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Click chemistry-derived bivalent quinine inhibitors of P-glycoprotein-mediated cellular efflux

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ABSTRACT

P-glycoprotein (P-gp) effluxes a diverse set of drug substrates out of cells in an ATP dependent manner, thereby limiting the effective accumulation of therapeutic agents. Herein we demonstrate the use of click chemistry to rapidly generate bivalent quinine dimers, containing an intervening triazole ring, as potential inhibitors of P-gp mediated efflux. Calcein-AM substrate accumulation assays were performed in an MCF7/DX1 cell line that overexpresses P-gp to monitor the inhibitory activity of the clicked quinine dimers. A small library of potent P-gp inhibitors with varying tether lengths is reported, with the best dimer demonstrating low micromolar efficacy.

P-glycoprotein (P-gp) is an integral membrane protein that is a member of the ATP binding cassette (ABC) super family of transporters.[1] P-gp is over expressed at a range of anatomical and cellular sites, such as brain capillary endothelial cells of the blood–brain barrier (BBB), epithelial cells of the small intestine and cancer cells.[2,3] P-gp effluxes a large and highly diverse set of therapeutically-relevant substrates such as the anticancer agents taxol[4] and gleevec[5], and the antipsychotic agents olanzapine[6,7] and quetiapine[8], thereby precluding the accumulation of these drugs at the site of therapeutic action. Because of this efflux of therapeutics, the development of P-gp inhibitors has been a target of intense interest.[9–13]

Numerous biochemical studies have pointed to the presence of at least two distinct binding pockets within P-gp.[14–16] The recent X-ray crystal structure of mouse P-gp, which has 87% homology with its human counterpart, has further confirmed the presence of two binding sites that are capable of stereochemical discrimination of substrates.[17] Occupying the two binding sites in the transporter region of P-gp is a potential strategy for designing inhibitors. As such, dimerization of P-gp substrates has been shown to afford substantial P-gp inhibition in cells.[18–23] For instance, we have demonstrated that cross-linking two molecules of the P-gp substrate quinine via ester linkages leads to potent P-gp inhibitors.[21] When co-administered with taxol, these agents reversed the drug resistant phenotype of cells. The nature of the tether in such bivalent inhibitors is of crucial importance for inhibition and may also be used to interrogate the region between the transporter binding sites within P-gp. For instance, the tether length has been found to significantly influence the efficacy of P-gp inhibition within emetine homo-dimers[20] whereas using highly rigid tethers within quinine dimers limits P-gp inhibition.[21]

In an effort to more rapidly screen a variety of tethering moieties, we turned to the copper(I) catalyzed Huisgen 1,3-dipolar cycloaddition reaction between azides and alkynes (commonly known as the click reaction[24]) as a way to quickly generate dimeric P-gp inhibitors and probes from monomeric P-gp substrates, such as quinine. The click reaction has shown great utility in bringing together a wide range of functional molecules,[25–27] including in situ reactions.[28] As shown in Figure 1, utilizing the pendant alcohol functionality of quinine, we developed a series of alkyne- (**QnA**) and azide-modified (**Qn′Z**) quinine analogs, of varying tether lengths. Using these functionalized quinine monomers, the click reaction was used to synthesize a small library of unique quinine dimers containing a triazole ring within the tether. The shortest tether combination of **Q2A** and **Q2′Z** resulted in a dimeric agent **Q(2,2′)** with two methylene units flanking either side of the triazole ring, whereas the longest tether combination resulted in **Q(8,8′)**. A representative synthesis of the clicked quinine dimer **Q(4,4′)** is shown in Figure 2. The azido ester, **Q4′Z**, and the alkynyl ester, **Q4A**, were synthesized from quinine with 5-azidopentanoic acid[29] and 6-heptynoic acid, respectively, using standard 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) coupling conditions. The click reaction between **Q4A** and **Q4′Z** was performed using copper sulfate and sodium ascorbate in a 1:1 dichloromethane (DCM)–water solvent mixture to afford **Q(4,4′)**. All clicked dimers were purified to homogeneity on silica gel and

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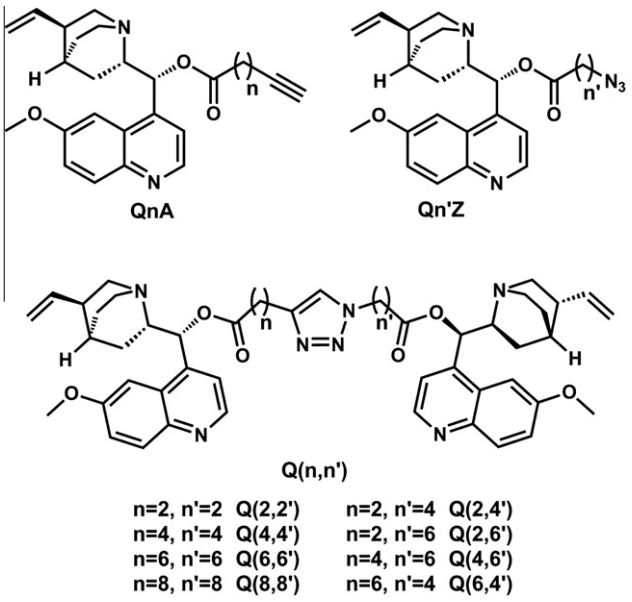


