fullerene **2**. The studies were performed on mice (ddY female, 18–20 g) by a single intraperitoneal (i.p.) injection (200–500 mg kg^{-1} doses, in 0.2 % Tween 80/Saline containing dimethylsulfoxide). Although the mice showed symptoms of discomfort

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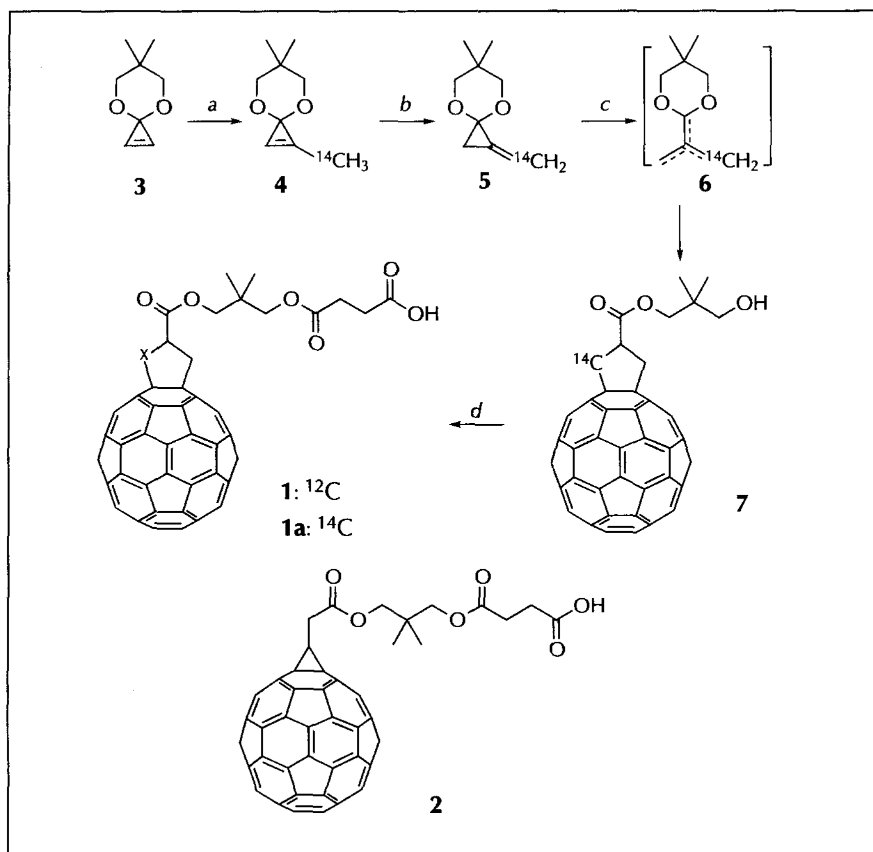

Fig. 1. Synthesis of the ^{14}C-labeled fullerene **1a**. For conditions used, see Materials and methods.

after dosing, such as writhing with stretching of the trunk, and some weight loss (5–10 % weight loss after a day at a dose of 500 mg kg^{-1} of compound **2**), all mice survived for a period of one week. As the approximate lethal dose must thus be more than 500 mg kg^{-1} i.p., and given similar data for the parent C_{60} [6–8], fullerenes may be regarded as compounds with relatively weak acute toxicity provided that they are manipulated with reasonable care. A full evaluation of the toxicity of these compounds awaits further studies.

To address basic issues of the pharmacokinetics and distribution of these compounds, a synthetically viable route to a labeled fullerene derivative was needed. Of the two possible strategies for making labeled fullerenes, the simplest conceptually is the labeling of the C_{60} core carbons with ^{14}C. This route, however, is difficult to perform practically [7,8]. It involves a several-step synthesis of ^{14}C-enriched graphite, and requires the handling of a large amount of radioactive soot, from which the labeled C_{60} is separated. The second route, involving attachment of a labeled carbon fragment to the fullerene, may be far less hazardous, more isotopically economic, and may also be applicable to fullerenes other than C_{60}.

Among the reported methods for fullerene functionalization, the trimethylenemethane (TMM) route [9,10] appeared to be ideal for rendering the second labeling strategy practical. The synthesis of labeled compound **1a** was achieved in four synthetic steps (Fig. 1). The readily available cyclopropenone, compound **3** [11], was lithiated and allowed to react with $^{14}CH_3I$ (>97 % isotopic purity). The methylated product, compound **4**, was isomerized with *tert*-BuOK to the methylenecyclopropane **5**, and then allowed to react with C_{60} at 80 °C via the trimethylenemethane **6**. The cycloadduct **7** was purified and esterified to obtain the labeled succinate **1a**. The mechanism of the TMM reaction, previously studied for a ^{13}C-labeled compound [12], ensures that the ^{14}C label is placed unambiguously at the position next to the C_{60} core, where the probability of the premature biological loss of the label is smallest.

