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Original article

A liposomal method for evaluation of inhibitors of H^+ — coupled multidrug transporters

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ABSTRACT

Introduction: This paper describes a novel technique, Fluorosomes, applied to investigating the interaction of antimicrobials with proton driven microbial efflux transporters. These transporters remove toxic compounds from the cytoplasm, including antibiotics and are involved in antibiotic resistance.
Methods: To assess transporter activity we developed a methodology to generate a proton gradient across Fluorosome membranes into which selected purified fully active efflux transporters were reconstituted. The interior of the Fluorosome particle (a unilamellar liposome) contains a fluorescent drug sensing probe whose fluorescence is quantitatively quenched by transporter substrates. Using an injecting fluorescence plate reader to initiate a proton gradient and to monitor subsequent fluorescence change, real time transport kinetics can be followed and transport inhibition characterized.
Results: Fluorosomes containing the *Escherichia coli* EmrE efflux pump demonstrated transport of two known EmrE substrates, ethidium and methyl viologen upon creation of a proton gradient. For Fluorosomes containing the inactive EmrE mutant, E14Q, no transport was observed. When the gradient was fully collapsed by the addition of nigericin, full inhibition of substrate transport was observed. The IC_{50} for nigericin inhibition of ethidium was shown to be 0.71 μM.
Discussion: We have for the first time prepared and validated a single bacterial efflux pump assay, Fluorosome-*trans*-EmrE, that faithfully mimics properties of the transporter in vivo. It is faster than whole cell screens, simple to use, amenable to robotics, and reports on very specific targets. We have demonstrated proof of principle with EmrE and have created the first of an intended series of proton driven Fluorosomes.

1. Introduction

Many antibiotics are ineffective because they are actively removed (effluxed) from the target organism by multidrug efflux transporters (MDTs) (Li, Plesiat, & Nikaido, 2015; Nikaido, 2009; Piddock, 2006; Poole, 2005). This is the case for bacteria such as *Escherichia coli* (Ec), a major Gram-negative pathogen responsible for enteric and urinary infections. Candidate inhibitors of the MDTs have been reported, but none of them have yet reached the market, probably because they are limited by their lack of selectivity and also that they inhibit specific mammalian pumps (Lomovskaya & Bostian, 2006; Lomovskaya et al., 2001; Nakayama et al., 2003; Opperman et al., 2014; Van Bambeke, Pages, & Lee, 2006). Here we provide a novel tool to tackle some of the existing problems by looking at the direct action of inhibitors on critical bacterial transport proteins.

We have adopted the Fluorosome platform to study proton driven bacterial transport systems. The Fluorosome method was originally developed to measure the passive diffusion of compounds across membrane bilayers (Fix & Melchior, 2002). It was then extended to determine the interaction of drugs with mammalian ATP-driven multidrug resistant pumps such as the p-glycoprotein (Melchior et al., 2012) and recently the ABCG2 (BCRP) and ABCB11 (BSEP) transporters.

To adapt the Fluorosome platform to proton driven bacterial pumps, we chose the EmrE transporter as a model transporter. Numerous efflux transporters in Gram-negative bacteria, especially the AcrAB–TolC system in EC and the MexAB-OprM system in *Pseudomonas aeruginosa* (Pa), have been studied as targets for the reversal of specific antibiotics (Lomovskaya & Bostian, 2006; Lomovskaya et al., 2001; Nakayama et al., 2003; Opperman et al., 2014; Van Bambeke et al., 2006). While in Gram-negative bacteria, the AcrAB–TolC (and MexAB-OprM) efflux systems are considered to be the major multidrug resistance transporters (MDTs) (Li et al., 2015; Nikaido, 2009; Piddock, 2006; Poole, 2005; Pos, 2009), it is estimated that in a bacterium such as Ec there are additionally 20 different proteins or protein complexes that function as MDTs, each of them conferring resistance to a slightly divergent, but partially overlapping set of drugs (Nishino & Yamaguchi, 2001). These proteins, based on amino acid sequence similarity, belong to five classes of transporters and have been designated as the: 1) ATP-binding cassette (ABC); 2) resistance nodulation–division (RND);

