Uptake and distribution of fullerenes in human mast cells

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Abstract:

Fullerenes are carbon cages of variable size that can be derivatized with various side chain moieties resulting in compounds that are being developed into nanomedicines. Although fullerene use in several preclinical in vitro and in vivo models of disease has demonstrated their potential as diagnostic and therapeutic agents, little is known about how they enter cells, what organelles they target, and the time course for their cellular deposition. Fullerenes (C_{70}) that have already been shown to be potent inhibitors of mast cell (MC)–mediated allergic inflammation were conjugated with Texas red (TR) and used in conjunction with confocal microscopy to determine mechanisms of uptake, the organelle localization, and the duration they can be detected in situ. We show that C_{70}-TR are nonspecifically endocytosed into MCs, where they are shuttled throughout the cytoplasm, lysosomes, mitochondria, and into endoplasmic reticulum at different times. No nuclear or secretory granule localization was observed. The C_{70}-TR remained detectable within cells at 1 week. These studies show that MCs endocytose fullerenes, where they are shuttled to organelles involved with calcium and reactive oxygen species production, which may explain their efficacy as cellular inhibitors.

From the Clinical Editor: Fullerenes are carbon cages of variable size that have already been shown to be potent inhibitors of mast cell (MC)-mediated allergic inflammation. These were conjugated with Texas red (TR) and used in conjunction with confocal microscopy to determine mechanisms of uptake, the organelle localization, and duration, demonstrating that MCs endocytose fullerenes, which are shuttled to organelles involved with calcium and reactive oxygen species production. This intracellulac trafficking may explain the efficacy of fullerenes as cellular inhibitors.

Keywords: Fullerene | Nanomedicine | Mast cell | Intracellular trafficking

Article:

The use of nanomaterials to diagnose and treat disease has been a goal for scientists ever since the advent of methods to manipulate molecules at the nanometer level. Fullerenes are one type of nanomaterial that are being investigated for diagnostic and therapeutic modalities.[1] Our studies have already suggested that certain derivatives possess anti-inflammation properties,[2, 3] and other groups have demonstrated their use in neurological disorders.[4] We additionally showed that certain fullerenes could potentiate hair growth.[5] However, their use as new nanomedicines has not yet fully been realized because of the lack of clearly identified candidates for a particular disease. Once these candidates are more thoroughly characterized, relevant pharmacological and biodistribution data must be assessed. Fullerenes' full capabilities as new nanomedicines has been additionally hampered by the inconsistency in how the water-insoluble fullerenes are derivatized into biologically acceptable agents; most studies thus far have used highly impure and uncharacterized material, which makes interpretation of data difficult.

To begin to address the issue of in situ biodistribution, earlier characterized fullerene derivatives that have been shown to have anti-inflammatory activity[3] were modified by chemically adding reporter dyes. Using this technique we were able to study the cellular uptake and distribution of fullerenes using a human mast cell (MC) model. It is shown that these fullerene derivatives are endocytosed into the cell and trafficked to different organelles at different time points. The fullerene conjugate localized with a particular predominance to calcium and reactive oxygen species (ROS)–controlling organelles, which may help explain how they exert their cellular-inhibitory capabilities.

Methods

Fullerene derivatives

All fullerene derivatives were synthesized at Luna Innovations Inc. Texas red (TR) was attached to the functionalized 70-carbon fullerene (C_{70}), earlier shown to demonstrate anti-inflammatory properties,[3] as shown in Supplementary Figure S1 (available in the online version of this article). Monoprotected octa(ethyleneglycol)diamine (Supplementary Figure S1, step 1, available in the online version of this article) (1 mmol) was reacted with ethylmalonylchloride (1 mmol) in the presence of triethylamine (1.2 mmol) in anhydrous dichloromethane (DCM, 30 mL) for 3 hours at 23°C, and the mixture was washed twice with brine before subjecting it to column chromatographic purification (silica gel). The isolated oily product (Supplementary Figure S1, step 2, available in the online version of this article) was characterized by both proton nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-NMR. Malonamide 2 (10 μmol) was reacted with one equivalent of iodine, 2.3 equivalents of 1,8-diazabicycloundec-7-ene, and 1.0 equivalent of the C_{70} monoadduct (Supplementary Figure S1, step 3, available in the online version of this article), which was prepared from $C_{70}$and di(dodecyl)lmalonate under Bingel conditions (reaction to create extensions to the fullerene sphere). The reaction mixture was stirred for 4 hours in toluene, washed with brine, and concentrated for silica column purification. The isolated red band from silica column, characterized by both NMR and matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS), was treated with 20% trifluoroacetic acid in DCM for 2 hours, and the solvent was evaporated using a rotary evaporator and pumped under vacuum for 6 hours. The resulting amine-functionalized C_{70} bisadduct (Supplementary Figure S1, step 4, available in the

online version of this article) (10 mg) in its trifluoroacetic acid salt form was dissolved in 5 mL dimethylformamide (DMF) and stirred with two equivalents of triethylamine for 10 minutes. Next, 2 mg of TR *N*-hydroxysuccinimide ester (Biotium, Inc., Hayward, California) dissolved in 0.2 mL amine-free DMF (Sigma Aldrich, Milwaukee, Wisconsin). was added to the mixture and stirred for 6 hours at room temperature. DMF was removed under vacuum, and the residue was subjected to preparative thin-layer chromatography (silica phase) for purification. After the thin-layer chromatography plate was spotted with the residue, it was eluted with a mixture of DCM and methanol. There were three bands: trace amounts of the dye on the top, product (Supplementary Figure S1, step 5, available in the online version of this article) in the middle, and unreacted C_{70} (Supplementary Figure S1, step 4, available in the online version of this article) at the bottom. The plate was dried in a hood. The product band was scraped off the plate, redissolved in a mixture of methanol and DCM (1:1), filtered and concentrated to dryness, yielding pure 70-carbon fullerene-TR (C_{70}-TR) conjugate, which was characterized by MALDI-MS, ultraviolet-visible, and fluorescence spectrometry.