Absorption of the water-soluble fullerene **1a** administered orally to male Fischer rats was found to be poor. Only trace radioactivity was found in the liver and other tissues after 3 and 6 h periods, and virtually all radioactivity (97 % of the total) was excreted in the feces within 48 h (Fig. 2). It is notable, however, that trace amounts of fullerene derivatives were identified in the urine, indicating that a small amount of compound **1a** did pass through the gastrointestinal wall. In the 0–12 h period, a small amount of compound **1a** itself was tentatively detected (using HPLC) in the urine, and, in the 24–30 h period, trace amounts of **1a** and **7a** were detected after acidification of the urine. In the 24–30 h sample, we could not initially detect any CH_2Cl_2-soluble radioactive material in the urine sample (pH 8), but found, upon acidification to pH 1 with HCl, that one third of the radioactivity did move to the CH_2Cl_2 extract. Two radioactive zones were separated by HPLC (Buckyprep column, CH_2Cl_2). The

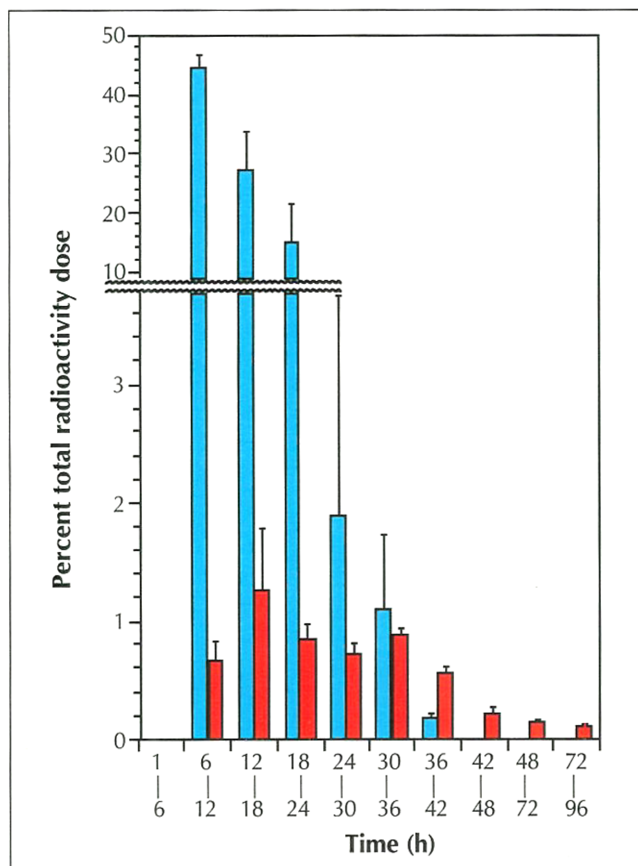

Fig. 2. Fecal excretion of radioactivity after oral (blue) and intravenous (red) dosing of compound **1a**.

retention times of these compounds were consistent with those of compounds **7a** and **1a**, as determined by comparison with the retention times of authentic samples (in a 7.5:1 ratio, giving retention times of 10 and 18 min, respectively). The fact that compounds **1a** and **7a** could be extracted only at pH 1 suggested that, in the urine, they existed as acid-labile metabolites, as is often observed for lipophilic drugs. It is also possible that compound **1a** may have been present as the carboxylate salt at pH 8, in which case the lack of extraction may simply reflect a lack of solubility in the organic solvent used.

When the dose was delivered intravenously (i.v.), compound **1a** was excreted extremely slowly (Fig. 2). After 160 h, only 5.4 % was eliminated into the feces and the remainder stayed in the body. In both p.o. and i.v. experiments, virtually all of the excretion occurred via the feces, and little radioactivity was seen in the urine. The strong retention of the i.v.-dosed fullerenes is consistent with the high lipophilicity of the molecule. Compound **1** is unusually lipophilic for a carboxylic acid derivative, as evidenced by its high log P_{ow} value (the partition coefficient in octanol/water [13]) of 4.5 (free acid form), which is in the same range as that of phenanthrene (log P_{ow} = 4.5) and 1,2-diphenylethane (log P_{ow} = 4.8).

