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Letter

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Peptide Targeting of an Antibiotic Prodrug toward Phagosome-Entrapped Mycobacteria

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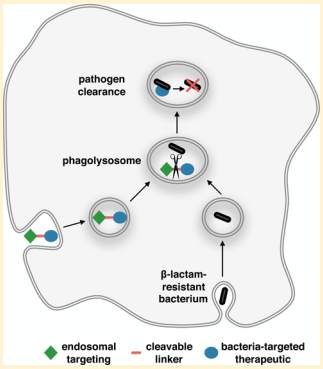
Supporting Information

ABSTRACT: Mycobacterial infections are difficult to treat due to the bacterium's slow growth, ability to reside in intracellular compartments within macrophages, and resistance mechanisms that limit the effectiveness of conventional antibiotics. Developing antibiotics that overcome these challenges is therefore critical to providing a pipeline of effective antimicrobial agents. Here, we describe the synthesis and testing of a unique peptide−drug conjugate that exhibits high levels of antimicrobial activity against *M. smegmatis* and *M. tuberculosis* as well as clearance of intracellular mycobacteria from cultured macrophages. Using an engineered peptide sequence, we deliver a potent DHFR inhibitor and target the intracellular phagosomes where mycobacteria reside and also incorporate a β-lactamase-cleavable cephalosporin linker to enhance the targeting of quiescent intracellular β-lactam-resistant mycobacteria. By using this type of prodrug approach to target intracellular mycobacterial infections, the emergence of antibacterial resistance mechanisms could be minimized.



KEYWORDS: *Mycobacterium tuberculosis, methotrexate, peptide, β-lactamase, prodrug, cephalosporin*

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), is one of the world's deadliest infectious diseases, infecting one-third of the world's population and causing 1.5 million deaths annually.[1] When managed properly, tuberculosis has a high cure rate; however, the treatment regiment requires a cocktail of antibiotics and can last up to a year, resulting in high patient noncompliance and leading to the development of antibiotic-resistant strains of *Mtb*. First-line antibiotics, such as rifampicin and isoniazid, are becoming less effective with the emergence of multidrug-resistant (MDR) TB.[1,2] Thus, there is an urgent need for the development of new antibiotics that can kill *Mtb* quickly and effectively.

There are several drug design challenges in targeting antimicrobials for *Mtb*. Mycobacterial infections, such as those caused by *Mtb*, are difficult to treat due to the bacterium's slow growth kinetics, natural resistance mechanisms, and ability to localize within intracellular compartments in cells. Conventional antibiotics are unsuccessful against mycobacteria due to the highly hydrophobic and impermeable mycolic acid cell wall that surrounds the bacterium.[3] An additional challenge inherent in targeting *Mtb* arises because it is typically sequestered within phagocytic cells, such as macrophages and neutrophils.[4,5]

Prodrugs such as isoniazid have been particularly effective in treating *Mtb* infections,[2] but resistance continues to develop against this particular compound, which targets InhA. Here, we describe an engineered peptide that delivers a potent antibacterial agent to mycobacteria as a highly specific prodrug. Our drug conjugate targets dihydrofolate reductase (DHFR), an essential enzyme in the production of reduced folate cofactors.[6] These cofactors are necessary as one-carbon donors in the biosynthesis of a number of essential building blocks including nucleic acids, certain amino acids, and formyl-methionyl tRNAs. A recent study has attributed antifolate activity in *Mtb* to decreased downstream methionine levels, impacting *S*-adenosylmethionine synthesis.[7] While DHFR inhibitors have been identified as useful antibiotics against *Mtb*,[8] prior efforts to target this activity have remained unsuccessful due to insufficient penetration of the mycolic acid barrier. We deliver methotrexate, a potent DHFR inhibitor, to the phagolysosomes, where intracellular mycobacteria reside. To attenuate the activity of the drug in the absence of bacteria, we use a β-lactamase-dependent release system that exploits the constitutive expression of β-lactamases by mycobacteria.[9] Through linking of a charge-shielding peptide to the Mtx delivery peptide by a β-lactamase-cleavable cephalosporin, we have developed a prodrug that is effectively endocytosed and translocated to phagolysosomes. Upon growth of organelle-entrapped bacteria, the antimicrobial is activated, releasing the potent antimetabolite Mtx, which has been engineered to effectively translocate across the mycobacterial cell envelope and eradicate the bacterial population within phagolysosomes (Figure 1A).

