CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.201000241

Multi-tasking with Single Platform Dendrimers for Targeting Sub-Cellular Microenvironments

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The fabrication of multi-functional biocompatible materials with a high degree of complexity at the molecular level constitutes the key to the future of biotechnology.[1] Much remains to be done to develop efficient physiologically or therapeutically active nanostructures that can not only transport biologically active cargo across cell membranes, but also ensure delivery to specific intracellular locations.[2] Macromolecular architectures with well-defined size and shape are of eminent interest for biomedical applications, such as the delivery of therapeutics and tissue imaging.[3–7] Dendrimers are monodisperse and hyperbranched macromolecules with unique architecture and properties that can be tailored for specific applications.[8] One of the key challenges in the development of dendrimers for applications in medicine is to incorporate complementary biological tasks, including imaging and therapeutic delivery, in the same dendrimer architecture.[9] However, introducing multiple functionalities into a single dendrimer platform has presented significant synthetic challenges.[10,11] Another important issue related to the biomedical applications of dendrimers pertains to their cytotoxicity,[12,13] and the need for versatile, clean and high yielding chemistry with essentially no side products, continues to be the key tenet and a topical area of research.[14] We demonstrate herein that orthogonally functionalised dendrimers with diverse groups such as a fluorescent dye, useful for organelle imaging, and a therapeutic agent can be easily constructed by using a "click" chemistry[15–17] approach. These multi-tasking dendrimers show no significant cytotoxicity, and represent the first example of the simultaneous direction of a drug and a fluorophore to lipid droplets.

Lipid droplets play a crucial role in lipid metabolism and might have a much broader repertoire of "tasks", such as managing the availability of proteins and serving as generic sites of protein sequestration.[18] α-Lipoic acid (LA) is an essential cofactor for many enzymes, particularly in aerobic metabolism.[19,20] It is readily taken up by cells and reduced to its potent dithiol form, dihydrolipoate, much of which is rapidly effluxed out from cells. We demonstrate that by linking LA to a dendrimer, its intracellular retention could be increased, thereby providing an enhanced effectiveness with smaller therapeutic doses. Dipyrromethene boron difluoride (BODIPY) PM605, abbreviated here as PM, is an efficient lipophilic dye that has characteristic narrow absorption and emission bands in the visible region of the spectrum with high fluorescence quantum yields, and it readily diffuses into cells and accumulates in lipid bodies.

The synthetically tailored linkage of PM and LA to the same assembly was made possible by the design and synthesis of an ABB′ building block that facilitates performing Cu^{I}-catalysed click reactions in a sequential manner, and eventually the construction of multi-valent dendrimers. It contains two acetylenes (BB′) protected with two different groups (trimethylsilyl (TMS) and triisopropylsilyl (TIPS)), which are individually removed when desired. It was constructed by starting from 3-bromo-5-iodobenzylalcohol, and performing Sonogashira coupling to introduce triisopropylacetylene at the iodo position at room temperature (Scheme 1), and the substitution of bromo group with trimethylsilylacetylene under reflux. It was subsequently followed by bromination of the benzylalcohol, and a mild deprotection procedure to eliminate the trimethylsilyl group. The resulting compound (**4**) is a highly versatile unit that can carry multiple functionalities on itself, or introduce them on the dendritic macromolecule at a later stage. We used its free acetylene arm to click a long-chain alcohol upon reaction with 11-azido-undecan-1-ol that was subsequently used to covalently link LA. The benzyl bromide was then activated by using a simple azidation (**6**).

