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Design and Synthesis of a Mitochondria-Targeting Carrier for Small Molecule Drugs

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Junyan Han,[a,b,*] Tae Hoon Lee,[a] Ching-Hsuan Tung,[a,†] and Daniel Lee[a,c,*]

A novel mitochondria-targeting carrier QCy7HA was developed. QCy7HA transported the covalently attached doxorubicin (DOX) to mitochondria specifically. The conjugate limited the effects of P-glycoprotein (Pgp) efflux pumps of multidrug-resistant cells on DOX, indicating that diverting drugs to mitochondria is a potential promising method for treatment of drug-resistant cancers.

Mitochondria represent interesting targets for drug development.[1] Mitochondrial dysfunction appears to contribute to a wide range of pathologies including neurodegenerative diseases;[2] cardiovascular disorders;[3] diabetes;[4] and cancers.[1a] Mitochondria have been postulated to have a critical role in the natural process of aging.[5] Delivery of therapeutic substances to mitochondria may interfere with specific mitochondrial molecules involving in the initiation and progression of diseases, leading to desired therapeutic effects.[6] However, targeting drugs to mitochondria remains challenging as it mandates that molecular species cross two distinct barriers, namely the plasma membrane and the relatively impermeable, protein-dense, and hydrophobic mitochondria membranes.

One of the best studied strategies to contend with this challenge is to covalently attach therapeutic agents to a mitochondria-targeting carrier (MTC), thereby resulting in delivering "cargos" to mitochondria.[6c] Well-studied MTCs include delocalized lipophilic cationic compounds, e.g. rhodamine B,[7] and lipophilic cationic triphenylphosphonium salts,[6d] that traverse the mitochondria lipid membrane driven by its negative membrane potential gradient. Other classes of MTCs are based on either proteins with natural occurring mitochondria-targeting amino acid sequences, or hydrophobic and cationic short peptides, e. g. tetrapeptides with 2',6'-dimethyltyrosine residues and hexapeptide $(CR)_3$ (C and R represent synthetic cyclohexylalanine and D-arginine respectively), or peptides and peptitomimetics derived from the sequence of the membrane-active gramicidin S antibiotics.[6c, 8] These MTCs have been shown to transport a variety of small molecular drugs into mitochondria including antioxidant agents, photosensitizers and antineoplastic agents.[6c, 7a, 8]

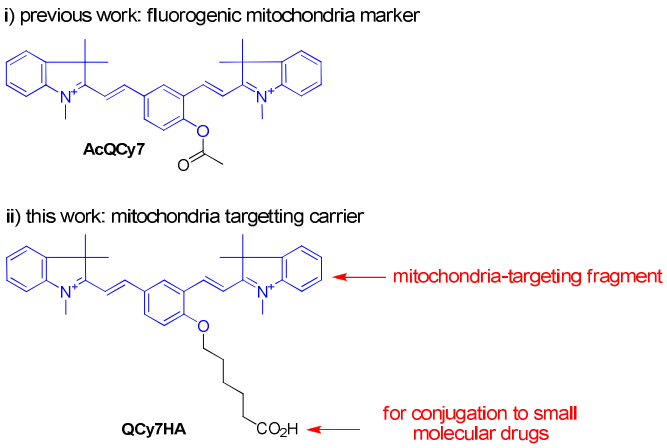
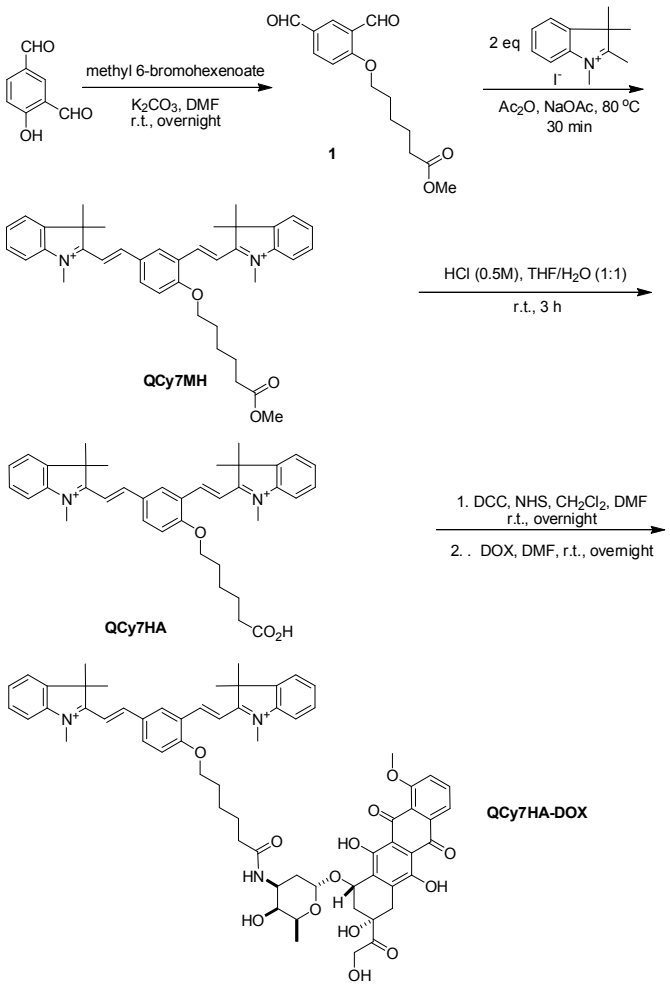


