Video Article

Visualization of Bacterial Resistance using Fluorescent Antibiotic Probes

M. Rhia L. Stone[1], Wanida Phetsang[1], Matthew A. Cooper[1], Mark A. T. Blaskovich[1]

[1]Centre for Superbug Solutions, Institute for Molecular Biosciences, The University of Queensland

Correspondence to: Mark A. T. Blaskovich at m.blaskovich@imb.uq.edu.au

URL: https://www.jove.com/video/60743
DOI: doi:10.3791/60743

Keywords: Immunology and Infection, Issue 157, antibiotic, resistance, bacteria, efflux, fluorescent, microscopy

Date Published: 3/2/2020

Citation: Stone, M.R.L., Phetsang, W., Cooper, M.A., Blaskovich, M.A.T. Visualization of Bacterial Resistance using Fluorescent Antibiotic Probes. *J. Vis. Exp.* (157), e60743, doi:10.3791/60743 (2020).

Abstract

Fluorescent antibiotics are multipurpose research tools that are readily used for the study of antimicrobial resistance, due to their significant advantage over other methods. To prepare these probes, azide derivatives of antibiotics are synthesized, then coupled with alkyne-fluorophores using azide-alkyne dipolar cycloaddition by click chemistry. Following purification, the antibiotic activity of the fluorescent antibiotic is tested by minimum inhibitory concentration assessment. In order to study bacterial accumulation, either spectrophotometry or flow cytometry may be used, allowing for much simpler analysis than methods relying on radioactive antibiotic derivatives. Furthermore, confocal microscopy can be used to examine localization within the bacteria, affording valuable information about mode of action and changes that occur in resistant species. The use of fluorescent antibiotic probes in the study of antimicrobial resistance is a powerful method with much potential for future expansion.

Video Link

The video component of this article can be found at https://www.jove.com/video/60743/

Introduction

Antimicrobial resistance (AMR) is a rising crisis which poses a major threat to human health around the world. Resistance to most antibiotics has been reported, and infections caused by bacteria resistant to all clinically available drugs are emerging. In order to combat the rise of AMR, we need to increase our understanding of this multifaceted phenomenon and the underlying mechanisms and interactions between antibiotics and bacteria. One aspect that has been historically poorly understood is the permeation of antibiotics into bacteria, along with the phenomena of accumulation and efflux. This knowledge is crucial in designing new drugs and understanding mechanisms of resistance. Hence, this plays a critical role in AMR research.

There are two main approaches that may be taken in order to measure antibiotic concentration: measuring the drug directly or tagging with a moiety designed to facilitate quantification. Although tagging the antibiotic improves detection, this can perturb the biological activity of the drug, such as antimicrobial activity and permeability. This is not a problem for untagged methods; however, detection can be challenging. In the past few years, technological advancements have led to a boom in research utilizing mass spectrometry (MS) to directly measure the antibiotic concentration in bacteria[1,2,3,4,5,6,7]. These studies have shown that it is possible to study intracellular accumulation in a variety of bacteria, with gram-negative bacteria the most widely studied. Quantification of molecule permeability has then been linked to activity and used to inform drug development[2,3,4], though caution must be taken when directly conflating accumulation and target activity[5]. Prior to MS development, the only antibiotics whose concentration could be directly measured were those possessing intrinsic fluorescence, such as tetracycline and the quinolones[8,9,10,11]. Although obviously limited in scope, accumulation and efflux were examined and quantified, illustrating the usefulness of fluorescence-based quantification.

Tagged antibiotics have been used for many decades to study distributions, modes of action, and resistance, with radioactive and fluorescent tags being common. Radio-tagged probes have the advantage of being almost identical to the parent compound, hence the biological activity is unlikely to be significantly different. Isotopes such as ^{3}H, ^{14}C, and ^{15}N have been frequently used due to the prominence of these elements in antibiotics, and a variety of antibiotic scaffolds have been examined[1,10,12,13]. While the detection of radio-probes is simple, there are a number of logistical concerns (e.g., safety, isotope half-life) that have limited the use of this approach. Another strategy is fluorescently-tagged antibiotics. These probes can be used to examine the distribution and modes of action and resistance of the parent drug, using simpler technology than MS and without the logistical problems of radiation[8]. The main drawback to this approach is that antibiotics are generally relatively small molecules, hence the introduction of a fluorescent moiety poses a significant chemical change. This alteration can impact physiochemical properties and antibacterial activity. Therefore, care must be taken to assess these factors in order to generate results representative of the parent antibiotic.

In this work, a method is described to synthesize, assess, and use fluorescent antibiotics, as in our previous publications[14,15,16]. Through previous work, a number of fluorescent antibiotics have been prepared and used for a variety of purposes (see Stone et al.[8]). In order to minimize the likelihood of impacting biological activity, very small fluorophores are used in this work: nitrobenzoxadiazole (NBD, green) and 7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl (DMACA, blue). Further, the assessment of antibacterial activity using the microbroth dilution minimum inhibition concentration (MIC) assay is described, so that the effect of modifications on activity can be measured. These fluorescently-tagged probes can be used in spectrophotometric assays, flow cytometry, and microscopy. The range of possible applications is where the advantage

of fluorescent antibiotics lies. Cellular accumulation can be quantified, categorized, and visualized, something not possible using MS alone. It is hoped that the knowledge gained through the use of fluorescent antibiotics will aid in our understanding of resistance, and the fight against AMR.