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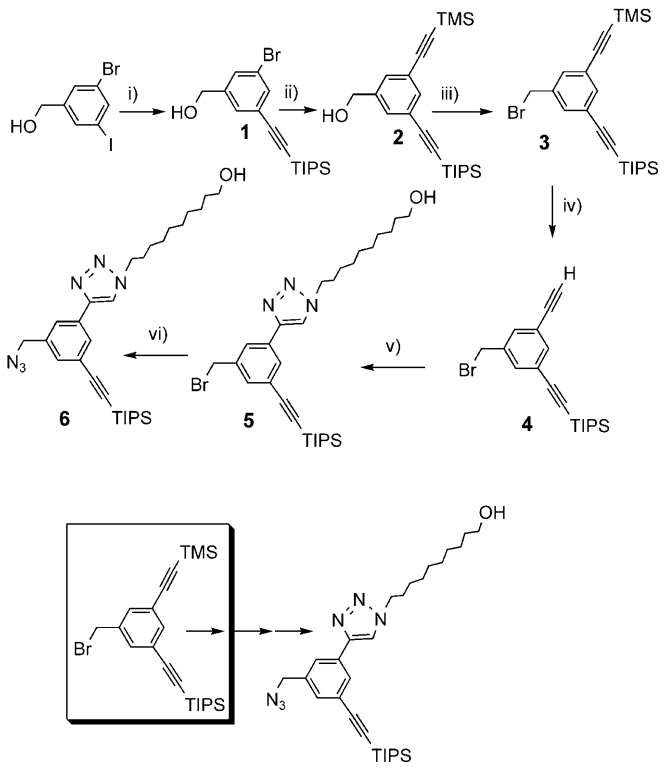
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201000241.



Scheme 1. Synthesis of building blocks: i) TIPS-acetylene, $[PdCl_2(PPh_3)_2]$, CuI, NEt_2H, RT, 48 h; ii) TMS-acetylene, $[PdCl_2(PPh_3)_2]$, CuI, NEt_3/benzene, reflux, overnight; iii) CBr_4, triphenylphosphine (TPP), THF, 2 h; iv) acetone/water, K_2CO_3, overnight; v) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, H_2O/THF, 50 °C, overnight; vi) NaN_3, DMF, RT, 2 h.

To synthesise the desired carrier, building block **6** was clicked to the 1,3,5-triethynylbenzene core, followed by removal of the TIPS groups (Scheme 2). To covalently link the PM dye to the resulting free acetylenic centres, it was functionalised with an azide group (PM-N_3), by forming an ester linkage between the alcohol form of the dye with 6-azido-hexanoic acid (Scheme 3). The hydroxyl terminated arms were then treated with LA by using an esterfication reaction to obtain the desired asymmetrically functionalised dendrimer (**7**; Scheme 2). The synthetic goals of this study included examining the role of the dendritic structure on the internalisation process. For this purpose, we synthesised a monofunctional dendrimer (**8**; Scheme 3 by clicking three azide terminated PM-N_3 dye molecules to the 1,3,5-triethynylbenzene (TEB) core.