Human mast cell cultures and addition of fullerene derivatives

Human skin was received from the Cooperative Human Tissue Network. All studies were approved by their Human Studies Institutional Review Board. Tryptase-chymase positive MCs (MC_{TC}) type[6] were purified and cultured as described,[7, 8, 9] MCs were incubated with 5 μg/mL of C_{70}-TR in normal medium for 5 hours, washed, and photodocumented, or placed back in culture for up to 1 week at 37°C in a 6% CO_2 incubator. Preliminary experiments demonstrated that the C_{70}-TR was maximally taken up within the MCs at 5 hours; longer incubation times did not increase fullerene uptake. Thus, this time point was used in all subsequent experiments.

Endocytosis studies of fullerene derivatives

To determine the mechanisms of fullerene uptake MCs were first pretreated with or without various inhibitors of cellular uptake (10 μg/mL each of filipin, inhibitor of the raft/caveolae endocytosis pathway; amiloride, inhibitor of clathrin-independent pathways; and indomethacin, inhibitor of caveolae pathways). Cells were incubated for 30 minutes at 37°C in a humidified atmosphere with 6% CO_2, washed, and the fullerenes added for 5 hours before being washed and uptake monitored using confocal microscopy.

Confocal microscopy

Mast cells were incubated with C_{70}-TR for 5 hours, washed, and incubated with the indicated organelle-specific dyes (Mito-Tracker, Lyso-Tracker, and ER-Tracker; Invitrogen, Carlsbad, California) for the final 30 minutes. In some experiments cells were treated as above and cytospins prepared at various times after washing. The MC-specific antibody, G3 (ref. 10), attached to fluorescein isothiocyanate, was used to examine secretory granule co-localization. Cells were visualized using a Yokogawa multiple-lens Nipkow spinning-disk scanner (CSU10) (Perkin-Elmer Laboratories, Waltham, Massachusetts) illuminated by a ~100-mW krypton/argon laser mounted to a Nikon TE2000U inverted confocal microscope (Nikon Instruments, Inc., Melville, New York). Images were captured using a Hamamatsu Orca ER (Hamamatsu

Photonics, Bridgewater, New Jersey) and SimplePCI software (Hamamatsu Corp., Sewickley, Pennsylvania).

Calcium and ROS measurement

MCs were pretreated with or without C_{70}-TR overnight, washed with Tyrodes buffer supplemented with bovine serum albumin, and incubated with Fura-2 AM (2 μM; Invitrogen) for 30 minutes at 37°C. Cells were washed, stimulated with an FcεRI activator (3B4), and calcium flux measured in real time on a Perkin-Elmer LS55 Spectrofluorometer (Perkin-Elmer Laboratories). For ROS production cells were exposed to fullerenes as above. After washing, cells were resuspended in normal medium containing 5 μM dichlorodihydrofluorescein[2] at 37°C for 30 minutes, washed, and FcεRI activation-induced changes in mean fluorescence measured using spectroscopy with excitation at 502 nm and emissions at 523 nm for 15 minutes. The data are presented as fluorescence intensity of the 523-nm emission over time. All experiments were performed in triplicate, and degranulation was measured in parallel. Separate experiments were performed to ensure that the fullerenes do not interfere with indicator dye binding (not shown).

Results

Design and synthesis of dye-conjugated fullerenes

A C_{70}-based fullerene was selected for dye conjugation that is similar in structure to those determined to be potent anti-inflammatories and inhibitors of FcεRI-mediator release from MCs.[3] The strategy for designing this C_{70}-TR dye conjugate is shown stepwise in Supplementary Figure S1. Dye conjugation to the fullerene compound was confirmed by MALDI-MS and absorption spectra (data not shown). All free, unconjugated dye was dialyzed to separate from the C_{70}-TR conjugate.

Fullerenes enter MCs through endocytosis

Little is known about how fullerenes enter cells and where fullerenes localize in situ. To examine the mechanisms of cellular entry we preincubated MCs with several broad-acting endocytosis inhibitors and examined for fullerene localization within cells. In general, no fullerene localization was observed when cells were pretreated with the endocytosis inhibitors filipin and amiloride (Figure 1). The use of other endocytosis inhibitors such as indomethacin demonstrated a similar lack of fullerene uptake, which was not affected by higher fullerene concentrations or extended incubation times (not shown). Thus, fullerenes are taken up by human MCs through an endocytosis pathway.