Protocol

1. Synthesis of Alkyne-fluorophores

1. Synthesis of NBD-alkyne (7-nitro-*N*-(prop-2-yn-1-yl)benzo[*c*][1,2,5]oxadiazol-4-amine)
    1. Dissolve 1,031 mg of 4-chloro-7-nitro-benzofuran (5.181 mmol) in 60 mL of tetrahydrofuran (THF). Add 1,857 mg of $CsCO_3$ (5.696 mmol), then 0.39 mL of propargylamine (6.1 mmol). Heat the reaction to 50 °C for 2 h, which will turn from brown to green, then cool to room temperature (RT).
    2. Filter the reaction using a filtering aid (see **Table of Materials**), and wash with ethyl acetate (EA). Concentrate the filtrate under reduced pressure, then dissolve the residue in 150 mL of EA and move to a 500 mL separating funnel.
    3. Wash the EA solution with 100 mL with water and brine respectively. Then combine the aqueous phases and wash 2x with 100 mL of EA.
    4. Dry the combined organic phases over anhydrous magnesium sulfate, then filter and concentrate under reduced pressure.
    5. Purify the crude product by flash chromatography on silica gel (20–30% EA in petroleum ether [PE]), checking purity by liquid chromatography mass spectrometry (LCMS, $[M+H]^+$ = 219.1) and/or nuclear magnetic resonance (NMR) spectroscopy, chemical shifts as follows:
    ^{1}H NMR (CD_3OD, 600 MHz) δ 8.54 (d, *J* = 8.7 Hz, 1H), 6.35 (d, *J* = 8.4 Hz, 1H), 4.31 (dd, *J* = 5.7 Hz, *J* = 2.5 Hz, 2H), 2.43 (t, *J* = 2.5 Hz, 1H); ^{13}C NMR (CD_3OD, 150 MHz) δ 144.3, 143.7, 142.2, 135.8, 125.7, 100.0, 76.5, 74.2, 33.4.
    NOTE: When performing purification by silica gel chromatography, prepare the column using the less polar of the solvents listed. Crude compound may either be loaded as a concentrated solution or adsorbed onto the silica if solubility does not allow it. After the compound has been added to the top of the silica, run through 1–2 column volumes of the same solvent used for silica wetting. Then start with the solvent ratio listed, running through at least 1 column volume of each solvent, and making sure to not make large jumps in solvent composition. Collect fractions, and check purity/identity by LCMS or TLC (thin layer chromatography). Combine pure fractions and concentrate under reduced pressure by rotary evaporation.

2. Synthesis of DMACA-alkyne (2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)-*N*-(prop-2-yn-1-yl)acetamide).
    1. Dissolve 5.02 g of 3-(dimethylamino)phenol (36.6 mmol) in 30 mL of ethanol, then add 6.7 mL of diethyl 1,3-acetonedicarboxylate (36 mmol). Add 10.5 g of $ZnCl_2$ (77.2 mmol), then reflux the red solution for 42 h. Add an additional 9.20 g of $ZnCl_2$ (67.6 mmol), then reflux for 8 h.
    2. Cool the reaction and concentrate under reduced pressure. Disperse the resulting red solid in 200 mL of EA, filter, then transfer to a 500 mL separating funnel.
    3. Wash the EA with 200 mL each of water and brine, then dry over anhydrous magnesium sulfate. Filter the dried organic phase and concentrate under reduced pressure.
    4. Purify the red solid (ethyl 2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetate) by flash chromatography on silica gel (0–100% EA in PE), checking purity by LCMS ($[M+H]^+$ = 275.1) and/or NMR, chemical shifts as follows:
    ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.30 (d, *J* = 8.9 Hz, 1H), 6.52 (dd, *J* = 8.9 Hz, *J* = 2.6 Hz, 1H), 6.40 (d, *J* = 2.6 Hz, 1H), 5.87 (m, 1H), 2.96 (m, 8H), 2.25 (d, *J* = 1.2 Hz, 3H).
    5. Dissolve 488 mg of ethyl 2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetate (1.18 mmol) in 10 mL of THF, then add a solution of 157 mg of $LiOH{\cdot}H_2O$ (3.74 mmol) in 15 mL of water. Stir the reaction at RT for 3 h, then move to a separating funnel and dilute with an additional 50 mL of water.
    6. Wash the reaction mixture 2x with 50 mL of diethyl ether (Et_2O), then wash the combined organic phase 2x with 25 mL of water. Take any yellow precipitate with the organic layer. Concentrate the organic phase under reduced pressure using a rotary evaporator.
    7. Acidify the aqueous phase to pH = 2 with concentrated HCl, and cool to 4 °C overnight. Filter the acidified aqueous phase and add the yellow solid to the concentrated organic phase.
    NOTE: The 2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetic acid can be used without further purification, but this can be checked by LCMS ($[M+H]^+$ = 247.1) and/or NMR, chemical shifts as follows:
    ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.41 (d, *J* = 9.0 Hz, 1H), 6.62 (dd, *J* = 9.2 Hz, *J* = 2.8 Hz, 1H), 6.52 (d, *J* = 2.8 Hz, 1H), 5.98 (d, *J* = 0.9 Hz, 1H), 3.05 (s, 6H), 2.35 (d, *J* = 0.9 Hz, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 162.2, 155.7, 152.9, 152.8, 125.3, 109.7, 109.3, 109.1, 108.8, 98.3, 40.2, 18.5.
    8. Dissolve 466 mg of 2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetic acid (1.89 mmol) in 7 mL of dry *N*,*N*-dimethylformamide (DMF) and place under an atmosphere of nitrogen.
    9. Dissolve 0.33 mL of propargylamine (5.1 mmol) in 7 mL of dry DMF under nitrogen. Add 1.30 mL of di-isopropylethyl amine (DIPEA, 7.50 mmol) to the dye solution, then 535 mg of *O*-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU, 1.29 mmol). Stir the activated dye solution for 15 min at RT, then add the amine solution dropwise, and leave to stir overnight.
    10. The next day, dilute the reaction with 35 mL of water then concentrate under reduced pressure.
    11. Partition the resulting orange solid between EA and brine (100 mL each) in a 250 mL separating funnel. Separate the layers (run the two layers into different flasks), and wash the aqueous phase with 100 mL of EA.
    12. Concentrate the combined organic phases under reduced pressure, then redissolve the orange solid in 3 mL of 1:1 acetonitrile (ACN)/water (v/v). Purify the crude product by injecting onto a reverse phase medium pressure liquid chromatography (MPLC) system equipped with a C18 cartridge column (solvent A: water, solvent B: ACN).
    13. Check fractions for purity by LCMS ($[M+H]^+$ = 284.1, NMR chemical shifts given below), then combine and lyophilize appropriate fractions to give 2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)-*N*-(prop-2-yn-1-yl)acetamide, NMR chemical shifts as follows:

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.65 (t, *J* = 5.4Hz, 1H), 7.52 (d, *J* = 9.0Hz, 1H), 6.72 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.55 (d, J = 2.6 Hz, 1H), 6.00 (s, 1H), 3.88–3.87 (m, 2H), 3.62 (s, 2H), 3.13 (t, *J* = 2.5 Hz, 1H), 3.01 (s, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 167.7, 160.7, 155.4, 152.9, 151.0, 126.0, 109.4, 109.1, 108.1, 97.5, 80.9, 73.3, 39.7, 38.4, 28.2.

