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P-Glycoprotein-Dependent Trafficking of Nanoparticle-Drug Conjugates

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Supporting Information

Detailed synthetic/experimental methods and supporting data. Supporting Information is available on the WWW under <http://www.small-journal.com> or from the author.

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Resistance to chemotherapy contributes to treatment failure in over 90% of patients with metastatic cancer.^[1] Although, many tumors initially respond well to chemotherapies, selective pressure can lead to the proliferation and dissemination of cell subpopulations exhibiting either de novo or adaptive drug resistance, often accompanied by cross-resistance to a range of structurally diverse small molecule drugs. MDR1 P-glycoprotein (P-gp) is considered to be the most prevalent and single most important cause of multidrug-resistance (MDR) in humans,^[2] where the protein facilitates recognition, intracellular trafficking, sequestration,^[3] and/or cellular efflux of up to 50% of all cytotoxic chemotherapeutics (e.g. doxorubicin, paclitaxel, vinblastine, etoposide), as well as antibiotics (e.g. erythromycin, azithromycin, ketolides), and other therapeutic small molecules.

Gold nanoparticles are promising candidates for targeted anti-cancer drug delivery and laser photothermal therapy.^[4] Phase I clinical trials have been successfully completed for the former^[5] and human pilot studies^[6] are currently in progress for the latter, both for the treatment of solid tumors in the US. Likewise, gold nanoparticle-based biodiagnostic platforms are rapidly accelerating towards the clinic.^[4a,b,7] Although, the sequestration or efflux of small molecules by P-gp is well-documented, it is currently unclear whether P-gp plays a role in the cellular trafficking of nanoscale drug carriers. In prior work,^[8] we developed a gold nanoparticle delivery platform that preferentially targeted tumor stromal cells through surface presentation of macrolide small molecules, polarizing tumor associated macrophages towards an anti-tumor phenotype. Here, we use these novel nanoscale constructs to investigate the effects P-gp substrate presentation on the cellular trafficking of PEGylated gold-nanorods.

To investigate P-gp ligand-dependent cellular trafficking of nanoparticles, a series of colloidal gold nanorods were synthesized and conjugated with substrates of P-gp that exhibit varying degrees of susceptibility to P-gp-mediated efflux, as reported previously.^[9] Figure 1a illustrates the composition of these model nanoscale drug carriers, each comprised of $50 \pm 8 \times 13 \pm 2$ nm gold nanorods (Figure 1b) surface functionalized with mixed (9:1) self-assembled monolayers of thiolated poly(ethylene glycol) (PEG) and one of the three thiol PEGylated macrolide antibiotics: azithromycin (Zithromax®), clarithromycin (Biaxin®), or tricyclic ketolide (TE-802). These gold nanorod (AuNR) conjugates are abbreviated hereafter as Azith-AuNRs, Clarith-AuNRs, and TriKeto-AuNRs, respectively. The macrolide ligands were synthesized by 'N-alkynylation of the corresponding desmethyl desosamine analogs, followed by Cu-catalyzed Huisgen cycloaddition (click) using an azide-modified PEG-thiol (Supporting Data, Schemes S1–4). The gold nanorods were synthesized^[8,10] and conjugated^[11] as described previously (see Supporting Information for detailed methods). Photon correlation spectroscopy, laser Doppler electrophoresis measurements, and surface plasmon extinction spectra from the purified nanoparticle conjugates indicate stable surface ligation that was maintained in 10% serum-containing cell growth media over the time course of the experiments (Supporting Data, Figures S1,2).

Cellular uptake of the nanoparticle conjugates was assessed using a lung macrophage cell line previously shown to exhibit P-gp-dependent accumulation of macrolide compounds,^[9b]

where recognition has been shown to modulate pharmacokinetic and pharmacodynamic profiles of these drugs.^[12] Consistent with known tissue disposition profiles of these ligands in lung macrophage cells,^[13] macrolide-AuNRs exhibited dose-dependent accumulation in RAW264.7 cells that was significantly greater than PEGylated control nanoparticles (t = 24 h, Figure 1c), where trends in nanoparticle accumulation qualitatively agree with the reported efficacies of these drugs in treating drug-resistant infections.^[9c] Confocal microscopy of fluorescently-labeled nanoparticles (Figure 1d) further found that uptake and intracellular colocalization of the nanoparticles occurred in a manner consistent with that previously reported for both macrolide^[14] and P-gp^[9a] accumulation in phagocytic cells.