Intracellular trafficking of C_{70}-TR

Time course studies were performed to determine the localization of the fullerenes in human MCs. As seen in Figure 2, *A–F* the majority of the C_{70}-TR was observed in the endoplasmic reticulum (ER) and lysosomes at 4 hours after washout (9 hours total). Minimal co-localization was observed within the mitochondria (Figure 2, *G–I*) at this time point. Similar localization was observed at 24 and 96 hours after washout with less co-localization within the lysosomes

(Figures 3, *A–F* and 4, *A–F*). The fullerenes were detected up to 1 week within the cells, although there was notably less C_{70}-TR within the cell (Figure 5, *A–F*). As with earlier time points there was a predominance of fullerene localization with the ER. The presence of the fullerenes in the cells at any of the times examined did not affect cell viability, which was consistently greater than 90% and not significantly different compared to nontreated cells (not shown). This suggests that fullerenes are not quickly shuttled out of the cell but persist within organelles involved in FcεRI-dependent mediator release.

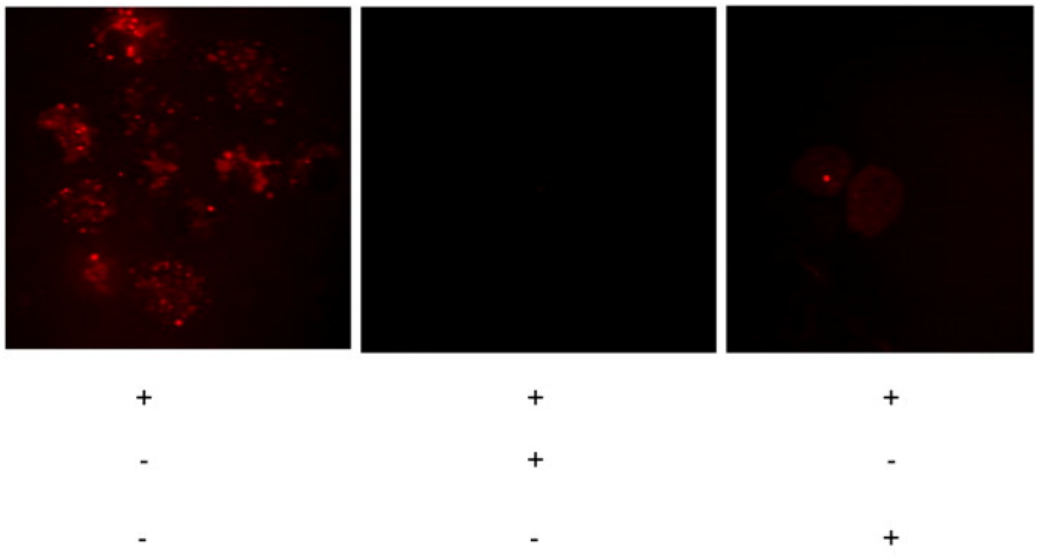


Figure 1. Endocytosis inhibitors block fullerene entry into cells. Skin mast cells were incubated with or without the indicated endocytosis inhibitors for 16 hours in the tissue culture incubator. The next day cells were incubated with the Texas red–conjugated C_{70} fullerenes for 5 hours, washed, and analyzed by confocal microscopy. Pictures (63× oil; cropped as needed) are representative of two separate experiments.

C_{70}-TR fullerene co-localized in ER reduces calcium release and ROS production

The activation of MC FcεRI leading to degranulation is calcium-dependent and induces elevated cellular levels of ROS.[11, 12] Given that calcium and ROS are produced within the ER and mitochondria, and we and others have demonstrated that these organelles accumulate fullerenes with varying affinities, we hypothesized that the co-localization of fullerene derivatives developed herein would inhibit these cellular mediators. The intracellular calcium and ROS levels in FcεRI-challenged cells with or without preincubation with fullerenes were measured to determine the impact that this fullerene has on both calcium stores and ROS levels. As seen in Figure 6, the C_{70}-TR inhibited calcium (Figure 6, *A, B*) and ROS levels (Figure 6, *C, D*) as compared with the untreated MC control. In general, a higher inhibition of release of intracellular calcium stores occurred as compared with ROS levels.