Figure 1. The structure of mitochondria targeting carrier QCy7HA.

Our laboratory recently developed a fluorogenic probe AcQCy7 for specific mitochondria labelling in live cells (see Figure 1).[9] We assumed that we can convert AcQCy7 into a mitochondria-targeting carrier by attaching a drug-loading linker to it. The mitochondria-targeting ability of AcQCy7 is due to its hydrophobic and cationic Cy-7 like structure. Mitochondria have large negative membrane potential. Consequently, they uptake positive charged hydrophobic molecules involuntarily. Both QCy7HA and AcQCy7 features identical Cy7-like structures (see blue fragment in QCy7HA in Figure 1), which have hypothetical mitochondria-targeting strength due to its hydrophobic and cationic properties (two positive charges on each molecule). In our design, we attached the hexanoic acid linker to the phenolic oxygen of the Cy7-like moiety through an ether bond. The terminal carboxylic acid may be activated and conjugated to payloads. The C6 unbranched hydrocarbon chains increase the hydrophobicity of the carrier and increase its mitochondria targeting capability.[10] AcQCy7 is a fluorogenic compound and its fluorescence was turned on immediately inside live cells due to the fragility of the acetate ester group toward intracellular esterases, resulting in release of the fluorescent compound QCy7.[9a] In contrast to AcQCy7, compound QCy7HA is a nonfluorescent carrier as the mitochondria-targeting fragment and the linker were connected via a stable ether bond and the Cy7-like

structure are disrupted. Therefore, no fluorescent QCy7 will be produced inside live cells for the new carrier QCy7HA.

We chose the well-studied antineoplastic anthracycline drug, doxorubicin (DOX), as a prototype drug for conjugation to QCy7HA. DOX provides two practical advantages:[10] first, it has a free $–NH_2$ functional group that can easily react with the activated carboxylic acid group on the carrier QCy7HA to efficiently form the QCy7HA-DOX conjugate (see Scheme 1); and second, DOX is intrinsically fluorescent thus allowing for visualizing its intracellular localization by fluorescence microscope.



Scheme 1. Synthesis of the mito-targeting conjugate QCy7HA-DOX. DMF: N, N'-dimethyl formamide; DCC: N, N'-dicyclohexylcarbodiimide; NHS: N-hydroxyl succinimide; DOX: doxorubicin.