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3) multidrug and toxic compound extrusion (MATE); 4) small multidrug resistance (SMR); and 5) major facilitator (MFS) superfamilies (Li et al., 2015; Nikaido, 2009; Piddock, 2006; Poole, 2005). We have shown that such a large variety of these multidrug transporters (MDTs) work in a concerted mode rather than independently (Tal & Schuldiner, 2009). Transporters such as the EmrE, MdfA and MdtM supply the substrates to the AcrB–TolC complex — they move drugs from the cytoplasm to the periplasm or the outer leaflet of the membrane from where the AcrB–TolC complex expels them directly into the external medium (Tal & Schuldiner, 2009). The central role of the AcrB–TolC complex stems from the fact that it catalyzes the last step in a multistep process where a large variety of MDTs take part. Indeed, we have shown that in EmrE and mdfA double chromosomal knockouts, the AcrB–TolC complex cannot provide resistance to a spectrum of toxic compounds (Tal & Schuldiner, 2009). The specificities of the MDTs are very broad and overlap each other like the slats of a fan, so that they form a very strong and efficient barrier that prevents accumulation of a wide variety of xenobiotics. The rationale of our approach is to develop a medium to high throughput liposomal assay to identify inhibitors of MDTs that can be used as adjuvants to existing antibiotics. For the above reasons, we have chosen one of the above MDTs, the EmrE, as a model transporter to develop our new Fluorosome assay.

Criteria for a useful antimicrobial screening assay for inhibitors of antibiotic efflux pumps would include the following; specificity, speed, simplicity of instrumentation and application, amenability to robotics, high throughput and low cost. Using the EmrE as a prototype transporter, we have developed a novel screening platform that fulfills the abovementioned criteria. This assay also obviates analytical problems arising from the presence of a periplasmic space.

2. Materials and methods

2.1. Fluorosome manufacture

EmrE and its inactive mutant E14Q were isolated and, together with total *E. coli* lipids (Avanti Polar Lipids, Alabaster, AL), were reconstituted as proteoliposomes essentially as previously described (Yerushalmi, Mordoch, & Schuldiner, 2001) at a protein to lipid ratio of approximately 1.1 μg protein/mg lipid (wt/wt). These proteoliposomes were converted into Fluorosomes by procedures described in the literature (Melchior et al., 2012). For this study, Fluorosomes were manufactured in "Loading buffer" (0.15 M $(NH_4)_2SO_4$, 0.015 M Tris, pH 7.5). Fluorosome size was determined by photon correlation spectroscopy (PDDLS/Cool Batch, Precision Detectors, Franklin, MA) to be ~200 nm. Their bilayer integrity was confirmed by measurement of the passive permeability coefficients (PC) of test drugs amiloride (PC ~ 0.5×10^{-7} cm/s) and propranolol (PC ~ 12×10^{-7} cm/s).

2.2. Measurements

Measurements were made on Fluorosome-*trans*-EmrE, containing reconstituted wild type EmrE in their bilayers, and Fluorosome-*trans*-E14Q, containing reconstituted inactive mutant, E14Q, in their bilayers, with the drug sensor BSA–fluorescein (Sigma-Aldrich) in the aqueous interior of the Fluorosome. Transport was monitored in 384 well plates with a NOVOStar injecting fluorescence plate reader (BMG Labtech, Cary, NC). Measurements were made at 23 °C in well mode with excitation at 485 nm and emission monitored at 520 nm. To initiate proton driven substrate transport, 2.5 μl of Fluorosomes in Loading buffer were rapidly injected into 97.5 μl of "Reaction buffer" (140 mM K_2SO_4, 5 mM MgCl2, 10 mM Tricine, 10 mM Tris (pH 8.5)) containing either 25 μM ethidium plus 1 μM valinomycin or 500 μM methyl viologen as substrates. To quantitate the results, initial curve slopes (rate of substrate transport) were calculated after Fluorosome injection using BMG's Mars analysis package.