2. Synthesis of Fluorescent Antibiotics

1. Prepare an azide-derivative of an antibiotic as described previously[14,15,16].
   NOTE: The procedure is specific to each antibiotic and requires careful examination of the structure activity relationship (SAR) of the parent molecule to ensure that the functionalized antibiotic retains activity comparable to the parent. (e.g., ciprofloxacin[16], linezolid[14], and trimethoprim[15]). See **Figure 1** for examples of published fluorescent antibiotics, and the general synthesis scheme.
2. Click reaction procedure A
   NOTE: For most of the antibiotics, follow the procedure detailed here for copper catalyzed Huisgen [2+3] cycloaddition of azide (step 2.1) and fluorescent alkyne (prepared in step 1).
   1. Place the azide-antibiotic in a round bottom flask and add *tert*-butanol (^{t}BuOH) and water (1:1 v/v, 25 mL each per mmol azide).
   2. Add the fluorophore-alkyne prepared in step 1.1 (3 eq.) and heat the reaction to 50 °C. Then add copper sulfate (100 mM in water, 0.6 eq.) to the reaction, followed by ascorbic acid (500 mM in water, 2.4 eq.).
   3. Stir the reaction at 50 °C for 1 h, or until analysis by LCMS upon indication of the reaction completion (complete consumption of starting azide).
   4. Cool the reaction and purify as appropriate for the antibiotic scaffold and proceed for purification either by step 2.3 or 2.4.
      NOTE: Several different purification methods are possible, depending on the polarity and stability of the scaffold.

3. Click reaction procedure B
   NOTE: Follow this procedure for peptide-based antibiotics, to provide stronger reaction conditions (unpublished work, Phetsang, 2019).
   1. Place the peptide azide-antibiotic in a round bottom flask and add enough DMF (750 mL/mmol azide) to dissolve.
   2. Add the fluorophore-alkyne prepared in step 1 (5 eq.) and heat the reaction to 50 °C for 1 h.
   3. Add copper (I) iodide (20 eq.), then DIPEA (120 eq.), then acetic acid (240 eq.).
   4. Stir the reaction at 50 °C for 1 h, or until the analysis by LCMS indicates reaction completion (i.e., complete consumption of starting azide). Cool the reaction and proceed for purification by method 1 (see step 2.3).

4. Purification method 1 (used for ciprofloxacin, trimethoprim, and linezolid)
   1. Inject the cooled click reaction directly onto a MPLC C18 cartridge column.
   2. Incorporate a long wash phase (roughly 10 min) at the beginning of the run (100% solvent A), then run a gradient up to 100% solvent B, followed by a return to solvent A.
      NOTE: Solvent A can be chosen from water, 0.05–0.1% formic acid (FA) in water, 0.05–0.1% trifluoroacetic acid (TFA) in water, depending on solubility, stability, and the best resolution of peaks. Solvent B can be chosen from acetonitrile (ACN), 0.05–0.1% FA in ACN, 0.05–0.1% TFA in ACN, to match solvent A. If elution proves difficult, methanol may be used in place of ACN.
   3. Collect and combine appropriate fractions, as indicated by LCMS and color (correct mass seen, singular peak), then lyophilize to give the (semi)pure fluorescent antibiotic.
   4. Further purify the product if needed. Assess purity by NMR and/or LCMS and high-pressure liquid chromatography (HPLC), using a column and method appropriate for the scaffold.

5. Purification method 2
   NOTE: If solubility allows, prepurification may be carried out by the aqueous workup (used for macrolides, unpublished work, Stone 2019).
   1. Dilute the cooled click reaction with water and Et_2O (1:1 v/v, approximately 10-fold dilution from initial reaction volume), transferring to an appropriately sized separating funnel.
   2. Separate the layers and wash the aqueous phase twice with Et_2O.
   3. Wash the combined organic phases 2x with water (equal volume with organic phase), then dry over Na_2SO_4.
   4. Filter the dried organic phase and concentrate under reduced pressure.
   5. Purify the crude product by MPLC and/or HPLC as described in step 2.4.4.
      NOTE: See **Figure 2** for examples of incomplete, complete, and purified click reaction LCMS traces. Typical purified yields for the antibiotic-fluorophore click reactions range from 30–80%.
      CAUTION: Most of the chemicals used in these syntheses possess specific safety hazards. Caution must be taken at all times, including the use of personal protective equipment. Et_2O, ^{t}BuOH, FA, acetic acid, PE, EA, THF, ACN, DMF, EtOH, DIPEA, propargylamine, and HCTU are all flammable; avoid contact with heat or spark sources. THF, propargylamine, DIPEA, ^{t}BuOH, FA, DMF, and PE are all toxic; avoid exposure. Propargylamine, $CsCO_3$, $ZnCl_2$, LiOH-H2O, DIPEA, CuI, FA, acetic acid, and HCl are all corrosive; avoid contact and be aware of surface contact. $ZnCl_2$, $CuSO_4$, CuI, and PE present environmental hazards; be mindful of disposal conditions. THF can form explosive peroxides; take care with storage conditions. Organic azides are explosive; take care especially with large scale production.

3. Evaluation of Antimicrobial Activity

NOTE: All work involving bacteria should be carried out under sterile conditions to avoid contamination of either the assay or the laboratory. All media should be autoclaved before use, and plasticware and equipment such as pipettes must be kept sterile. It is recommended that work be done in a biocontainment hood (type 2).

1. Streak glycerol stocks of bacterial strains appropriate for the antibiotic scaffold onto lysogeny broth agar (LB, prepared per manufacturer's instructions), and grow overnight at 37 °C.

NOTE: The choice of bacteria to test antibacterial activity must be made based on the antibiotic scaffold being used. A representative range of 5–10 bacteria should be chosen from the species that are known to be susceptible to the antibiotic, with consideration given to the logistical capabilities of the lab. If possible, resistant bacteria should also be tested. The protocol given below will work on most bacteria, but check if special conditions are required (e.g., CO_2, special media) and make alterations as necessary. Bacteria successfully assayed using these conditions include *Staphylococci, Streptococci, Bacilli, E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Enterococcus faecium*.

2. Pick a single colony from the plate, and culture overnight in 5 mL of cation adjusted Mueller-Hinton Broth (CAMHB, prepared per manufacturer's instructions) at 37 °C.
3. Dilute the overnight cultures ~40-fold in CAMHB and grow to a mid-log phase, optical density at 600 nm (OD_{600}) = 0.4–0.8, volume 5 mL).
4. Prepare stock solutions of each fluorescent antibiotic at 1.28 mg/mL in sterile water, and pipette 10 µL of antibiotic to the first column of a 96 well plate.
5. Add 90 µL of CAMHB to the first column and 50 µL to all other wells. Then, perform serial 2-fold dilution across the plate.
6. Thoroughly mix, then dilute the mid-log phase cultures to ~10^6 colony forming units (CFU)/mL and add 50 µL to all wells, to provide a final concentration of ~5 x 10^5 CFU/mL.
   volume of culture (mL) = (media volume in mL)/(OD_{600} x 1,000)
   e.g., for an OD_{600} = 0.5 culture in a desired media volume of 12 mL, add (12/(0.5 x 1,000) = 0.024 mL of culture to 12 mL of media
7. Cover the plates with lids and incubate at 37 ˚C for 18–24 h without shaking.
8. Visually inspect the plates, with the MIC being the lowest concentration well with no visible growth.
   NOTE: See **Table 1** for some examples of active and inactive fluorescent antibiotics.