Figure 1. Structures of the alkyne- (**QnA**) and azide- (**Qn′Z**) modified quinine derivatives, and clicked quinine dimers **Q(n,n′)**.

characterized by mass spectrometry. Purity was assessed by reverse phase HPLC.[30]

To investigate the ability of the clicked quinine dimers to inhibit P-gp mediated efflux, calcein-AM accumulation assays were carried out using the MCF7/DX1 cell line. This sub-line is 200-fold more resistant to doxorubicin and has been shown to over express P-gp by immunoblot analysis.[21,31] Calcein-AM is a known P-gp substrate whose cellular internalization is possible only if P-gp efflux is inhibited.[32] Calcein-AM undergoes ester hydrolysis within cells generating fluorescent calcein. Therefore, flow cytometry can be used to quantify cellular fluorescence, and a measure of inhibitory activity can be obtained. As shown in Figure 3a, there is a marked increase in fluorescence upon incubating MCF7/DX1 cells with calcein-AM in the presence of the clicked quinine dimer **Q(6,6′)**.[30] The known P-gp inhibitor GF120918 was used as a positive control in these experiments.

As seen in Figure 3b all of the clicked dimers are capable of inhibiting P-gp in MCF7/DX1 cells with fairly similar potencies while being much more potent than quinine itself (IC_{50} ~100 μM). Maximal efficacy is observed with **Q(4,6′)**, **Q(6,4′)** and **Q(6,6′)**. Equipotent P-gp inhibition is observed with **Q(4,6′)** and **Q(6,4′)**, two dimers that differ only in the orientation of the triazole ring. As a consequence of these results, only tether length

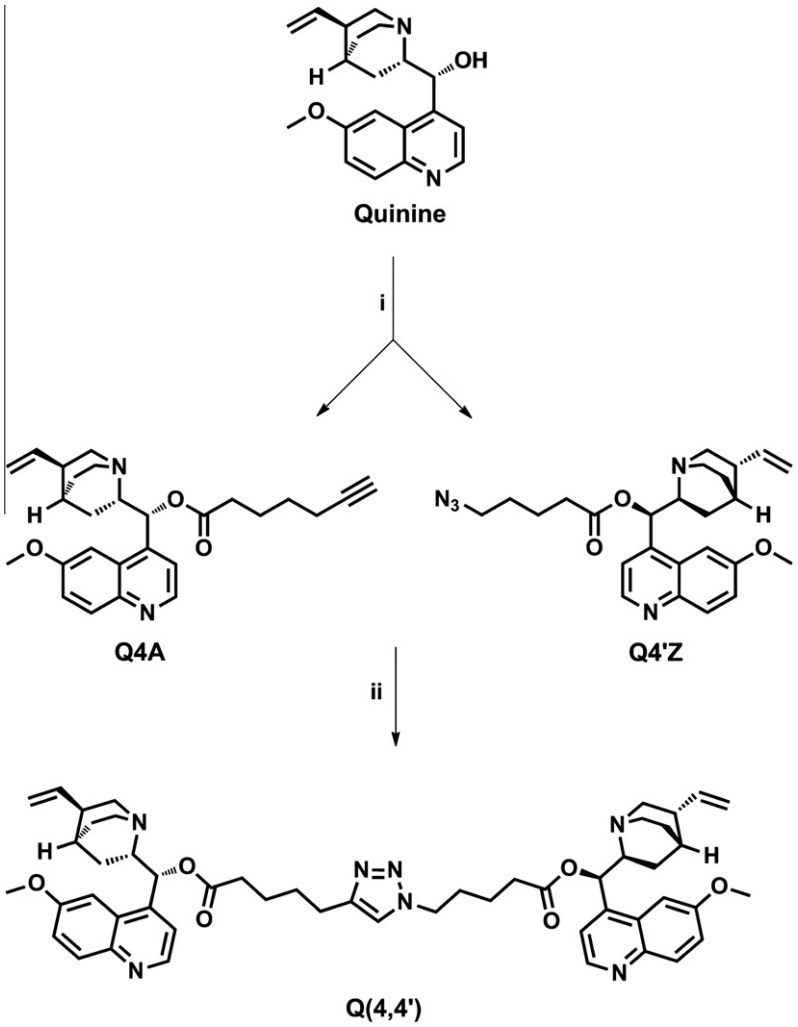
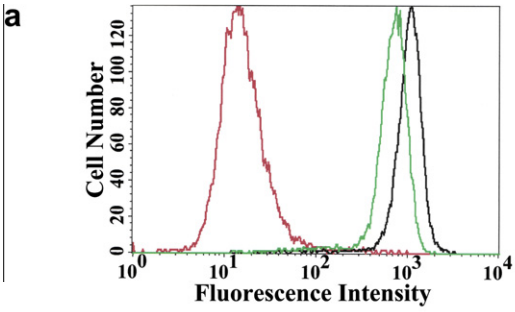