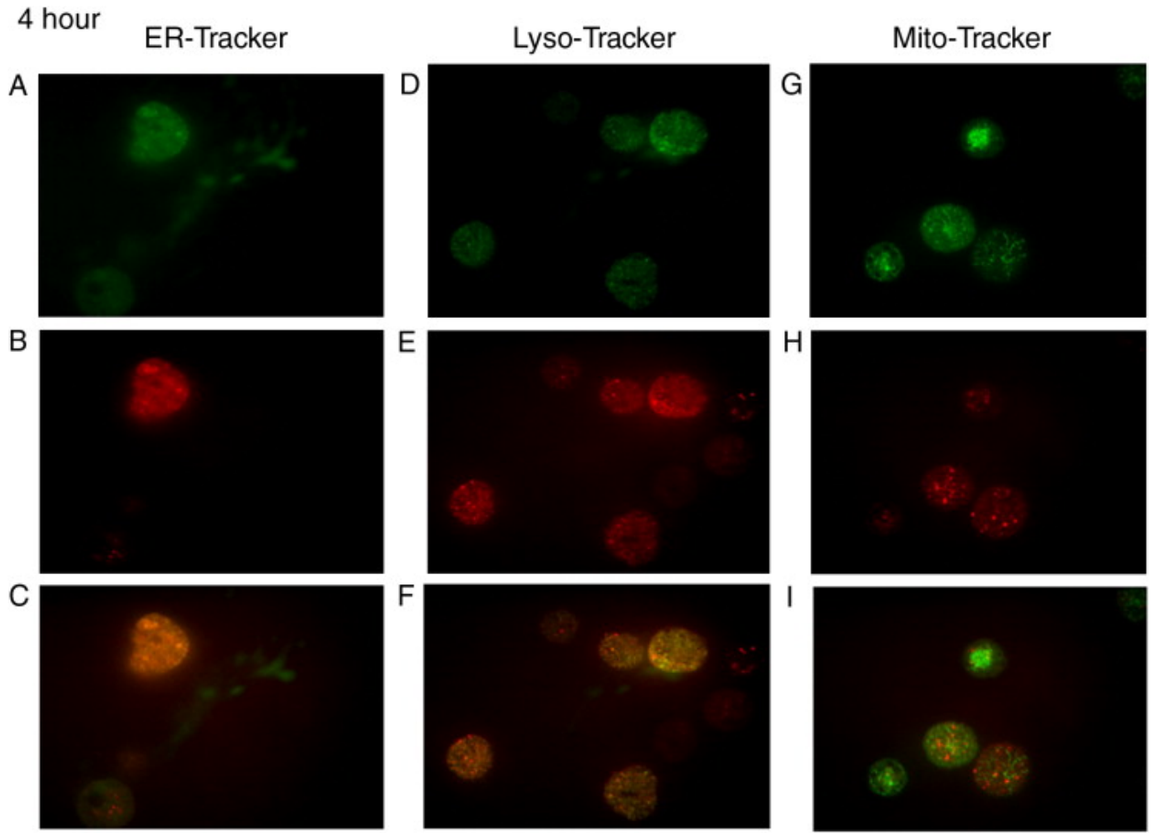


Figure 2. Time course of organelle localization using Texas red–conjugated C_{70} (C_{70}-TR) fullerenes in human mast cells (MCs). Cells were incubated with C_{70}-TR fullerenes (5 μg/mL) for 5 hours, washed, and returned to culture for the indicated times: Figure 2, 4 hours; Figure 3, 24 hours; Figure 4, 96 hours; Figure 5, 1 week. For the final 30 minutes cells were incubated with **(A–C)** ER-Tracker (500 nM), **(D**,**E)**Lyso-Tracker (25 nM), or **(F–H)** Mito-Tracker (20 nM), washed, placed on a coverslip, and imaged using confocal microscopy. Pictures (63× oil; cropped as needed) representative of two separate MC cultures.