4. Analysis of Probe Accumulation by Spectrophotometry and Flow Cytometry

NOTE: These centrifugation times have been optimized for *E. coli*, so slight alterations may be needed for other species. Representative data for probe accumulation is reported for the NBD-labeled ciprofloxacin probe.

1. Streak glycerol stocks of the bacterial strains onto LB agar and grow overnight at 37 °C.
2. Pick a single colony from the plate and culture overnight in LB at 37 °C.
3. Dilute overnight cultures ~50-fold in media and grow to mid-log phase (OD_{600} = 0.4–0.8).
4. Centrifuge the cultures at 1,470 x *g* for 25 min and decant the media.
5. Resuspend the bacteria in 1 mL of phosphate-buffered saline (PBS), then centrifuge at 1,470 x *g* for 15 min.
6. Decant the media and resuspend the washed pellets in PBS to a final OD_{600} = 2.
7. If desired, add 10.1 µL of carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 mM in PBS) to 1 mL of bacteria (final concentration 100 µM) and incubate at 37 ˚C for 10 min.
   NOTE: CCCP is an efflux pump inhibitor. Addition of CCCP will allow the examination of the impact of efflux.
8. Centrifuge the cultures at 18,000 x *g* for 4 min at 20 °C and decant the media.
9. Add 1 mL of fluorescent antibiotic solution (10–100 µM in PBS) to the pellet, and incubate at 37 °C for 30 min.
10. Centrifuge the cultures at 18,000 x *g* for 7 min at 4 °C and decant the media.
11. Resuspend the bacteria in 1 mL of cold PBS, and repeat step 4.9.
12. Repeat step 4.10 a total of 4x.
13. If desired, lyse bacteria by adding 180 µL of lysis buffer (20 mM Tris-HCl, pH 8.0, and 2 mM sodium EDTA) then 70 µL of lysozyme (72 mg/mL in H_2O).
14. Incubate at 37°C for 30 min, then freeze-thaw 3x (−78 °C for 5 min, then 34 °C for 15 min).
15. Sonicate the samples for 20 min, then heat to 65 °C for 30 min.
16. Centrifuge the lysed samples (18,000 x *g*, 8 min), then filter through a 10 kDa filter membrane.
17. Wash the filter the 4x with 100 µL of water.
18. Transfer the lysate to a flat-bottom black 96 well plate and measure the fluorescence intensity on a plate reader with excitation and emission wavelengths appropriate for the fluorophore (i.e., DMACA: λ_{ex} = 400 nm, λ_{em} = 490 nm; NBD: λ_{ex} = 475 nm, λ_{em} = 545 nm).
    NOTE: See **Figure 3** for examples of spectrophotometric analyses of bacteria using the fluorescent NBD-labeled ciprofloxacin antibiotic.
19. For analysis by flow cytometry, the same growth and staining conditions may be used (steps 4.1–4.17), with changes solely in the final preparation.
    1. Bring the total volume to 1 mL of PBS.
    2. Read samples on a flow cytometer at a flow rate of approximately 60 µL/min, using logarithmic amplification for the data acquisition (F1 excitation = 488 nm; emission = 525/20 nm).
    3. Record a total of 10,000 events, then analyze the data using appropriate software.
    4. Plot the fluorescence intensity from F1 against the number of events counts, estimating the median fluorescence intensity from the histogram peaks after the bacteria was stained.
       NOTE: See **Figure 4** for examples of flow cytometry analyses of bacteria using the NBD-labeled ciprofloxacin antibiotic.

5. Preparation for Microscopic Analysis

1. Grow subcultures to OD_{600} = 0.4, as for MIC assessment, then divide into 1 mL aliquots and centrifuge at 18,000 x *g* for 3–5 min.
2. Decant and discard media, then resuspend bacterial pellet in 500 µL of HBSS.
3. Centrifuge at 18,000 x *g* for 3 min, then decant and discard media.
4. Prepare solutions of antibiotic probes in HBSS at concentrations of 1–100 µM.
5. Resuspend the washed bacteria in 500 µL of the probe solution, and incubate at 37 °C for 30 min.
6. Repeat step 5.3. For a multiple labeling experiment, resuspend the pellet in 500 µL of an orthogonally colored nucleic acid dye. For green, use Syto9 (5 µM in HBSS); for blue, use Hoechst 33342 (20 µg/mL in HBSS). Incubate at RT for 15–30 min.
7. Repeat step 5.3, then resuspend in 500 µL of FM4-64FX (5 µg/mL in HBSS) and incubate at RT for 5 min.

8. Repeat step 5.3, then resuspend in 500 µL of HBSS, and repeat step 5.3 once more.
9. Repeat step 5.8, then finally suspend the washed, dyed bacteria in 15 µL of mounting medium (see **Table of Materials**).
10. Pipette mounting medium on a microscope slide and top with a high-performance cover slip, then seal edges with clear nail polish.
NOTE: See **Figure 5** for examples of confocal microscopy images taken with NBD-labeled ciprofloxacin and trimethoprim antibiotics, and **Figure 6** for DMACA-labeled oxazolidinone (linezolid) antibiotic.

Representative Results

Figure 1 Illustrates the key click chemistry reaction (**A**) for the preparation of the fluorescent antibiotics, and with (**B**) examples of structures of our published fluorescent antibiotics based on ciprofloxacin (cipro), trimethoprim (TMP), and linezolid. These probes were all synthesized from the corresponding antibiotics via an azide intermediate. They were then coupled to the NBD and DMACA fluorophores, each functionalized with an alkyne.

Figure 2 shows example LCMS traces from a ciprofloxacin-N_3 and NBD-alkyne click reaction, where the azide eluted at 3.2 min and the product at 3.8 min. Comparing **1** and **2** shows how the progress of the click reaction could be followed by the disappearance of the azide peak (by UV or MS detector). Spectra **3** demonstrate the impact of purification, with erroneous peaks disappearing from the MS and UV traces. Both purity and reaction progress could be quantified by the integration of the product peak and any impurity peaks.

Figure 3 demonstrates typical results from the assessment of intracellular accumulation by fluorescence spectroscopy in the presence and absence of efflux. In this experiment, *E. coli* was treated with TMP-NBD with or without the addition of CCCP, which collapses the proton motive force (PMF). The intracellular fluorescence of the bacteria was significantly higher when pretreated with CCCP, indicating that efflux reduced the accumulation in these bacteria. This experiment was repeated using bacteria deficient in *tolC*, displaying the capacity of this assay to examine the impact of individual efflux pump components. In this case, although there was an increase in intracellular fluorescence compared to the wild type bacteria, CCCP accumulation still increased. These findings indicate that *tolC* takes part inTMP efflux but is not the sole PMF-drive pump involved.