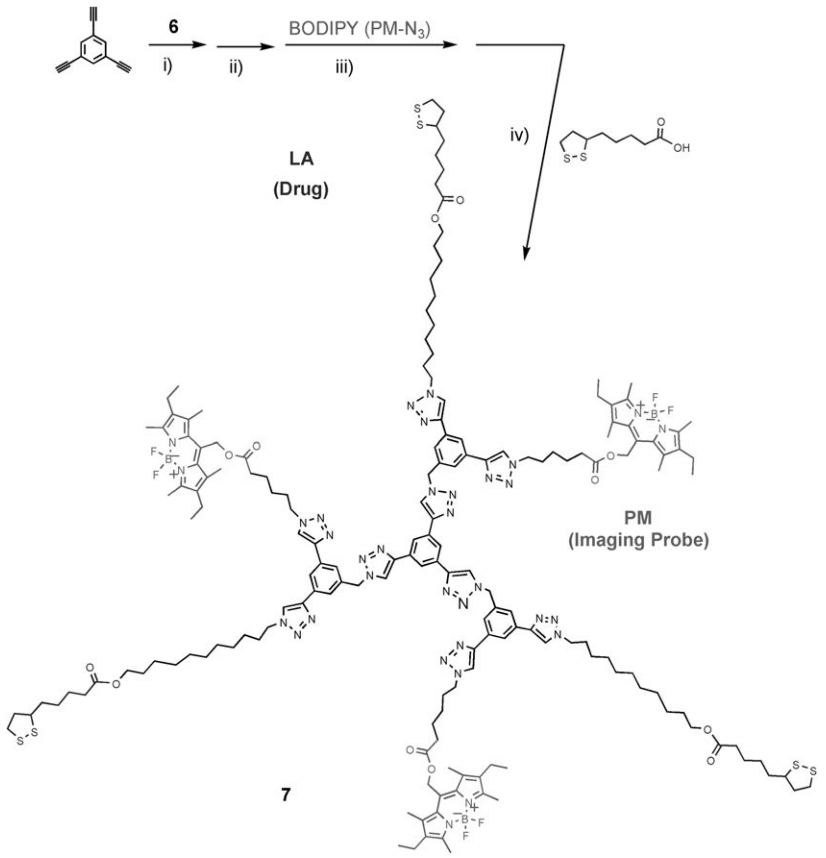
We had also anticipated that the structure of the carrier may influence its overall behaviour. Therefore, a linear analogue of the dendrimer containing the PM dye and LA (**9**; Scheme 4) was constructed by using (4-ethynyl-phenylethynyl)triisopropylsilane as the central unit on which two Cu-catalysed click reactions were carried out in sequence. The free acetylene arm of (4-ethynyl-phenylethynyl)triisopropylsilane was first functionalised with a long-alkane-chain alcohol by using 11-azidoundecan-1-ol. Upon subsequent deprotection, the other acetylene unit was made available for the second click reaction with PM-N_3. The free primary alcohol was subsequently used for attaching LA through a quantitative esterfication reaction.

We first assessed the cellular internalisation kinetics of the carriers reported herein, and whether they exert any cytotoxicity in human cells in culture. We demonstrated that the compounds of interest were internalised in human hepatocytes, however, the quantity and rate of entry varied significantly for each compound (Figure 1a). The free PM dye rapidly entered the cells and reached a maximum intracellular fluorescence within an hour before gradually decreasing to approximately 11 % of its initial value. Compounds **7** and **8** were also rapidly internalised by the cells, however, to a lesser degree and with slower kinetics. Interestingly, the mean fluorescence intensities remained significantly higher in cells exposed to the bifunctional dendrimer incorporating both LA and dye (**7**), as well as the monofunctional dendrimer containing the dye alone (**8**), for up to 48 h of incubation, in comparison with the free dye.

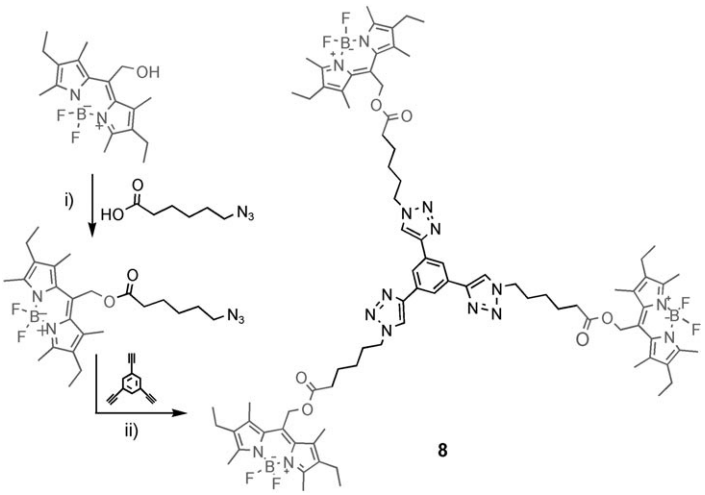
The PM dye and dendritic compounds functionalised with PM (in 0.1–10 μM concentrations) did not markedly reduce mitochondrial metabolic activity or alter the cell morphology of human hepatocytes (data not shown). When the compounds were assessed at equal concentrations of 1 μM, for an extended period, the corresponding metabolic activity did not decrease post 24, 48 and even 72 h of incubation (Figure 1b), thus indicating the neither the free dye, nor the dendrimers, **7** and **8**, are cytotoxic under the conditions evaluated.