Ligand-dependent cellular accumulation of the macrolide-AuNRs was next assessed following concurrent incubation with the P-gp competitive inhibitors, cyclosporine and verapamil, using both P-gp-expressing and P-gp-null cell lines. No significant changes in the cellular accumulation of control PEG-AuNRs were observed following treatment with either inhibitor in P-gp(−) COLO 205 cells^[15] or in P-gp(+) J774.2 cells^[9a] (Figure 2a,b). In contrast, while P-gp-dependent cell accumulation of macrolide-AuNRs was not observed in P-gp-null COLO 205 cells, accumulation of Azith- and Clarith-AuNR was significantly increased in P-gp(+) J774.2 cells following competitive inhibition, with no significant changes in TriKeto-AuNR accumulation. These findings agree well with previous reports indicating i) macrolide-competitive P-gp binding by verapamil and cyclosporine,^[16] ii) diminished recognition of TriKeto (TE-802) by P-gp,^[9f] iii) enhanced in vivo efficacy of third-generation tricyclic ketolides,^[9d,9e] and iv) P-gp-dependent accumulation/cytotoxicity of free macrolide ligands (Supporting Data, Figure S3). These data suggest that P-gp-dependent trafficking can significantly affect the cellular accumulation of nanoscale drug carriers to which P-gp substrates are appended (e.g. cytotoxic chemotherapeutics,^[1,17] macrolide/ketolide antibiotics,^[9a,18] fluorescent imaging agents,^[18] etc.).

Concurrent optical dark-field scattering microscopy^[19] (DFSM) of nanoparticle- and P-gp inhibitor-treated cells (Figure 2c) further corroborate the findings in Figure 2a–b, showing augmented cellular accumulation of Azith- and Clarith-AuNRs in the presence of both P-gp inhibitors and no significant change in the accumulation of TriKeto-AuNRs in P-gp-expressing J774.2 cells. DFSM also illustrated that uptake of the nanoconjugates occurred in a manner competitive with the free ligands (Figure S4).

Finally, P-gp-dependent efflux of macrolide-AuNRs was quantitatively evaluated by DFSM live-cell imaging, as described previously.^[19b,20] Following incubation of P-gp(+) J774.2 cells with 0.01 nM Azith-AuNRs, cell solutions were replaced with verapamil-spiked growth media and nanoparticle efflux kinetics (i.e. cell associated surface plasmon scattering^[11b,21]) were monitored over time (Figure 3a). In the absence of competitive P-gp inhibition, nanoparticle efflux rates were notably slower than that previously reported for free azithromycin in J774.2 cells ($k = 0.14 \pm 0.03 \text{ h}^{-1}$ v. ca. 0.7 h^{-1} ,^[9a] respectively), in agreement with the notion that nanoparticle conjugation can mitigate multidrug resistance through decreased P-gp-dependent cell efflux (Figure 3b). With competitive P-gp inhibition, the rate of nanoparticle efflux was further decreased ($k = 0.036 \pm 0.01 \text{ h}^{-1}$), suggesting a

role for P-gp in ligand-dependent cellular trafficking of multivalent nanoparticle-drug conjugates.

P-glycoprotein is expressed on the plasma membrane, [9b-f] as well as in lysosomes, and in both early and recycling endosomes. [22] where it is known to mediate the cellular trafficking of small molecule drugs (host detoxification) through both transmembrane efflux [9f,23] and trapping in acidic vesicles, [3,24] respectively. Moreover, P-gp is also known to modulate cholesterol transport from the plasma membrane to the endoplasmic reticulum via endosomal recycling, [25] as well as plasma membrane reorganization through flippase-mediated depletion of cholesterol-interacting sphingomyelin, [25a,26] processes which could both significantly alter the intracellular trafficking of non-polar P-gp substrate compounds and their respective nanoparticle-drug conjugates. Confocal microscopy experiments (Figure 1d) found that both macrolide- and PEG-AuNRs co-localize in pericellular vesicles with fluorescently-labeled dextran, consistent with ligand-dependent (Figure 1c) endocytotic uptake of the nanoparticles and also in agreement with subcellular co-localization patterns previously reported for both P-gp [9a] and macrolides [14] in J774 cells. These findings suggest a partial role for P-gp-mediated lysosomal sequestration [3,24] or cholesterol trafficking [25,26] in the cellular accumulation of macrolide-AuNRs. P-gp substrate-competitive nanoparticle accumulation data (Figure 2a-c) showed that inhibition of P-gp increased the net accumulation of P-gp substrate-conjugated nanoparticles (Azith- and Clarith-AuNRs), while the lower affinity TriKeto conjugate was unaffected. DFSM efflux experiments further indicated a significant role for P-gp in the retention of the nanoscale conjugates, where competitive binding (inhibition) decreased both the total amount and rate of nanoparticle efflux. Again, the lack of P-gp competitive accumulation for TriKeto-AuNRs in Figure 2b relative to free TriKeto ligand in Figure S3 suggests a significant role for P-gp in the endosomal trafficking of multivalent nanoparticles. Together, accumulation and efflux data support a role ligand-dependent intracellular trafficking of nanoparticles bearing P-gp substrates. Although the effects observed here may be fully attributable to P-gp alone, inhibitors such as the verapamil [27] and cyclosporine [28] have also been shown to enhance autophagic vesicle formation that could either augment lysosomal trapping or impair endosomal recycling, partially contributing to the observed changes in cellular trafficking. The fact that changes in accumulation occurred in both a ligand- and cell phenotype-selective manner however, do support a significant role for the protein in the uptake and retention of nanoparticle-drug conjugates. While studies to determine the precise mechanism by which P-gp may facilitate direct/indirect cellular trafficking of nanoscale drug carriers are currently underway, subcellular localization, ligand-dependent accumulation, and efflux data provide important insights into these contributions and their relevance to nanoscale drug delivery.