The mitochondria-targeting version of DOX (QCy7HA-DOX) was synthesized by following the steps in scheme 1. The active intermediate phenoxide anion, produced through deprotonation of compound 4-hydroxylisophthalaldehyde on inorganic base K_2CO_3, reacted with methyl 6-bromohexanoate in anhydrous DMF solvent to give the o-alkylation product 1. The key compound QCy7MH was obtained by the Knoevenagel condensation reaction of dialdehyde **1** with 1, 2, 3, 3-tetramethyl-3H-indolium iodide (two equivalents). The hydrochloric acid-catalyzed hydrolysis of the methyl ester existing in QCy7MH produced the corresponding carboxylic acid QCy7HA, which was firstly activated by forming the N-hydroxyl succinimide ester, and then coupled to the primary amine $–NH_2$ of the sugar motif in DOX to afford the mito-targeting conjugate QCy7HA-DOX. Alternative hydrolysis of the methyl ester in QCy7MH under basic conditions, such as using NaOH in aqueous environment, caused the decomposition of the compound.

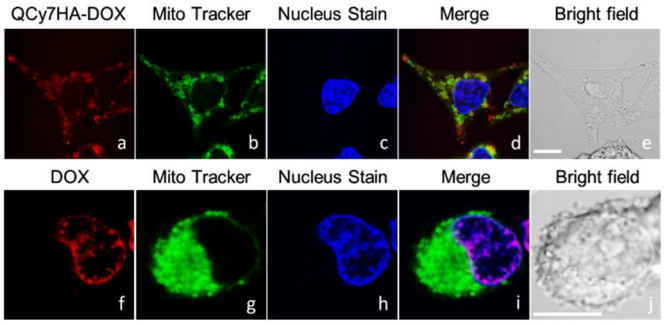


Figure 2. The subcellular localization of QCy7HA-DOX. Top panel: Triple labeling of MES-SA cells with QCy7HA-DOX (4 μM), MitoTracker Deep Red (MDR) (300 nM), and nucleus stain Hoechst 33342. Bottom panel: MES-SA cells were triple-stained with DOX (4 μM), MitoTracker MDR (300 nM) and Hoechst 33342. From left to right in both panels are the fluorescence signals produced by QCy7HA-DOX/DOX, MDR, and Hoechst 33342, the merged images, and the bright field images, respectively. Scale bar 10 μm.

To ascertain whether the QCy7HA-DOX conjugate targets mitochondria specifically over other cellular organelles, we investigated co-localization of the fluorescence of known Mito Tracker MDR with QCy7HA-DOX in a live drug-sensitive human uterine sarcoma MES-SA cells. As shown in Figure 2b, the morphology of mitochondria was very well preserved as shown by the fluorescence of MDR. The red fluorescence of QCy7HA-DOX exhibited a distribution similar to that found with MDR (Figure 2a and 2b). The presence of both MDR and QCy7HA-DOX in one region will be shown as yellow color in merged images shown in Figure 2d. The predominant yellow-color fluorescence in merged images indicated MDR and QCy7HA-DOX co-localized completely in mitochondria. We also studied the intracellular localization of the parent DOX. As shown in the bottom panel of Figure 2, the compound DOX was preferentially kept in cell nucleus over other subcellular organelles, confirmed by observing the fluorescence co-localization of DOX with the commercially available nucleus marker Hoechst 33342. It is very obvious that no DOX was distributed into mitochondria. These results clearly demonstrated that QCy7HA is able to deliver the conjugated DOX to mitochondria efficiently.

DOX commonly accumulates in cell nucleus, and the mechanism of its antitumor action involves intercalation into double helical DNA, resulting in blockade of topoisomerase II action.[11] DOX is a remarkable substrate for P-glycoprotein (Pgp) efflux pumps, which exist in the plasma and nuclear membranes of multidrug-resistant cancer cells.[12] Therefore, DOX is ineffective at eliminating a wide range of multidrug-resistant tumours in clinical applications. We hypothesized that diverting DOX to mitochondria, where mitochondrial DNA exists in high copy numbers[1f, g], can also lead to cell death. Moreover, we assumed that mitochondria-targeted doxorubicin would have an impact on demolishing multidrug-resistant cancer cells that overexpress Pgp efflux pumps.