The therapeutic potential of the LA-containing dendrimer **7**, and free LA (at equimolar concentrations of LA) was evaluated for cytoprotectivity against H_2O_2 insult in human breast cancer (MCF-7) cells (Figure 1c). The bifunctional dendrimer, after both 1 and 48 h of pre-incubation, was able to completely protect the cells against H_2O_2 cytotoxicity (*** $p < 0.0001$). Free LA at equimolar concentrations was only able to partially protect cells from the oxidative stress (** $p < 0.01$). These results imply that when bound to dendrimers, LA may be therapeutically effective at reduced concentrations, perhaps due to the greater retention time permitted by the dendrimer structure.

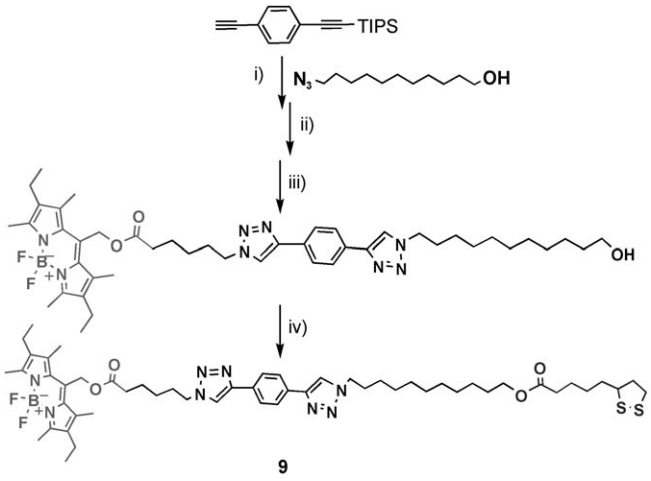
Since most of the cell-labelling protocols call for short times of exposure to the fluorophore, and our initial internalisation experiments showed a rapid increase in fluorescence intensities in the treated cells, we assessed the fluorophore entry by fluorescence microscopy. The imaging of single cells (see Figure S7 in the Supporting Information,) clearly supported the spectrofluorometric measurements and showed that the PM dye and the dendritic molecules were internalised at different rates, and that their sub-cellular distribution patterns were different. Fluorescent microscopy studies indicated that the red PM and the lipid droplet tracker, green BODIPY dyes, were at least partially co-localising in lipid droplets, as indicated by the yellow fluorescent signal resulting from the overlay of red and green fluorescence. In the case of the bifunctional dendrimer (**7**), the yellow fluorescent signal was most intense and clearly detectable as early as two minutes post-treatment as a bright



Scheme 2. Synthesis of the bifunctional dendrimer **7**: i) $CuSO_4 \cdot 5H_2O$/sodium ascorbate, H_2O/THF, 50 °C, 48 h; ii) tetrabutylammonium fluoride (TBAF)/THF; iii) same as in i); iv) dicyclohexylcarbodiimide (DCC)/ 4-dimethylaminopyridine (DMAP), CH_2Cl_2, 2 h.



Scheme 3. Synthesis of monofunctional dendrimer **8**: i) DCC/DMAP, CH_2Cl_2, 2 h and ii) $CuSO_4 \cdot 5H_2O$/sodium ascorbate, H_2O/THF, 50 °C, 48 h.



Scheme 4. Synthesis of linear analogue **9**: i) $CuSO_4 \cdot 5H_2O$/sodium ascorbate, H_2O/THF, 50 °C, overnight; ii) TBAF/THF; iii) PM-N_3, same as in i); iv) LA, DCC/DMAP, CH_2Cl_2, 2 h.

yellow signal, depicting almost perfect overlapping with the lipid droplet stain.

To more precisely assess dendrimer sub-cellular localisation, confocal microscopy was employed (Figure 2). Organelles were fluorescently labelled, the lipid droplets with green BODIPY, the mitochondria with Mitotracker Deep Red 633, and the lysosomes with Lysotracker DND 26.