Figure 4 shows the result of the same experiment as **Figure 2**, but with the accumulation measured by flow cytometry instead of spectroscopy. The same data trends were observed, demonstrating that either technique may be used to study the phenomenon of efflux mediated intracellular accumulation.

Figure 5 shows representative confocal microscopy images of gram-positive (*S. aureus*) and gram-negative bacteria (*E. coli*) labeled with TMP-NBD (**1**) and cipro-NBD (**2 + 3**) fluorescent probes, respectively. In both cases, the red membrane dye FM4-64FX was added in order to compare co-localization. For TMP-NBD, the blue nucleic acid dye Hoechst-33342 was also used. By overlaying these images, the localization of the antibiotic in the bacteria was visualized. Comparing panels **2** and **3** shows how the impact of efflux was examined, with the efflux inhibitor CCCP used in **2**, resulting in intracellular accumulation. In panel **3**, no CCCP was added. Hence, efflux is active and no probe accumulation was seen.

Figure 6 shows representative confocal microscopy images of Gram-positive (*S. aureus*) bacteria labeled with DMACA-labeled oxazolidinone probe Lz-NBD. The red membrane dye FM4-64FX was added in order to compare co-localization, and the green nucleic acid dye Hoechst-33342 was also used. By overlaying these images, the localization of the antibiotic in the bacteria was visualized, showing internal localization distinct from the membrane and nucleic acid.

Table 1 shows MIC values for three series of fluorescent antibiotics, ciprofloxacin, trimethoprim (TMP), and linezolid (Lz), with data presented for the parent antibiotic, NBD and DMACA derivatives of each. Representative species for each antibiotic were chosen, including both gram-positive and gram-negative. For the ciprofloxacin series, both fluorescent probes lost antibiotic activity compared to the parent drug, but retained some activity against all species. Similarly, the linezolid probes lost some activity, but remained a moderate to weak antibiotic. The TMP probes lost almost all activity against wild type bacteria, but were active against efflux deficient *E. coli*, indicating that the loss of antibacterial activity was due to lack of accumulation.

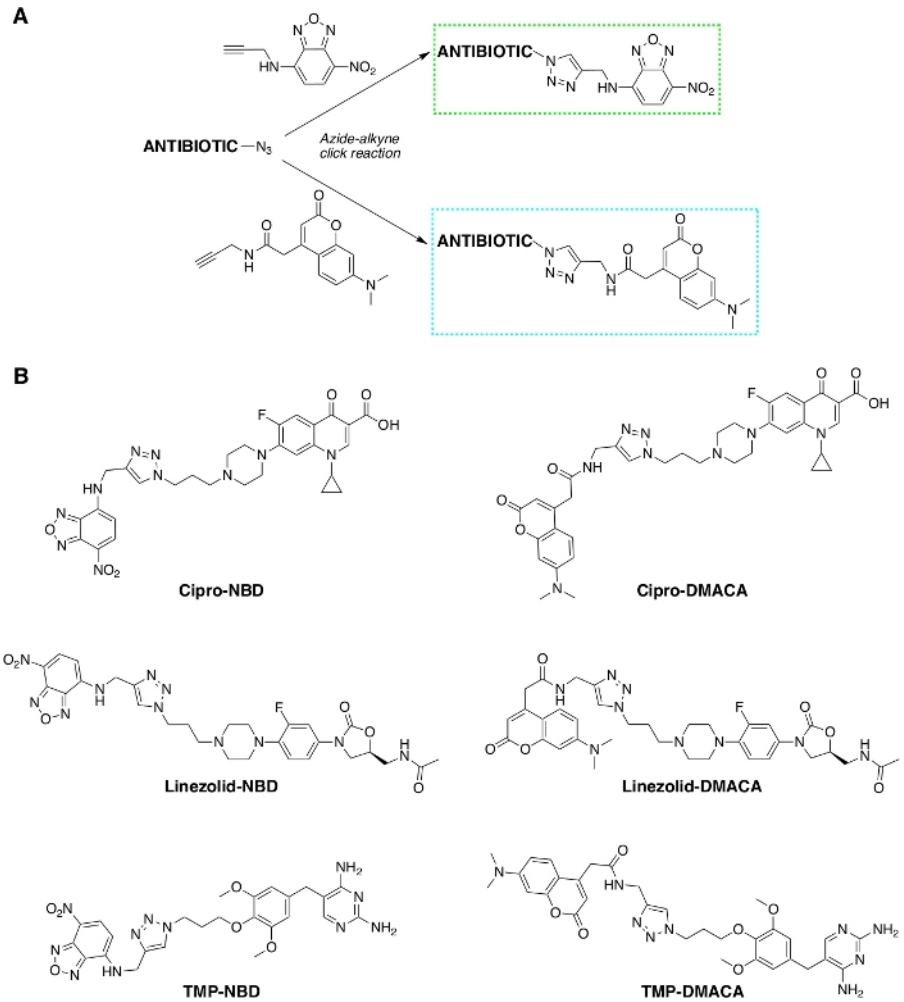

Figure 1: Synthesis and structures of antibiotic-derived probes. (**A**) The general reaction scheme for the synthesis of fluorescent antibiotic probes from azide-antibiotics and alkyne-fluorophores. (**B**) The structures of our published probes based on ciprofloxacin, trimethoprim, and linezolid. Please click here to view a larger version of this figure.

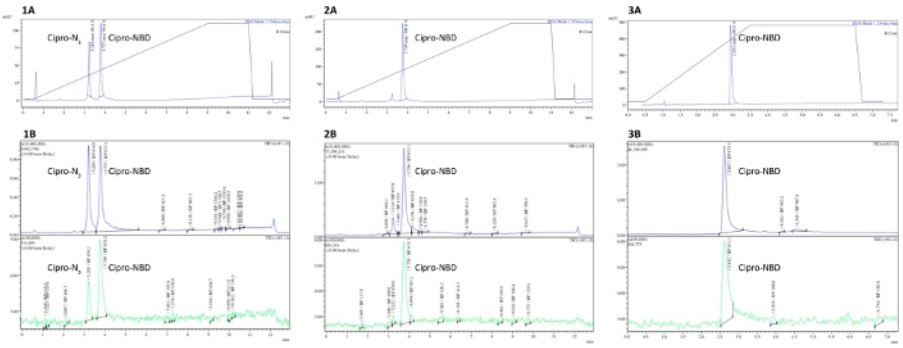

Figure 2: Measurement of antibiotic-derived probe purity by LCMS. Analytical LCMS traces from (1) incomplete, (2) complete, and (3) HPLC purified ciprofloxacin-N_3 + NBD-alkyne click reactions demonstrating the disappearance of starting material upon reaction completion, and miscellaneous peaks on purification. A = UV-Vis trace (absorbance at 250 nm), B = MS trace (positive and negative mode). Please click here to view a larger version of this figure.