The overall structure of mycobacteria-targeted drug/peptide conjugate P-dpMtx has several key design features (Figure 1B). The active drug is conjugated to a cationic and hydrophobic delivery peptide (dpMtx, Figure 2A) that increases drug uptake

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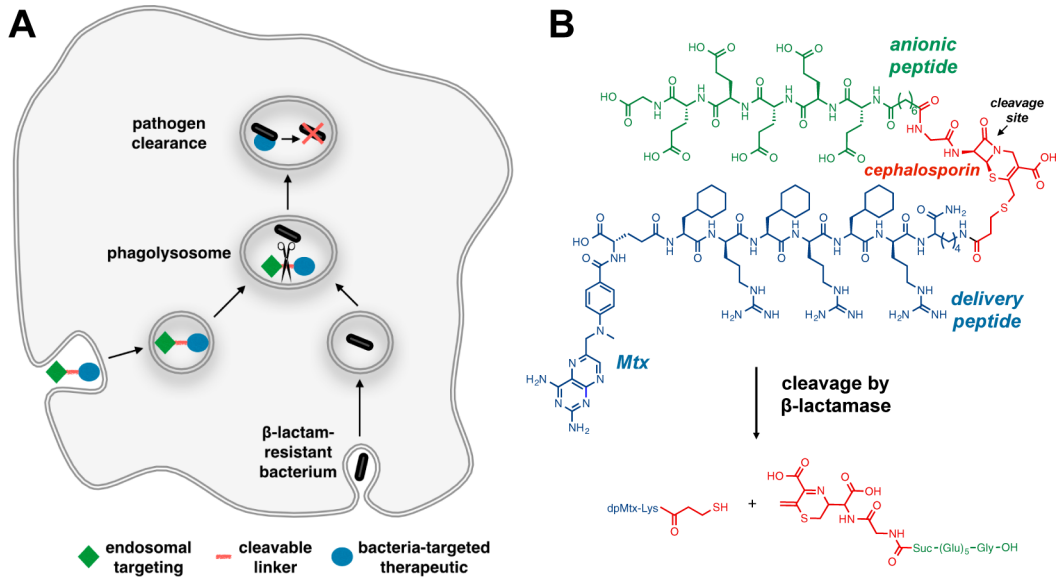

Figure 1. Design of methotrexate prodrug responsive to β-lactamase secretion by intracellular mycobacteria. (A) Endocytosis and intracellular trafficking of methotrexate prodrug and β-lactam-resistant bacteria to phagosomes in macrophages result in cleavage of the cephalosporin linker and subsequent Mtx release. (B) Schematic representation of β-lactamase activation of Mtx.

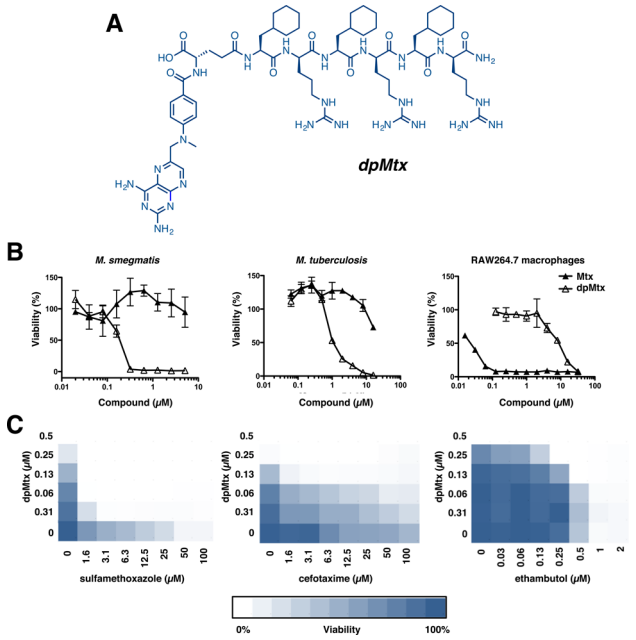

Figure 2. dpMtx exhibits antibacterial properties against mycobacteria and demonstrates synergy with antifolates and cell-wall inhibitors. (A) Chemical structure of dpMtx. (B) Toxicity of Mtx and dpMtx in *M. smegmatis*, *M. tuberculosis*, and RAW264.7 macrophages (*M. smegmatis* 48 h treatment, *M. tuberculosis* 14 day treatment, macrophages 72 h treatment). (C) Microdilution checkerboard analysis showing the combined effect of dpMtx with sulfamethoxazole (SMZ), cefotaxime (CET), and ethambutol (EMB). In each case, synergistic effects were evident. FIC values are dpMtx-SMZ 0.0913, dpMtx-CET 0.28, and dpMtx-EMB 0.5.