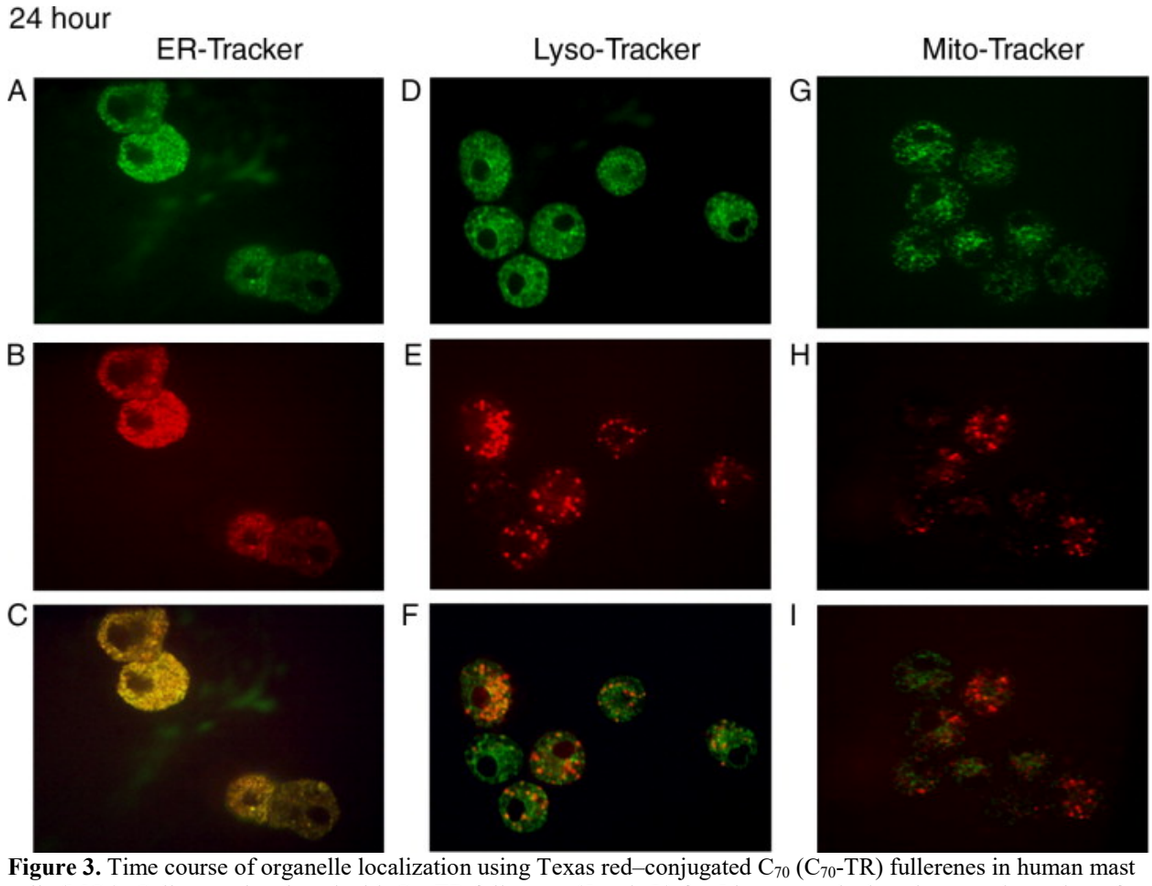


Figure 3. Time course of organelle localization using Texas red–conjugated C_{70} (C_{70}-TR) fullerenes in human mast cells (MCs). Cells were incubated with C_{70}-TR fullerenes (5 μg/mL) for 5 hours, washed, and returned to culture for the indicated times: Figure 2, 4 hours; Figure 3, 24 hours; Figure 4, 96 hours; Figure 5, 1 week. For the final 30 minutes cells were incubated with **(A–C)** ER-Tracker (500 nM), **(D**,**E)**Lyso-Tracker (25 nM), or **(F–H)** Mito-Tracker (20 nM), washed, placed on a coverslip, and imaged using confocal microscopy. Pictures (63× oil; cropped as needed) representative of two separate MC cultures.

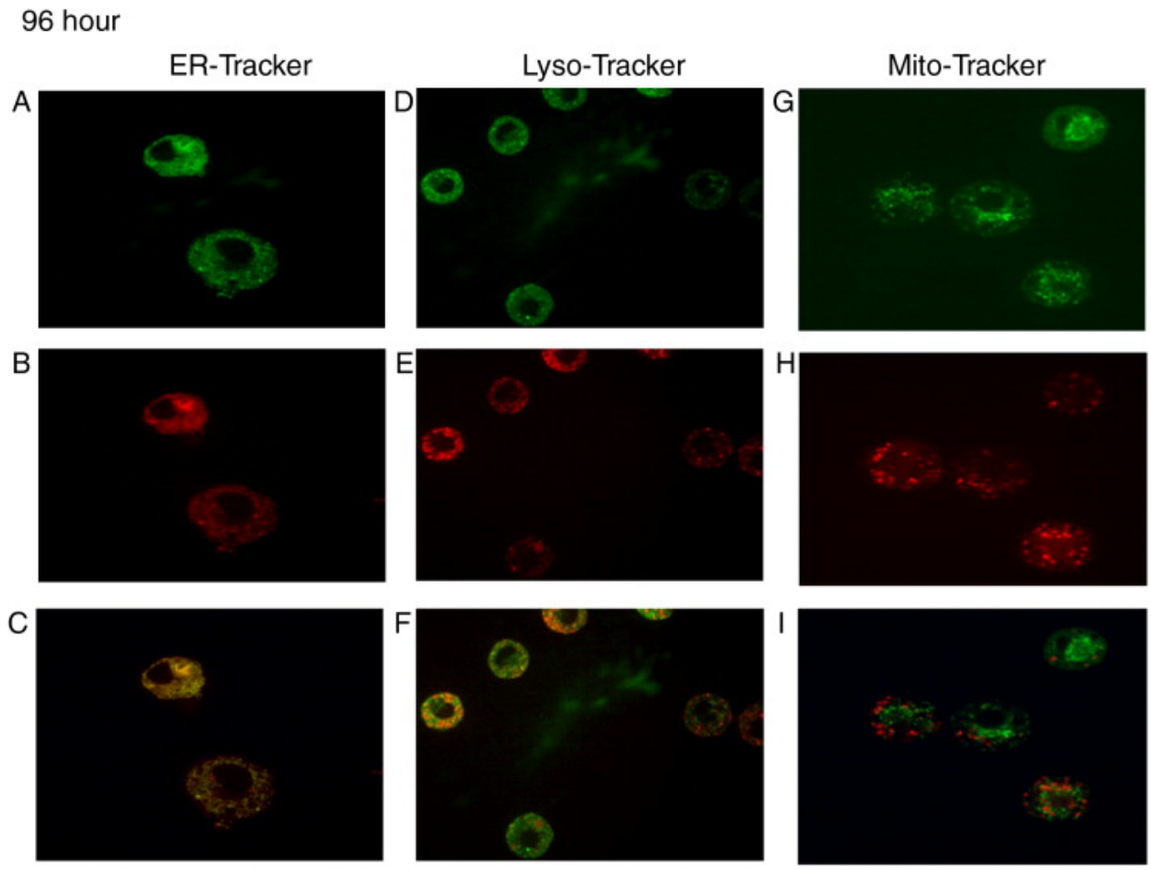


Figure 4. Time course of organelle localization using Texas red–conjugated C_{70} (C_{70}-TR) fullerenes in human mast cells (MCs). Cells were incubated with C_{70}-TR fullerenes (5 μg/mL) for 5 hours, washed, and returned to culture for the indicated times: Figure 2, Figure 4 hours; Figure 3, 24 hours; Figure 4, 96 hours; Figure 5, 1 week. For the final 30 minutes cells were incubated with **(A–C)** ER-Tracker (500 nM), **(D**,**E)** Lyso-Tracker (25 nM), or **(F–H)** Mito-Tracker (20 nM), washed, placed on a coverslip, and imaged using confocal microscopy. Pictures (63× oil; cropped as needed) representative of two separate MC cultures.