As the i.v.-administered fullerene **1a** is strongly retained in the body, we examined the time-dependent change of the concentration of the radioactivity in tissues in sacrificed animals (Table 1). The dosed fullerene quickly passed from the blood to the liver, leaving 1.6 % of the

Table 1. Distribution of radioactivity in rats.

	% total dosed radioactivity (concentrations of radioactivity (pg equiv/g))					
tissue	**1 h**	**3 h**	**6 h**	**16 h**	**30 h**	**160 h**
liver	72.9 ± 13.7 (57.7 ± 4.8)	84.7 ± 10.4 (57.3 ± 8.3)	86.7 ± 20.2 (62.2 ± 14.6)	91.7 ± 8.0 (61.9 ± 6.3)	80.1 ± 13.4 (54.5 ± 7.6)	1.62 ± 0.31 (0.29 ± 0.03)
spleen	0.74 ± 0.63 (11.5 ± 2.2)	1.4 ± 0.1 (10.6 ± 0.7)	1.2 ± 0.3 (8.7 ± 2.1)	1.6 ± 0.2 (11.4 ± 2.4)	2.0 ± 0.4 (12.4 ± 1.0)	0.05 ± 0.00 (0.12 ± 0.01)
lungs	1.4 ± 0.3 (5.5 ± 0.7)	5.0 ± 3.3 (22.9 ± 13.5)	1.4 ± 0.3 (5.9 ± 1.2)	1.0 ± 0.3 (4.2 ± 0.6)	3.2 ± 2.3 (12.7 ± 9.2)	0.05 ± 0.02 (0.06 ± 0.02)
kidney	0.50 ± 0.04 (1.3 ± 0.1)	0.58 ± 0.15 (1.5 ± 0.2)	0.99 ± 0.18 (2.5 ± 0.4)	1.0 ± 0.3 (2.7 ± 0.8)	3.2 ± 0.1 (1.8 ± 0.2)	0.41 ± 0.14 (0.35 ± 0.10)
heart	0.31 ± 0.25 (1.8 ± 1.3)	0.44 ± 0.65 (3.7 ± 5.7)	0.24 ± 0.08 (1.5 ± 0.5)	1.07 ± 0.16 (5.8 ± 1.3)	0.26 ± 0.13 (1.5 ± 0.8)	0.01 ± 0.00 (0.03 ± 0.00)
brain	0.20 ± 0.04 (0.30 ± 0.06)	0.15 ± 0.24 (0.24 ± 0.37)	0.84 ± 0.70 (1.2 ± 1.0)	0.57 ± 0.19 (0.85 ± 0.24)	0.10 ± 0.11 (0.14 ± 0.16)	0.00 ± 0.00 (0.00 ± 0.00)
testicles	0.03 ± 0.00 (0.12 ± 0.01)	0.04 ± 0.02 (0.14 ± 0.07)	0.06 ± 0.01 (0.20 ± 0.03)	0.09 ± 0.01 (0.26 ± 0.02)	0.08 ± 0.02 (0.21 ± 0.05)	0.07 ± 0.01 (0.05 ± 0.00)
blood	1.6 ± 0.6 (1.6 ± 0.3)	1.0 ± 0.2 (0.87 ± 0.05)	0.50 ± 0.12 (0.45 ± 0.11)	0.57 ± 0.27 (0.45 ± 0.20)	0.50 ± 0.08 (0.38 ± 0.04)	0.00 ± 0.00 (0.00 ± 0.00)

total radioactivity in the blood after 1 h and only 0.5 % after 6 h. In the liver, 73 % of the radioactivity was found after 1 h, and the concentration was maximal after 6–16 h. About 80 % of the total radioactivity was retained in the liver even after 30 h, but the radiolabel was mostly eliminated (1.6 %) after 160 h.

The concentrations of compound **1a** in the spleen and kidney were highest after 30 h and accounted for 2–3 % of the total radioactivity dosed initially. Notably, the radioactivity was also found in the brain. The high lipophilicity and the rather compact structure of the fullerene **1a** must be responsible for the transportation of this large molecule (molecular weight 995) through the blood–brain barrier. From 30 to 160 h, the radioactivity in the various organs decreased in a time-dependent manner and, after 160 h, the radioactivity found in the organs listed in Table 1 mostly disappeared without excretion from the body, and became distributed to skeletal muscle and hair (data not shown).

Significance

Fullerenes have attracted considerable interest since their first synthesis ten years ago, partly for the intrinsic interest of their unusual structure, but also because of increasing evidence that they can be biologically active themselves, and can be used as cages or carriers for other biologically active molecules. To determine whether fullerenes will ever be useful as drugs or drug delivery devices, it is necessary to understand the pharmacokinetics and toxicity of these compounds.