Dendrimer **8** and PM co-localisation with lipid droplets was partial; however, the bifunctional dendrimer (**7**) demonstrated complete co-localisation with the lipid droplets as revealed by merging of the individual photomicrographs. Similarly, dual labelling of either the mitochondria and dendrimers, or lysosomes and dendrimers (within 5 min), failed to show any significant co-localisation of these organelles and the dendrimers or free PM.

The model therapeutic compound, LA, easily diffuses in and out of the cell,[21] but when it is covalently bound to the dendrimer, its diffusion was more restrained. Thus, as concluded by Tirosh et al., conjugation of LA may improve cellular retention as well as enhance its therapeutic potential.[22] Results reported herein suggest that the residence time of LA can be enhanced by binding to the dendrimer, and its export from the cell can be slowed down. The latter could contribute to an enhanced antioxidant effect, as seen after cell treatment with H_2O_2.

The synthetic methodology developed herein is versatile and could be easily elaborated to other pharmacological

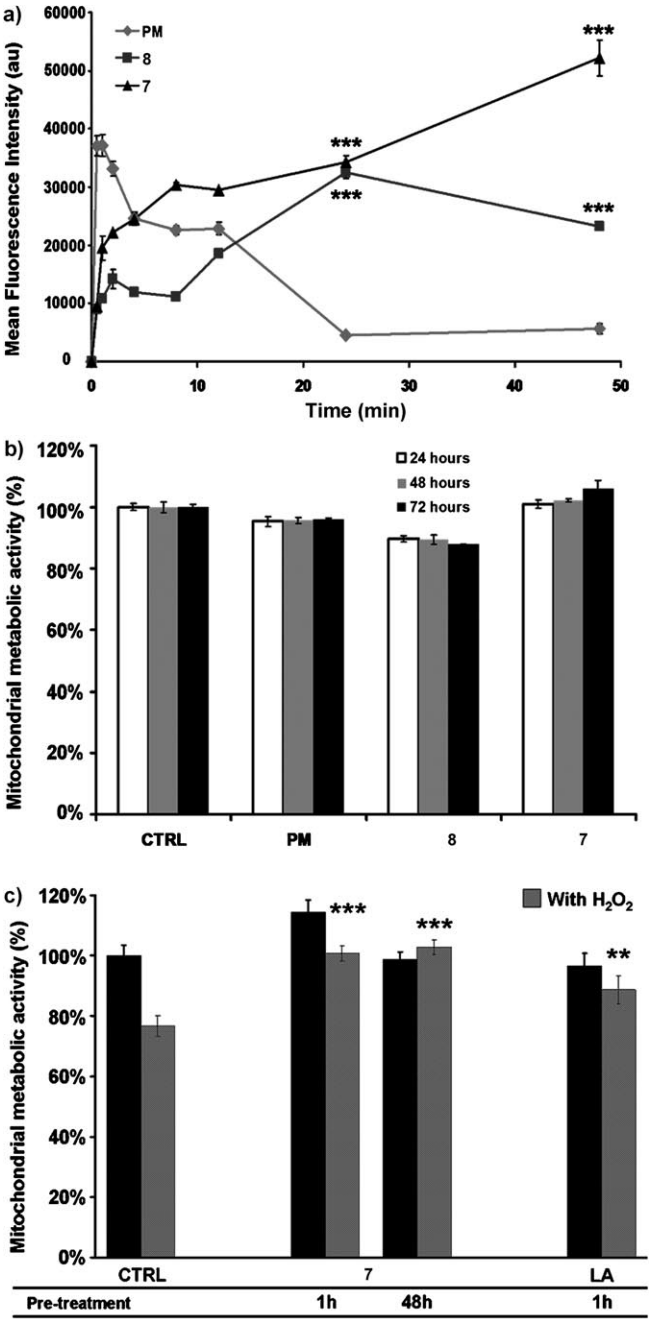


Figure 1. Cellular internalisation, cytotoxicity and cytoprotectivity of dendritic compounds. a) Time course of cellular internalisation of the dendrimers (**7**,**8**), and free dye (PM) were evaluated over 48 h. b) Mitochondrial metabolic activity of cells was assessed by the MTT assay (conversion of a tetrazolium salt to formazan by enzymes in living cells). None of the three compounds reduced mitochondrial metabolic activity after 24, 48 or 72 h of incubation. c) Cytoprotective effects of **7** in cells exposed to H_2O_2 (200 μM, for 24 h) were evaluated. For more details, see the Experimental Section.