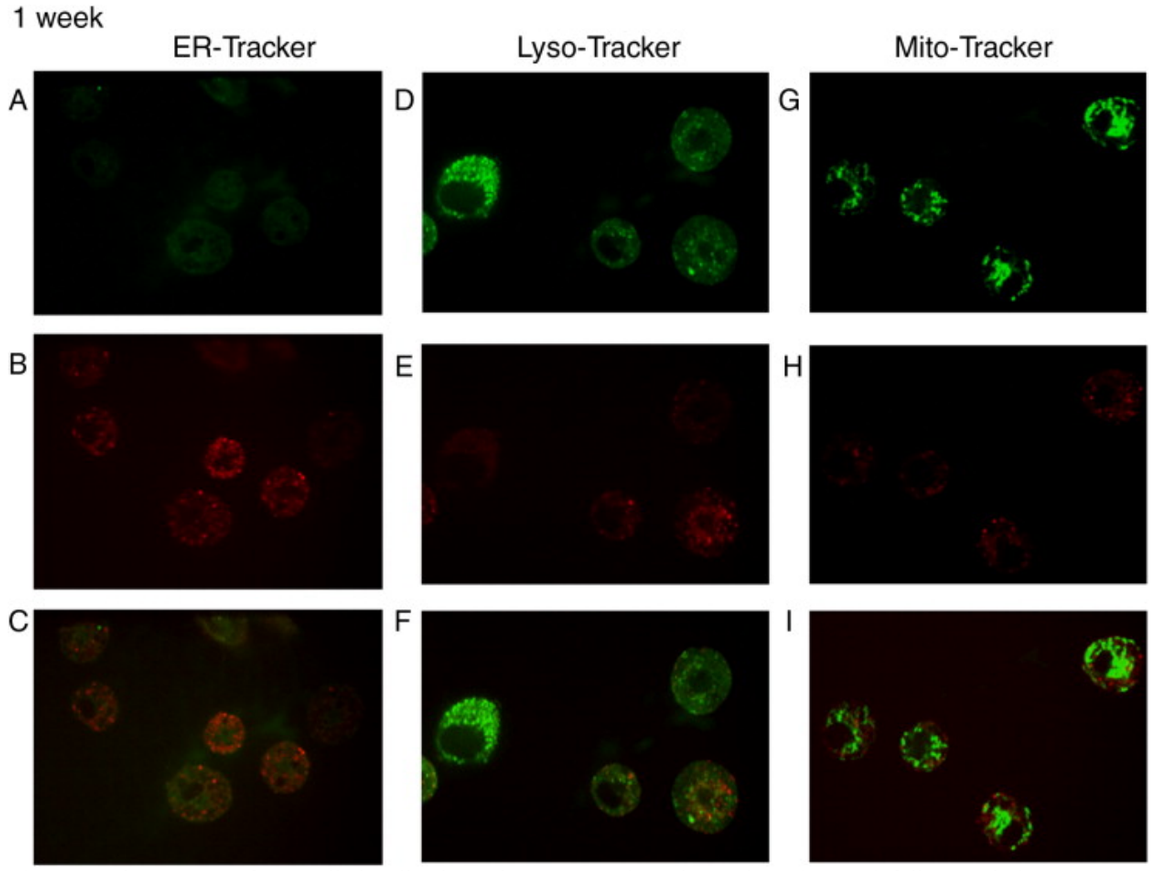


Figure 5. Time course of organelle localization using Texas red–conjugated C_{70} (C_{70}-TR) fullerenes in human mast cells (MCs). Cells were incubated with C_{70}-TR fullerenes (5 μg/mL) for 5 hours, washed, and returned to culture for the indicated times: Figure 2, 4 hours; Figure 3, 24 hours; Figure 4, 96 hours; Figure 5, 1 week. For the final 30 minutes cells were incubated with **(A–C)** ER-Tracker (500 nM), **(D**,**E)**Lyso-Tracker (25 nM), or **(F–H)** Mito-Tracker (20 nM), washed, placed on a coverslip, and imaged using confocal microscopy. Pictures (63× oil; cropped as needed) representative of two separate MC cultures.