through the bacterial cell envelope, thus increasing the antimicrobial activity, and also detoxifies the compound in human cells by sequestering it away from its target in human mitochondria.[10]

We hypothesized that by neutralizing the positive charge of the delivery vector with a short anionic peptide (D-E_5), phagocytosis would be facilitated since small anionic and uncharged macromolecules are unlikely to have cell-penetrating properties.[11] Intracellular mycobacteria secrete β-lactamases when active, and thus, we further proposed that the insertion of a β-lactamase-cleavable cephalosporin linker between dpMtx and the charge-shielding peptide would allow for the release of active drug dpMtx. It has been demonstrated that penicillin-binding proteins have suitable promiscuity in substrates to accept functionalized substrates,[12] a feature that is also observed in β-lactamases. Indeed, cephalosporin linkers have previously been used in fluorogenic probes to detect live *M. tuberculosis* in unprocessed human sputum.[13,14] Recent work has also demonstrated the utility of enzyme-cleaved counterion strategies for activating cell-penetrating peptides for the detection of enzyme activity in vivo.[15]

In order to determine the potential use of dpMtx against mycobacterial species, the unmodified drug, methotrexate, and the dpMtx were tested for antimicrobial activity in two *Mycobacteria* species. Methotrexate alone did not demonstrate antimicrobial activity against *M. smegmatis* and *M. tuberculosis* up to 10 μM but was toxic to RAW264.7 murine macrophages (IC_{50} = 24 nM) (Figure 2B). Conversely, peptide−drug conjugate dpMtx exhibited antimicrobial activity against both mycobacteria species (*M. smegmatis* IC_{50} = 160 nM, *M. tuberculosis* IC_{50} = 950 nM) and was significantly less toxic to macrophages (IC_{50} = 9600 nM). dpMtx but not methotrexate was able to clear intracellular *M. smegmatis* from macrophages but in an untargeted manner (Figure S1).

We tested the synergistic effects of the cotreatment of mycobacteria with dpMtx and other antibacterials to gain insight into the mechanism of dpMtx and to evaluate possible additive and synergistic actions with other antimicrobials (including first-line *Mtb* treatments) as DHFR inhibitors have been identified as promising cotreatments.[16,17] dpMtx exhibited synergy with sulfamethoxazole, an inhibitor of folic acid synthesis, as well as cefotaxime and ethambutol, inhibitors of bacterial cell-wall synthesis (Figure 2C). dpMtx did not exhibit strong synergistic effects with nalidixic acid but showed additive effects with rifampicin (FIC = 0.625) and isoniazid (FIC =

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0.75) (Figure S2). Synergy with antifolates and cell-wall inhibitors suggests that dpMtx can traverse the mycolic acid cell wall and interact with its target DHFR. The ability of dpMtx to synergize with cell-wall inhibitors is particularly interesting as antifolate and cell-wall inhibitor combinations could be investigated as a potential combination therapy. Together, these data demonstrate that dpMtx has enhanced antimicrobial efficacy against mycobacteria while reducing cellular toxicity to cultured macrophages.