Figure 2. Representative synthesis for **Q(4,4′)** (i) 5-azidopentanoic acid (3 equiv) or 6-heptynoic acid (3 equiv), EDC (3 equiv), DIEA (6 equiv), DMAP (0.4 equiv), DMF, 12 h, 0 °C to rt, 92–94% yield; (ii) copper sulfate (5 mol %), sodium ascorbate (10 mol %), DCM/water (1:1), rt, 12 h, 54% yield.

a



b

	Q2′Z	Q4′Z	Q6′Z	Q8′Z
Q2A	6.1±0.2 μM	6.2±0.2 μM	3.6±0.1 μM	
Q4A		4.6±0.1 μM	2.9±0.1 μM	
Q6A		2.8±0.3 μM	2.7±0.2 μM	
Q8A				4.5±0.1 μM

Figure 3. (a) Histograms displaying MCF7/DX1 fluorescence upon addition of calcein-AM alone (red) and with **Q(6,6′)** (black, 5 μM) or GF120918 (green, 1 μM) and (b) IC_{50} values for P-gp inhibition with the clicked quinine dimer library. All experiments were performed in duplicate.[33] Histogram plot shows the relationship between fluorescence intensity (FL1 channel) and cell count.

combinations with differing numbers of total intervening methylene groups were studied further. When comparing the series with equivalent numbers of methylene units flanking the triazole ring, such as **Q(2,2′)**, **Q(4,4′)** and so on, optimum inhibition was found with the **Q(6,6′)** dimer. A 2.3-fold increase in potency was observed when the tether length was increased from **Q(2,2′)** to **Q(6,6′)**. This effect was lost upon further increasing the tether to **Q(8,8′)**. When comparing these data to P-gp inhibition with other quinine-based dimers, we observe approximately equipotent inhibition between a dimer with a central phenyl moiety in the tether, as compared to triazole-linked dimers.[21] However, the triazole-containing inhibitors, such as **Q(6,6′)**, were found to be greater than 10-fold more potent against P-gp when compared to quinine dimers with an intervening naphthalene unit in the tether.[21]

In conclusion, we have developed a synthetic protocol for the rapid assembly of a small library of quinine dimers employing click chemistry. These bivalent agents encompass a range of tether length, incorporating the triazole ring. This approach allowed us to explore the chemical space in the P-gp substrate binding region and study inhibitory trends. Using calcein-AM as the P-gp substrate in MCF7-DX1 cells, we found that while the activity is broadly similar, P-gp inhibition is maximum for dimers with 10 to 12 methylene units flanking the triazole ring. Inhibition was found to drop off on lengthening or shortening the tether length further. Given the tolerance of the triazole moiety in the P-gp substrate binding region, this strategy may potentially be extended to other P-gp drug substrates. Thus both homo- and hetero-clicked dimers can be envisioned to explore the integral membrane P-gp transporter region and potentially discover novel inhibitors and probes.

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30. The click reaction was performed for all dimers as follows for **Q(4,4′)**. In a 1:1 mixture of DCM and water (2 mL) was added **Q4′Z** (0.13 mmol) and alkyne **Q4A** (0.14 mmol) to make an approximately 0.05 M solution in each. Sodium ascorbate (10 mol %) in water (14 μL) was added, followed by copper sulphate (5 mol %) in water (7 μL). The reaction mixture was stirred for 12 h at room temperature. The reaction mixture was extracted with DCM, the organic layer was dried with $MgSO_4$, filtered and concentrated under vacuum. The crude reaction mixture was purified by flash silica gel column chromatography using 5% methanol/DCM to provide the desired product in approximately 55% yield. Purity was determined as 95% by analytical RP-HPLC with a retention time of 13.6 min using a 5–95% acetonitrile–water/0.05%TFA solvent system monitoring at 254 nm. MALDI $[M+H]^+ = 882.1$ (expected), $[M+H]^+ = 882.6$ (observed).
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33. To study the inhibitory activity of the clicked quinine dimers a calcein-AM substrate accumulation assay was performed using 125,000 MCF-7/DX1 cells in suspension (1 mL Basal Medium Eagle) that were incubated for 15 min at 37 °C with 0.5 μM calcein-AM in DMSO alone or with increasing concentrations of the clicked dimers in DMSO (10 μL), to give a final 1% DMSO concentration. GF120918 (l μM) was used as a positive control. The cells were then harvested by centrifugation, the media removed and the cells resuspended in ice cold PBS (pH7.4). The cells were analyzed using a FACS Calibur flow cytometer equipped with a 488 nm argon laser and a 530 band pass filter. Ten thousand cells were counted for each data point and histogram plots were obtained. The IC_{50} values for the clicked dimers were acquired from the concentration-dependent data that were fitted using Sigma Plot software.