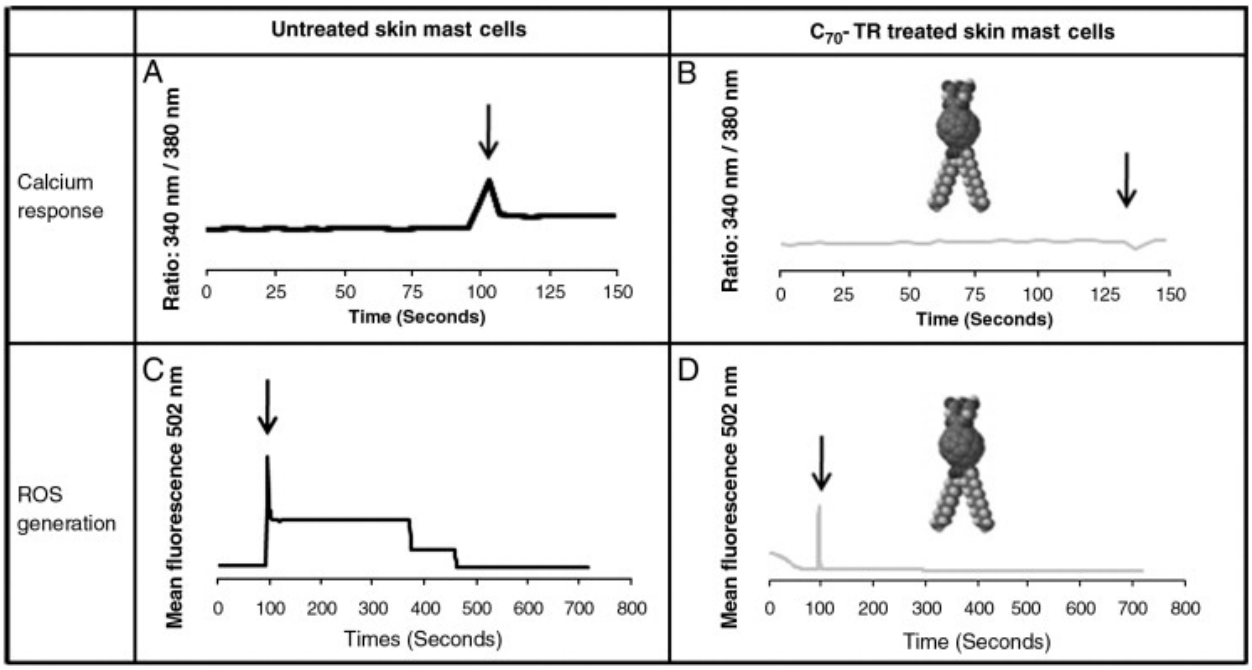


Figure 6. Texas red–conjugated C_{70} (C_{70}-TR) fullerenes co-localized in endoplasmic reticulum reduce calcium release and reactive oxygen species (ROS) production. FcεRI stimulation (3B4; 1 μg/mL) of fullerene-treated cells (20 μg/mL) was examined in parallel for calcium stores release as determined by the ratio 340 nm/380 nm and ROS measured by dichlorodihydrofluorescein detection. Results show average value (±SD) from at least three separate experiments and donors. Experiment represents three separate MC preparations.

Discussion

These are to our knowledge the first studies examining fullerene trafficking within human MCs. It was observed that the C_{70}-TR conjugated fullerenes were taken up through an endocytosis-dependent mechanism and persisted in the MCs for up to 1 week. The intracellular localization was found predominantly in the ER and to a lesser degree in mitochondria and lysosomes. No fullerenes were detected in the tryptase-containing secretory granules or in the nucleus at any time points measured. Taken together, it is clear that MCs actively take up fullerenes through an endocytotic pathway, where they remain mostly localized to ER and to a lesser extent in lysosomes and mitochondria.

The ER is the site for synthesizing and ensuring proper folding of proteins within a cell and shuttles misfolded proteins through a degradative pathway. Recent evidence suggests that this process involves the formation of disulfide bonds that stabilize the folding of nascent proteins resulting in an oxidizing environment and ROS generation.[13] In addition to the ER, mitochondria also produce ROS due to the production of adenosine triphosphate following oxidative phosphorylation. Our results using C_{70}-based derivatives are consistent with several publications examining fullerene localization in different cell types, in which deposition in different intracellular organelles depends on the moieties added to the carbon cage.[2, 14, 15, 16, 17] For example, $C_{63}(COOH)_6$ or $C_{61}(CO_2H)_2$ seem to localize to mitochondria,[14, 16] whereas C_{60} mixtures dispersed in tetrahydrofuran (and not purified from this solvent) localize to lysosomes and nuclei in macrophages.[17] Our results are also consistent with earlier studies suggesting fullerenes can easily penetrate into a lipid membrane, where they induce nontoxic changes in the

structural and elastic properties of the lipid bilayer at very high concentrations.[18] Taken together, fullerenes as a general class have a varied localization profile within cells; how the fullerene cage is modified determines where the cage localizes in situ. Given that no fullerene formulation has yet been identified for treating a particular disease, it is difficult to extrapolate in situ localization results from different fullerene preparations.

The results demonstrating that C_{70}-based fullerenes are endocytosed and localize to ER differ from earlier published results showing that endocytosed C_{60}-based fullerenes localize to the mitochondria and lysosomes.[19, 20] The ER accumulation of C_{70}-based fullerenes explains the data shown that preincubation of MCs with the same fullerenes caused a reduction of FCεRI-mediated calcium release and ROS generation. These findings may help explain fullerenes' mechanism of inhibition of FcεRI-activated mediator release from MCs.[2] The release of calcium stores and production of ROS occur in these organelles[21]; although release of calcium stores is absolutely required for FcεRI-mediator release, it is still not clear if increased ROS levels parallel mediator release or are a consequence of FcεRI crosslinking. Calcium stores release from the ER in response to FcεRI crosslinking is controlled by inositol 1,4,5-trisphosphate. Thus, the observation that fullerenes localize to this organelle fits well with our data showing that calcium stores release and ROS production are inhibited when FcεRI-challenged MCs are prechallenged with fullerenes. Current efforts are aimed at determining what ER-associated signaling molecules(if any) are bound to fullerenes, which would help explain how these molecules exert their inhibitory activities.