Although we have found that the fullerenes (and possibly their simple metabolites) are not acutely toxic, they are not free from undesirable effects such as pain and weight loss. Our observation that the administered fullerene is retained in the body for long periods may also raise new concerns about chronic toxicity. From a more positive perspective, however, our data suggest that it may in the future be possible to use fullerenes to mediate the delivery of highly polar drugs attached to the fullerene (such as a C_{60}–oligo DNA conjugate capable of site-specific DNA cleavage) [1,4] through membranes to a target tissue. In addition, these studies suggest that water-soluble derivatives may enable the delivery of metals into the brain in the form of endohedral metallofullerenes [14], offering new possibilities for imaging agents. Given the recent advances in fullerene synthetic chemistry [15–17], it should also soon be possible to alter fullerene absorption/excretion profiles.

Materials and methods

Synthesis of ^{14}C-labeled fullerene 1a

The labeled fullerene was synthesized according to the procedure previously reported for an unlabeled compound [1], as summarized in Fig. 1. Product identification was achieved using ^{12}C-authentic samples at the stage of both alcohol **7** and acid **1a** under two different chromatographic conditions (TLC (silica gel, 10 % EtOAc in toluene) and HPLC (Buckyprep column, CH_2Cl_2)). Reagents and conditions were as follows: *(a)* compound **3** (0.15 mmol), *n*-BuLi (0.15 mmol), hexamethylphosphoric triamide (0.30 mmol), tetrahydrofuran, –78 °C, 0.5 h, then $^{14}CH_3I$ (0.10 mmol, 0.21 GBq/mmol), –78 °C → room temperature (r.t.); *(b)* *t*-BuOK (0.05 mmol), Et_2O/dimethylsulfoxide, r.t., 1 h; *(c)* C_{60} (0.10 mmol), 1,2-dichlorobenzene, 80 °C, overnight; *(d)* succinic anhydride (1.0 mmol), 4-dimethylaminopiridine (1.0 mmol), CH_2Cl_2, r.t., overnight. Unoptimized yield 0.4 %. The low yield is due to inadvertent material loss at stage *(a)* and the remaining transformations took place cleanly, as monitored by chromatography. The optimized yield was 13 % for a sample of natural isotopic abundance. The radiolabeled sample contained 4±1 % of a faster-moving radioactive impurity as analyzed by HPLC (Buckyprep column, Nakalai tesque, Japan, elution with 30 % *i*-PrOH in CH_2Cl_2).

Fecal excretion of radioactivity after p.o. and i.v. dosing of compound 1a

The excretion of compound **1a** was studied for three male rats (Fischer rats; 85–95 g) in each case. The ^{14}C-labeled compound **1a**, dissolved in EtOH/PEG400/5 % aqueous albumin (1:1:23, 0.18 MBq ml^{-1}), was administrated p.o. (18 kBq) or i.v. (9.6 kBq). The radioactivity was measured using a liquid scintillation counter for samples of the feces, which were treated with 1N NaOH at 60 °C for 2 days. While the p.o. dosed rats excreted 97 % of the total radioactivity after 48 h, only 5.4 % of the radioactivity was excreted after 96 h in the i.v. dosed rats. Excretion into the urine was also measured using a liquid scintillation counter. In the p.o. dosing experiment, an initial (0–12 h period) urinary excretion of compound **1a** was found (taken up in CH_2Cl_2 by extraction), which accounted for ~0.2 % of the dosed radioactivity. Trace amounts of compounds **1a** and **7a** were also found in the acidified urine sample of the 24–30 h period, as described in the text. Compound identification was achieved by comparison of HPLC retention times against authentic samples (Buckyprep column, CH_2Cl_2).

Distribution of radioactivity in rats

The distribution of the labeled fullerene **1a** was investigated with male rats (Fischer rats; 85–95 g) for the liver, spleen, lungs, kidney, heart, brain, testicles and blood. A solution of the fullerene **1a** (14 kBq) in a mixture of ethanol (50 μL), polyethylene glycol (50 μL) and 4 % albumin (115 μL) was administered by i.v. injection. The distribution in each tissue was analyzed after 1, 3, 6, 16, 30 and 160 h periods (In the 160 h run, 18 kBq of compound **1a** was administered). The rats were dissected after the blood was removed with a hypodermic syringe. Each of the tissues was homogenized in 2N NaOH at 60 °C for 24 h. The radioactivity was measured using a liquid scintillation counter.

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