agents and organelle markers, thereby providing a wide platform for bi- and multi-functional materials for biomedical applications. For instance, dendrimers could carry inhibitors of lipid synthesis or storage, and provide clinically useful "nano-regulators" of cellular lipid homeostasis. Since lipid

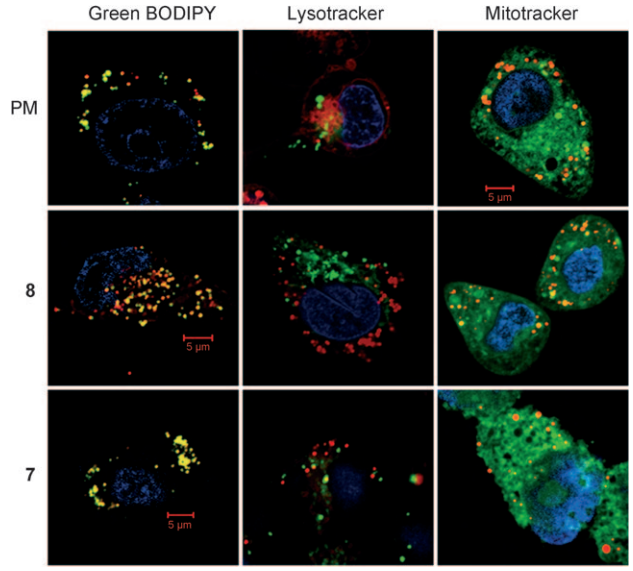


Figure 2. Intracellular localisation of fluorescent PM dye and dendrimers. Confocal photomicrographs of primary human hepatocytes stained with green BODIPY, or labelled with Lysotracker and Mitotracker in the presence of PM (10 min), **7** and **8**. Marked co-localisation was observed between lipid droplets and **7** compared with free PM and **8**. Note the absence of co-localisation between the lysosomes and any of the compounds in the early minutes post treatment when stained with Lysotracker. Note the absence of co-localisation between any of the compounds and mitochondria when stained with Mitotracker. For more details, see the Experimental Section.

bodies may function as dynamic and specialised intracellular sites, accommodating signalling molecules and storing lipids, approaches to normalise lipid accumulation and utilisation are of significant therapeutic value when lipid homeostasis is impaired (e.g., obesity, diabetes). Another application of such click chemistry materials targeting cells with an excessive number of lipid droplets could be in pathologies associated with inflammation in which non-steroidal anti-inflammatory drugs (NSAID) are linked with dendrimers.[23]

Experimental Section

Spectrophotometric measurements of internalised free and labelled dendrimers: Cells were seeded at a concentration of 150000 cells per well in a 24 well plate (Sarstedt). Prior to the addition of fluorescent dyes, the media was changed to serum-free RPMI 1640. Cells were treated with various forms of the red fluorescent dye (free dye, linear analogues and dendrimers), and allowed to incubate for several subsequent hours as indicated in Figure 2b. Upon termination of the incubation period, media from the cells was aspirated and the cells were gently rinsed with PBS. DMSO was added to each well and pipetted in triplicate to a black 96-well plate (Costar). Red fluorescence of the samples was then measured by using a BMG spectrofluorometer.

Cellular internalisation, distribution and cytotoxicity of the linear and dendritic compounds (Figure 1): Sub-cellular compartments and anticipated distribution of free lipoic acid, free PM dye and the bifunctional dendrimers. Hepatocytes were treated with equimolar concentrations (1 μM) of PM dye, of all three compounds; subsequent spectrofluorometric measurements of intracellular fluorescence show significant differences in the internalisation kinetics. Intracellular fluorescence intensities of

7 and **8** were found to be significantly different from the free dye, PM (***$p < 0.0001$) after 24 h of incubation. After 48 h, the two dendrimers showed statistically significant differences from the free dye, PM (***$p < 0.0001$).