	LD50 (μM)		
	DOX	QCy7HA-DOX	QCy7MH

MES-SA	0.1	5.0	1.2
MES-SA/Dx5	2.1	31.6	6.0

Table 1. Toxicity of DOX, QCy7HA-DOX, and the carrier QCy7MH to drug-sensitive MES-SA lines and multidrug-resistant counterpart MES-SA/Dx5 lines.

In order to determine effects of the Pgp efflux pumps on the cytotoxicity of QCy7HA-DOX, MES-SA and its multidrug-resistant subline MES-SA/Dx5 were chosen. Overexpression of Pgp efflux pumps in resistant but not in the sensitive cell lines has been reported.[13] The effects of dose escalation of DOX, QCy7HA-DOX and QCy7HA on cell viabilities is shown in Figure S1. The redirected DOX appeared to have significantly reduced toxicity in either cell lines compared to the unmodified DOX which resulted in approximately 50% cell death at 0.1 μM in the MES-SA versus 50% cell death at 2.1 μM in the MES-SA/Dx5. However, the free DOX exhibited toxicity against MES-SA lines 21 times as potent as MES-SA/Dx5 lines, while only 6 times were observed for mitochondria-targeting QCy7HA-DOX (see Table 1). Those results indicated that Pgp efflux pumps had much less effect on the mitochondria-targeting QCy7HA-DOX compared to free DOX whose toxicity is significantly reduced by Pgp efflux pumps. Similar results showed that redirecting DOX to mitochondria could limit the power of Pgp efflux pump using an alternative mitochondria-targeting carrier $(CR)_3$.[14] To our surprise, the methyl ester of the carrier QCy7HA was a little more toxic than the conjugate QCy7HA-DOX even Our previous study indicated that mitochondria-targeting moiety AcQCy7 was nontoxic.[9a] Those observations tell us that the toxicity of this new mitochondria carrier can be significantly altered after minor structure modification.

In summary, compound QCy7HA was designed, synthesized and tested as a novel mitochondria-targeting carrier. The new carrier can be easily prepared through three steps in gram scales and high yield from very cheap commercially available starting materials. Moreover, this new carrier QCy7HA contains a linker capable of activation and conjugating to small molecular drugs. QCy7HA is a Cy7-like hydrophobic molecule containing two positive charges. We clearly showed here that QCy7HA had exceptional ability to deliver covalently attached DOX, a commonly used drug in treatment of a wide range of cancer, to mitochondria, and this was confirmed by co-staining drug-sensitive MES-SA cells with QCy7HA-DOX and Mito Tracker MDR. QCy7HA-DOX showed less capability to kill both drug-sensitive MES-SA line and drug-resistant MES-SA/Dx5 line as against free DOX. However, the diversion of DOX from nucleus to mitochondria can significantly limit Pgp efflux pump effects, providing a promising method to kill multidrug-resistant cancer cells. Our results suggest that the robust accumulation of this targeting compound may be leveraged to potentiate cellular protective effects of mitochondria-specific protective agents such as free radical scavengers.[15] We envision that similar MTCs based on structure QCy7 can be designed and prepared, and in addition, the facile conjugation of DOX to QCy7HA can be used as a platform to rapidly produce and test rationally designed mitochondria-specific agents for treatment of a variety of human diseases in the future. The ability to redirect drugs to different compartments within the cell may present an exciting new approach to drug development.

Notes and references

[a] Department of Translational Imaging, Houston Methodist Research Institute, Weill Cornell Medical College, F-534, Houston, TX, 77030.
[b] Department of Neurology, Houston Methodist Hospital, 6565 Fannin street, F-553, Houston, TX, 77030. Email: jhan@houstonmethodist.org.
[c] Department of Radiology, Houston Methodist Hospital, 6670 Bertner Ave, RI1-309, Houston, TX, 77030. Email: dlee@houstonmethodist.org.
[†] Current address: Molecular Imaging Innovations Institute, Department of Radiology, Weil Cornell Medical College, New York, USA.

Electronic Supplementary Information (ESI) available: [Materials and experimental procedures, synthesis of compounds **1**, QCy7MH, QCy7HA, and QCy7HA-DOX; confocal microscopy; co-localization live cell imaging; MTT toxicity studies]. See DOI: 10.1039/c000000x/

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