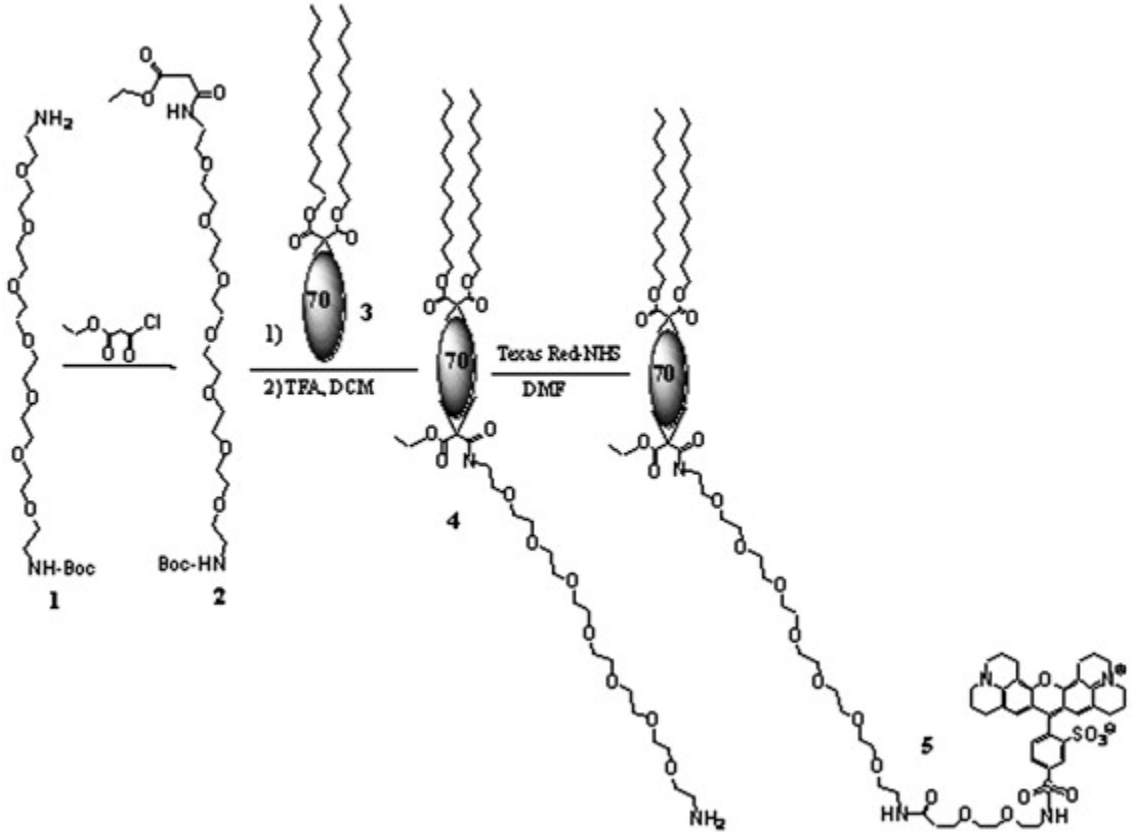
Fullerenes were also consistently found (but to a lesser extent as compared to ER accumulation) in lysosomes. Endocytosis from the plasma membrane can occur by a variety of mechanisms, and once uptake occurs the molecules are then routed through lysosomes to various places within cells. We observed lysosomal accumulation of C_{70}-TR predominantly at 4 hours, 96 hours, and 1 week after washout, perhaps indicating the cells shuffling the conjugates from the membrane early and to the membrane for possible excretion at the later times. Indeed, there seems to be a localization of fullerenes in the outer edges of the cell at later times (Figure 4, Figure 5), possibly indicating their imminent transport out of the cells.

As mentioned above, the mitochondria is another organelle that is best known for its role in ROS production, which is a result of the electrochemical membrane potential ($\Delta\Psi m$) across the inner mitochondrial membrane.[21] We found sporadic localization of the C_{70}-TR in the mitochondria at some of the times examined. Mitochondrial localization has been demonstrated before either directly or indirectly using other fullerene preparations.[14, 16, 22, 23] Given that fullerenes are potent antioxidants that can react with ROS, it has been speculated that much of their inhibitory capabilities are linked to this radical-scavenging ability. However, we show here that fullerenes that are effective anti-inflammatory agents mainly target ER, which is also a ROS-producing organelle. It is possible the C_{70}-TR may not reflect the same targeting specificity as the C_{70} derivative without TR. We are currently engineering fullerenes without bulky side chains that are structurally the same as those described before, so that results obtained on inhibitory capabilities can be extrapolated to organelle targeting more specifically.

In conclusion, we have identified the ER as a primary organelle for 70-carbon–based fullerene derivatives localization in human MCs as opposed to previous publications that show 60-carbon–

based fullerenes localize to the mitochondria This localization may help explain how the fullerenes exhibit their inhibitory activity through blunting of calcium and ROS spikes leading to subsequent reduction in histamine deganulation and cytokine production. Fullerenes are potent antioxidants and are being investigated as therapies for a wide range of diseases.[24, 25, 26] This may have clinical implications for developing future fullerene-based compounds; our efforts are focused on developing these lead candidates into novel ways to treat those diseases associated with MC activation including asthma, arthritis, and anaphylaxis.

Appendix A. Supplementary data



Supplementary Figure 1. Synthesis of fullerene-Texas Red conjugates. C_{70} was sequentially derivatized at the two poles with one lipophilic and one hydrophilic group. Texas red was covalently conjugated to the polar pole of C_{70} through a short polyethylene glycol (PEG7) spacer via an amide bond.

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