Time course of cellular internalisation of the dendrimers (**7**, **8**), linear compound (PM-LA) and free dye (PM) were evaluated over 48 h. Hepatocytes were treated with equimolar concentrations (1 μM) of PM dye, of all four compounds; subsequent spectrofluorometric measurements of intracellular fluorescence show significant differences in the internalisation kinetics. Intracellular fluorescence intensities of **7**, **8** and **9** were found to be significantly different from the free dye, PM (***$p < 0.0001$), after 24 h of incubation. After 48 h, only the two dendrimers showed statistically significant differences from the free dye, PM (***$p < 0.0001$). Of the four compounds, only **9** reduced mitochondrial metabolic activity after 24, 48 and 72 h of incubation (***$p < 0.0001$).

Mitochondrial metabolic activity of cells was assessed by the MTT assay. Cytoprotective effects of **7** in cells exposed to H_2O_2 (200 μM, for 24 h). Each compound was preincubated with the cells for either 1 h or the optimal time required to ensure maximal cellular uptake (24 h for **9**, 48 h for **7**). LA was preincubated with cells for 1 h prior to H_2O_2 addition. LA, **9** and the dendrimer **7** treatments were utilised in equimolar concentrations of LA (5.6 μM). Compound **9** regardless of incubation time was not protective, but significantly cytotoxic ($p < 0.0001$) and further enhanced the cytotoxicity derived from H_2O_2 (**$p < 0.01$). Compound **7** was cytoprotective ($p < 0.0001$) at both preincubation times, whereas LA, at these sub-therapeutic conditions was only mildly cytoprotective (**$p < 0.01$).

Confocal laser scanning microscopy: Confocal laser scanning microscopy was carried out by using a Zeiss LSM 510 microscope equipped with the following lasers: 1) HeNe LASOS LGK 7786 P/Power supply 7460 A: 543 nm, 1 mW, 2) Argon LASOS LGK 7812 ML-1/LGN: 458, 488, 514 nm; 25 mW; Laser class 3D and 3) Titanium:Sapphire The Coherent Mira Model 900-F Laser tuneable from 710 to 1000 nm for two photon microscopy set to pulse at 800 nm. Cells for imaging were grown in eight-well chambers (Lab-Tek, Nalge Nunc International, Rochester, NY, USA). Prior to staining the cells, the cell media was changed to serum free and fluorescent organelle dyes were added. Human primary hepatocytes were stained with the blue fluorescent nuclear dye, Hoechst 33342, and lipid droplets were labelled with 493/503 (20 μM, 10 min; λ_{ex} = 493 nm, λ_{em} = 503 nm). Lipid bodies were stained with green BODIPY (Invitrogen) (20 μM, 10 min; λ_{ex} = 644 nm, λ_{em} = 665 nm), mitochondria were stained with Mitotracker Deep Red 633 (1 μM, 1 min; λ_{ex} = 644 nm, λ_{em} = 665 nm), but then pseudo-coloured green, and nuclei were stained with Hoechst 33342 (Invitrogen) (10 μM, 1 h; λ_{ex} = 350 nm, λ_{em} = 461 nm). Red, linearised Red and dendrimers with red were added to designated wells and the cells were incubated for 10 min. Before imaging, cells were washed with PBS or with serum-free medium. No background fluorescence of cells was detected under the settings used. Images were also recorded of cells labelled with Lysotracker Red DND99 (500 nM; 3 min; λ_{ex} = 577, λ_{em} = 590) and Mitotracker Deep Red 633.

Acknowledgements

A.K. and D.M. would like to thank NSERC of Canada for financial assistance. R.H. thanks FQRNT (Quebec, Canada) and M.J. thanks FRSQ for a post-graduate scholarship.

Keywords: biological activity · click chemistry · dendrimers · lipids · multiple functionalities

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Received: January 27, 2010
Published online: May 3, 2010