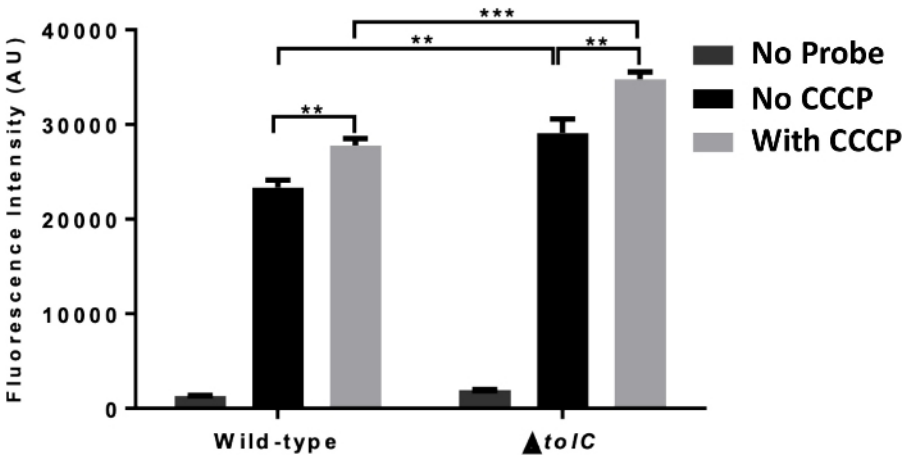

Figure 3: Plate reader measurement of antibiotic-derived probe accumulation. Fluorescence spectroscopic measurement of cellular accumulation of TMP-NBD (50 µM) in wild type (1, ATCC 25922) and Δ*tolC* (2, ATCC 25922) *E. coli* incubated (**A**) with and (**B**) without addition of CCCP (100 µM). Statistical significance (**p ≤ 0.01; ***p ≤ 0.001) is shown between the absence or presence of CCCP and between wild type and Δ*tolCE. coli*. Data reported are the mean ± SD for three experiments. This figure is adapted from our previous publication[15], and illustrates the use of spectroscopy to elucidate the role of efflux on intracellular accumulation. Please click here to view a larger version of this figure.

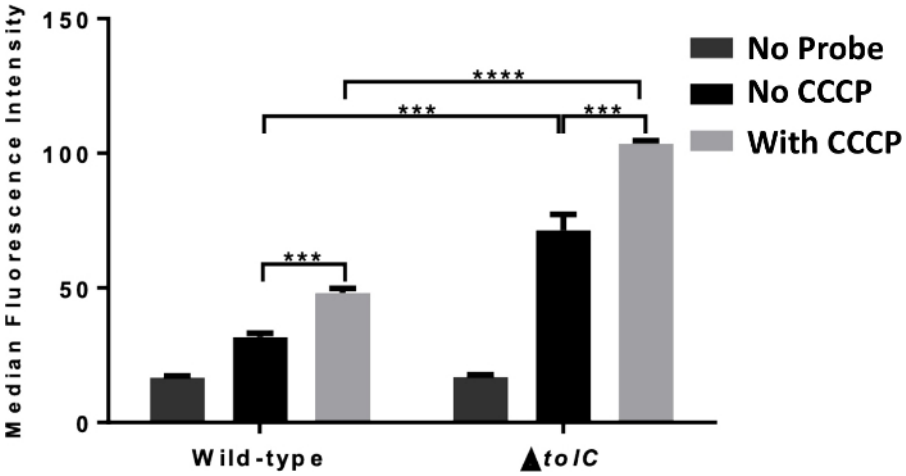

Figure 4: Flow cytometry measurement of antibiotic-derived probe accumulation. Flow cytometry measurement of cellular accumulation using TMP-NBD in in wild type (1, ATCC 25922) and Δ*tolC* (2, ATCC 25922) *E. coli* incubated with and without addition of CCCP (100 µM). Median fluorescence activity is shown from 10,000 bacterial events, Statistical significance (***, p ≤ 0.001; ****, p ≤ 0.0001) is shown between the absence and presence of CCCP and between wild type and Δ*tolCE. coli*. Data reported are the mean ± SD for three experiments. This figure is adapted from our previous publication[15], and illustrates the use of flow cytometry to elucidate the role of efflux on intracellular accumulation. Please click here to view a larger version of this figure.