In summary, we have demonstrated ligand-dependent accumulation and efflux of nanoscale drug carriers bearing substrate ligands for MDR1 P-glycoprotein (P-gp). Using PEGylated gold nanorod-drug conjugates, we found that the cellular accumulation of nanoparticles conjugated with substrates for P-gp was significantly enhanced following competitive inhibition of P-gp, while low-affinity P-gp substrate-conjugated nanoparticles were unaffected. Live-cell imaging experiments indicated that both the amount and rate of nanoparticle efflux could be significantly decreased following the administration of small

molecule inhibitors of P-gp. Although, the recognition and trafficking of numerous small molecule chemotherapeutics, antibiotics, and imaging agents by P-gp is well-described, interactions of the protein with nanoscale drug carriers is poorly understood. These findings suggest that nanoscale drug carriers incorporating P-gp substrates may benefit through minimization of surface presentation or the incorporation of P-gp inhibiting small molecules or siRNA.^[29] Rapid screening methods for P-gp-dependent cellular accumulation of newly developed nanoscale drug carriers may also contribute to improved chemotherapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

a) Illustration of model drug carriers used to investigate ligand-dependent cellular trafficking of nanoparticle-drug conjugates. Gold nanorods were functionalized with mixed self-assembled monolayers of thiolated poly(ethylene glycol) (PEG) and one of three thiol PEGylated substrates of P-gp that exhibit varying degrees of interaction with the protein. b) Spectroscopic and structural characterization (inset) of the nanoparticles indicating high purity and monodispersity by UV-Vis absorption spectroscopy and transmission electron microscopy, respectively. c) Cellular uptake of the nanoparticle conjugates in P-gp expressing RAW264.7 monocytes indicating concentration- and ligand-dependent cellular accumulation (24 h). d) Confocal fluorescence microscopy of dye-labeled nanoparticle conjugates (green) indicating co-localization with endo/lysosomal markers (red). L/T represents the intensity ratio between the longitudinal and transverse plasmon bands of the nanorods, a comparative metric which reflects the relative amount of impurity contributions from nanospheres. Error bars represent SD of ten technical replicates. Scale bar, (b) 50 nm, (d) 10 μm .

Figure 2.

P-gp- and ligand-dependent changes in nanoparticle accumulation following co-incubation with *P-gp*-competitive inhibitors. Net changes in nanoparticle accumulation in (a) *P-gp* null COLO 205 and (b) *P-gp*-expressing J774.2 cells incubated with *P-gp* substrate-conjugated gold nanorods and the inhibitor cyclosporine (red) or verapamil (blue). (c) Darkfield scattering microscopy of cells in (b) indicating ligand-dependent changes in particle accumulation in the presence of competitive *P-gp* inhibitors. Bright field images are overlaid in (c). Error bars represent SD of three biological replicates. P *0.05, **0.01. Scale bar, 10 μ m.

Figure 3. Real-time efflux kinetics of P-gp substrate-conjugated nanoparticles. J774.2 cells were loaded with 0.01 nM azithromycin-gold nanorods for 24 h, after which nanoparticle-containing media was removed and replaced by fresh media spiked with the P-gp-competitive inhibitor verapamil. a) Live-cell dark-field scattering microscopy and (b) quantitative image analysis of nanoparticle cell efflux kinetics in the presence and absence of verapamil. I_{SCA} represents the relative background-subtracted scattering intensity of the nanoparticles. Error in (b) is plotted as SEM of mean pixel intensity per field of view and rate constants represent mean $\pm$ SD of a one-phase exponential fit. Scale bar, 100 μm .