2.3. Compounds

Methyl viologen (paraquat), ethidium, nigericin, and valinomycin were purchased from Sigma-Aldrich. Methyl viologen and ethidium were added to buffer from water and nigericin and valinomycin from DMSO. The drug plate used for the nigericin IC_{50} study was formed using a SOLO Robotic Pipettor (Hudson Robotics, Springfield NJ). The IC_{50} value was calculated using Prism 5 software (GraphPad, San Diego, CA).

3. Results

3.1. Background

Fluorosomes are unilamellar vesicles containing selected transporters in their bilayers and drug sensors (i.e. BSA-fluorescein) in their aqueous interiors (Melchior et al., 2012). Fig. 1 illustrates the principles involved in measurements made for proton driven Fluorosome transport. In order to produce a Fluorosome system employing a proton gradient as the driving force for protein mediated transport, Fluorosomes were manufactured in "Loading buffer" (see Material and methods section). Use of this buffer results in the interior of the Fluorosomes containing ammonium in equilibrium with ammonia and protons. When the Fluorosomes are injected (diluted) into an ammonium-free medium "Reaction buffer" (Material and methods section), the free ammonia diffuses rapidly out of the Fluorosome interior, leaving behind a proton. This diffusion of ammonia out of the Fluorosome shifts the equilibrium promoting further generation of ammonia and protons, which in turn will diffuse out leaving behind further protons. Thus a proton gradient is created. EmrE utilizes the pH gradient thus generated to drive the uptake of a substrate in exchange for two protons (Schuldiner, 2009).

Two model substrates were used in this study, methyl viologen and ethidium (Schuldiner, 2009). Both of these compounds interact with the drug sensor BSA–fluorescein resulting in an immediate large decrease in fluorescence. In the case of the model substrate methyl viologen, a bilayer impermeant divalent cation, EmrE utilizes the pH gradient generated as described above to drive the uptake a molecule of methyl viologen in exchange for two protons. Fig. 2 shows in real time the fluorescence change as the substrate methyl viologen is transported into Fluorosome-*trans*-EmrE following Fluorosome injection into wells containing Reaction buffer. The consequent methyl viologen transport results in a large downward slope in fluorescence. This reflects the entrance of substrate into the aqueous interior of the Fluorosome, where it rapidly quenches the fluorescence of the sensor BSA–fluorescein contained in the particle (green and red lines). The change in fluorescence vs. time indicates the extent and speed at which transport occurs during a given run. In the absence of substrate transport, i.e. when the Fluorosome injection is made into a buffer with the same composition as the Loading buffer so that no pH gradient is generated (blue line), or when the gradient is collapsed by the addition of nigericin, an H^+ and K^+ ionophore, full inhibition of substrate transport is observed (brown line) as indicated by the lack of quenching. DMSO was added as a control (red line) because nigericin is added in DMSO.

To demonstrate the specificity of Fluorosomes containing EmrE, Fluorosomes were created under the same conditions as those employed in Fig. 2, but lacking EmrE. When injected into Reaction buffer, no fluorescence quenching was observed (not shown). Similarly, when Fluorosomes were prepared with the inactive EmrE mutant, E14Q, upon injection into Reaction buffer practically no substrate-induced quenching of the fluorophore was observed (Fig. 3).

We also tested transport of ethidium, a different model substrate, which is monovalent and more hydrophobic than methyl viologen. The transport of ethidium at 25 μM is shown in Fig. 4. The omission of valinomycin is seen to result in a reduced rate of transport. As the transport of monovalent substrates is electrogenic, this results in the