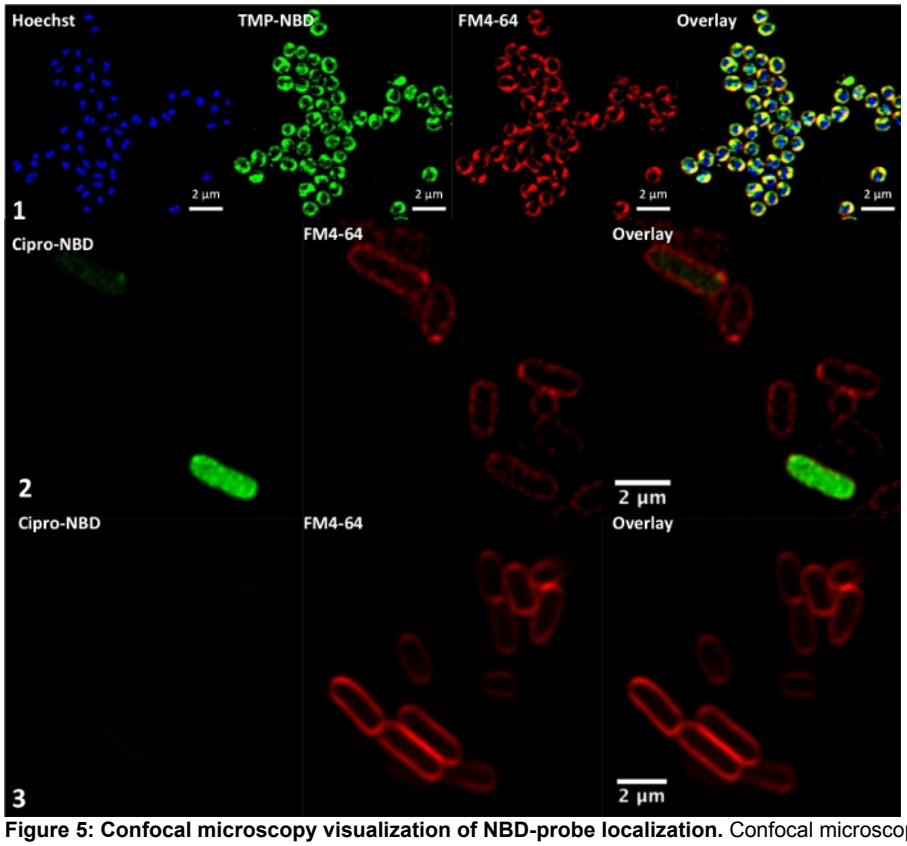

Figure 5: Confocal microscopy visualization of NBD-probe localization. Confocal microscopy images of 1) live *S. aureus* labeled with Hoechst-33342 (blue, nucleic acid), TMP-NBD (green), FM4-64FX (red, membrane), and overlaid; 2) live *E. coli* treated with CCCP (efflux inhibitor) labeled with cipro-NBD (green), FM4-64FX (red, membrane), and overlaid; 3) live *E. coli* labeled with cipro-NBD (green), FM4-64FX (red, membrane), and overlaid. This figure is adapted from our previous publications[15,16], and illustrates the use of microscopy to examine probe localization, including the impact of efflux. Please click here to view a larger version of this figure.

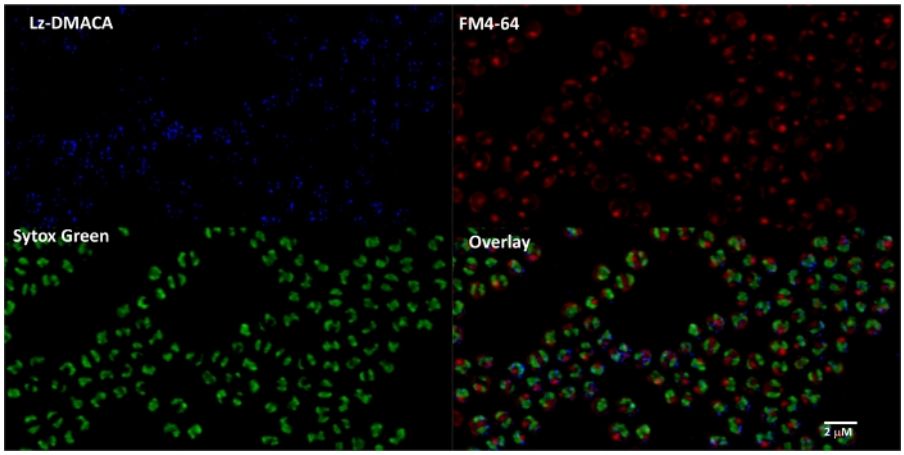

Figure 6: Confocal microscopy visualization of DMACA-probe localization. Confocal microscopy images of live *S. aureus* labeled with oxazolidinone probe Lz-DMACA (blue), Sytox green (green, nucleic acid), and FM4-64FX (red, membrane). Please click here to view a larger version of this figure.

		MIC (μg/mL)								
Species	**Strain**	**Cipro**	**Cipro-NBD**	**Cipro-DMACA**	**TMP**	**TMP-NBD**	**TMP-DMACA**	**Linezolid (Lz)**	**Lz-NBD**	**Lz-DMACA**
Staphylococcus aureus	ATCC 25923	0.125 - 0.5	32 - ≥64	16	1	16	>64			
	ATCC 43300							1	16	>64
Streptococcus pneumoniae	ATCC 700677							1	4	64
Enterococcus faecium	ATCC 35667	1 - 8	32	32 - ≥64						
	ATCC 51559							2	16	32
Klebsiella pneumoniae	ATCC 13883	0.015 - 0.06	8 - 16	8 - 32						
Pseudomonas aeruginosa	ATCC 27853	0.25 - 1	32 - ≥64	32 - ≥64						
Escherichia coli	ATCC 25922	≤0.004	8	2	0.5	>64	>64			
	Mutant ΔtolC				0.125	0.25	2			

Table 1. Antibiotic activities of fluorescent antibiotic probes based on ciprofloxacin, trimethoprim, and linezolid against appropriate clinically relevant bacterial strains, as measured by broth microdilution MIC assays. In most cases, the probes lost some activity compared to the parent drug, but retained some measurable antibiotic potency (sufficient to be useful in further studies).

Discussion

The creation of a successful fluorescent antibiotic probe must begin with careful planning and consideration of the SAR of the parent drug. If the SAR is not known or fully explored, several options may need to be tested to find a site which may be selectively modified without abolishing biological activity. Once a site/s have been identified, the installation of a linker moiety is often essential in order to provide steric spacing between the biological site of action and the inactive fluorophore. Care must be taken that the reaction used to attach the linker to the antibiotic leaves a bio-stable functional group, avoiding, for example, esters that are susceptible to cleavage by esterases in vivo. Depending on the pharmacodynamic and pharmacokinetic profile of the antibiotic, a simple alkyl linker may be used, or else a less lipophilic option such as a polyethylene glycol (PEG) linker should be considered. With the linker attached, the antibacterial activity should be assessed to ensure the MICs against relevant bacteria are similar to the parent compound.

In this work, we recommend the use of Huigsen azide-alkyne [3+2] dipolar cycloaddition (click chemistry, see **Figure 1**) to ligate fluorophore to antibiotic, for a number of reasons. Click reactions are highly selective, meaning that protection of reactive groups on the antibiotic is not necessary, and further, the reaction leaves a stable, biocompatible triazole moiety. The azide component is introduced to the antibiotic portion in our procedures, as this is generally more easily accomplished with a variety of structural types than introduction of an alkyne. The syntheses of two alkyne-derivatized fluorophores are described here, though others could be explored if desired. NBD and DMACA were chosen due to their small size, minimizing the possibility of interfering with cell penetration and target interaction. The click reaction itself is carried out using copper catalysis, where either Cu^{2+} ($CuSO_4$, with an ascorbic acid reducing agent) or Cu^{+} (CuI) may be used as the starting reagent. Following purification (**Figure 2**), the MICs should then be tested as with the azide. Even with careful consideration of fluorophore choice and site of attachment, it is possible that poor antibiotic activity will be observed. This does not, however, mean that an inactive probe is without use. As shown with the TMP probes, compounds with poor antibacterial activity may still bind to the same target as the parent drug. This can enable studies on the mode of action and examination of phenomena leading to resistance, such as efflux.

As outlined in the protocols section, it is possible to analyze bacterial labeling by the fluorescent antibiotics using either a simple spectrophotometry assay (**Figure 3**) or flow cytometry (**Figure 4**). Both methods are capable of quantifying cellular accumulation, and by lysing cells and examining fluorescence localization in lysate, it is possible to assess intracellular accumulation. In this protocol, the use of lysozyme for cell lysis is described, as this is a rapid, universal technique. Other lysis conditions, such as overnight treatment with glycine-HCl[7], have also been successfully used. Using this technique, it is possible to study the impact of efflux on antibiotic cellular accumulation, which is a major mechanism of resistance. If efflux is indeed present in the bacteria, a lack of intracellular accumulation will be observed, though this can be rescued using an efflux inhibitor like CCCP.

Microscopy may also be carried out to visually inspect probe localization in different bacteria, garnering information on mode of action, and potentially also resistance (see **Figure 5** for representative examples). In order to see localization within bacteria, a high resolution confocal microscope is required, equipped with capabilities such as SIM (structured illumination microscopy), SR-SIM (superresolution-SIM), Airyscan, or STED (stimulated emission depletion). Furthermore, high-performance cover slips should be used, and post-imaging analysis carried out on an appropriate software (e.g., FIJI, Zen, or Imaris). Localization of probes is compared to dyes that stain specific architectures, such as Hoechst-33342 (blue, nucleic acid), Syto-9 (green, nucleic acid), and FM4-64FX (red, membrane). The choice of dyes should be made to match the fluorescent antibiotic, so that each color used has minimal spectral overlap. In order to obtain the best possible images, optimization may be

required. For example, if bacteria are too crowded on the slide, take only part of the suspended pellet, then dilute with more mounting medium. In contrast, if bacteria are too sparse on the slide, simply start with more bacteria. In this protocol, the use of a thermo reversible gel that is compatible with live cells (e.g., Cygel) is recommended for live cell imaging, as it immobilizes bacteria (including motile bacteria), but other mounting media or agarose have also been successfully used.

Overall, despite challenges that may be faced in the preparation of a biologically active fluorescent antibiotic derivatives, the simplicity of their use and their versatility make these probes attractive tools for research in AMR. Future work using fluorescent antibiotics has the potential to provide insight into mechanisms of antibiotic resistance, improve our understanding of how current antibiotics operate, and aid the development of better drugs.

Disclosures

The authors have nothing to declare.

Acknowledgments

MRLS is supported by an Australian Postgraduate Award (APA) and an Institute for Molecular Biosciences Research Advancement Award. Wanida Phetsang was supported by UQ International Scholarship (UQI) and IMB Postgraduate Award (IMBPA). MAC is a NHMRC principle research fellow (APP1059354) and also holds a fractional professorial research fellow appointment at the University of Queensland, with his remaining time as CEO of Inflazome Ltd, a company developing drugs to address clinical unmet needs in inflammatory disease. MATB is supported in part by Wellcome Trust Strategic Grant WT1104797/Z/14/Z and NHMRC Development grant APP1113719. Microscopy was performed at the Australian Cancer Research Foundation (ACRF)/Institute for Molecular Bioscience Cancer Biology Imaging Facility, which was established with the support of the ACRF.

References

1. Cai, H., Rose, K., Liang, L.H., Dunham, S., Stover, C. Development of a liquid chromatography/mass spectrometry-based drug accumulation assay in Pseudomonas aeruginosa. *Analytical Biochemistry.* **385** (2), 321-325 (2009).
2. Bhat, J., Narayan, A., Venkatraman, J., Chatterji, M. LC–MS based assay to measure intracellular compound levels in Mycobacterium smegmatis: Linking compound levels to cellular potency. *Journal of Microbiological Methods.* **94** (2), 152-158 (2013).
3. Davis, T. D., Gerry, C. J., Tan, D. S. General Platform for Systematic Quantitative Evaluation of Small-Molecule Permeability in Bacteria. *ACS Chemical Biology.* **9** (11), 2535-2544 (2014).
4. Richter, M. F. et al. Predictive compound accumulation rules yield a broad-spectrum antibiotic. *Nature.* **545**, 299 (2017).
5. Iyer, R. et al. Evaluating LC–MS/MS To Measure Accumulation of Compounds within Bacteria. *ACS Infectious Diseases.* **4** (9), 1336-1345 (2018).
6. Prochnow, H. et al. Subcellular Quantification of Uptake in Gram-Negative Bacteria. *Analytical Chemistry.* **91** (3), 1863-1872 (2019).
7. Dumont, E. et al. Antibiotics and efflux: combined spectrofluorimetry and mass spectrometry to evaluate the involvement of concentration and efflux activity in antibiotic intracellular accumulation. *Journal of Antimicrobial Chemotherapy.* **74** (1), 58-65 (2019).
8. Stone, M. R. L., Butler, M. S., Phetsang, W., Cooper, M. A., Blaskovich, M. A. T. Fluorescent Antibiotics: New Research Tools to Fight Antibiotic Resistance. *Trends in Biotechnology.* **36** (5), 523-536 (2018).
9. Piddock, L. J. V., Johnson, M. M. Accumulation of 10 fluoroquinolones by wild-type or efflux mutant Streptococcus pneumoniae. *Antimicrobial Agents and Chemotherapy.* **46** (3), 813-820 (2002).
10. Mortimer, P. G. S., Piddock, L. J. V. A comparison of methods used for measuring the accumulation of quinolones by enterobacteriaceae, pseudomonas aeruginosa and staphylococcus aureus. *Journal of Antimicrobial Chemotherapy.* **28** (5), 639-653 (1991).
11. Li, X. Z., Nikaido, H., Poole, K. Role of MexA-MexB-OprM in antibiotic efflux in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy.* **39** (9), 1948-1953 (1995).
12. Vince, R., Weiss, D., Pestka, S. Binding of N-substituted erythromycylamines to ribosomes. *Antimicrobial Agents and Chemotherapy.* **9** (1), 131-136 (1976).
13. Vazquez, D. Antibiotics affecting chloramphenicol uptake by bacteria Their effect on amino acid incorporation in a cell-free system. *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis.* **114** (2), 289-295 (1966).
14. Phetsang, W. et al. An azido-oxazolidinone antibiotic for live bacterial cell imaging and generation of antibiotic variants. *Bioorganic and Medicinal Chemistry.* **22** (16), 4490-4498 (2014).
15. Phetsang, W. et al. Fluorescent trimethoprim conjugate probes to assess drug accumulation in wild type and mutant *Escherichia coli*. *ACS Infectious Diseases.* **2** (10), 688-701 (2016).
16. Stone, M. R. L. et al. Fluoroquinolone-derived fluorescent probes for studies of bacterial penetration and efflux. *MedChemComm.* **10** (6), 901-906 (2019).