Once phagocytosed by macrophages, mycobacteria reside within phagosomes. Mycobacteria are able to persist in phagosomes by blocking the maturation of phagosomes to lysosomes;[5] thus, it is important to ensure that mycobacterial antimicrobials target these intracellular compartments. Altering the charge and/or lipophilicity of macromolecules can lead to differences in cellular uptake and subcellular trafficking;[18,19] thus, we hypothesized that modifying the net charge of the compound would shift the cellular uptake mechanism from cell-penetrating to endocytosis. To determine if shielding the +3 net charge on dpMtx resulted in endocytosis and eventual trafficking to phagolyosomes, macrophages infected with *M. smegmatis* were treated with a tetramethylrhoadmine (TAMRA)-labeled peptide vector $(F_xr)_3$ (F_x = cyclohexylalanine, r = D-arginine) modified with a short anionic peptide (E_5) (E = D-glutamic acid) on the C-terminus and imaged for colocalization with *M. smegmatis* using fluorescence microscopy. As a control for peptide length and charge, a version of the peptide vector with a cationic peptide (K_5) (K = D-lysine) on the C-terminus was also tested. The original peptide vector demonstrated cell-penetrating ability in macrophages (Figure S3), similar to our previous results.[10,20] Adding an additional five charged residues (+8 net charge) was not sufficient to promote endocytic specific uptake of the peptide vector. This is consistent with a previous report showing positively charged (+8) proline-rich antimicrobial peptides with a distributed subcellular localization, including both endosomal and mitochondrial association.[21] However, modifying the net charge of the peptide vector from net positive (+3) to net negative altered the vector's uptake mechanism, as visualized by the colocalization of fluorescently labeled dextran with the TAMRA-labeled peptide vector. Additionally, the $(F_xr)_3E_5$ vector did not show colocalization with mitochondria (Figure S4). *M. smegmatis* also demonstrated colocalization with the peptide vector within phagolysosomes. These data demonstrate that the net-negative peptide vector can accumulate with intracellular mycobacteria within phagolysosomes.

Many antimicrobials must conserve net cationicity in order to maintain antimicrobial activity, but excess positive charge can be detrimental to mammalian cells.[22,23] In order to determine if the modified dpMtx was still effective at killing mycobacteria, the modified peptides were tested for antimicrobial activity and cytotoxicity against murine macrophages. Although the extra positive charges (+8) did not alter the EC_{50} of dpMtx against *M. smegmatis*, the compound became toxic to macrophages (IC_{50} = 1.1 μM) (Figure 3A). Upon neutralizing the positive charge of dpMtx to net negative, the modified compound was not toxic to macrophages but became ineffective at killing *M. smegmatis*. Furthermore, the anionic compound was unable to clear intracellular *M. smegmatis* from macrophages while the cationic compound maintained its efficacy (Figure 3B).

These data suggest that a prodrug approach to release dpMtx may be necessary to effectively clear intracellular mycobacteria. The antimicrobial prodrug isoniazid has shown effectiveness in

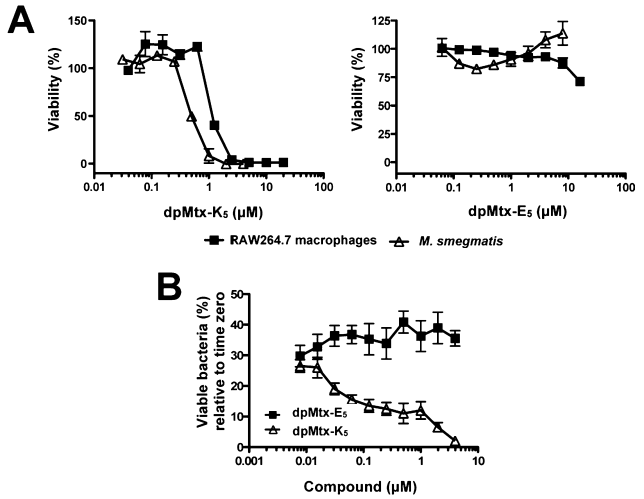

Figure 3. Modifying the net charge of dpMtx allows for phagolysosomal accumulation in macrophages. (A) Toxicity of charge-modified peptides dpMtx-K_5 and dpMtx-E_5 in RAW264.7 macrophages and *M. smegmatis* after 72 h of treatment. (B) Clearance of intracellular *M. smegmatis* in RAW264.7 macrophages with charge-modified peptides dpMtx-K_5 and dpMtx-E_5. Percent viability represents the viability of the internalized bacteria compared to bacterial numbers used for macrophage infection. Untreated control wells showed 33 and 31% viability compared to time zero for dpMtx-E_5 and dpMtx-K5 experiments, respectively.

the long-term treatment of *Mtb* due to its specific activation by bacterially secreted enzyme KatG,[2] thus rendering the compound active only in the presence of metabolically active bacteria. Another class of antimicrobials, β-lactam antibiotics, which include the cephalosporin subfamily, are widely used bactericidal agents that inhibit bacterial cell-wall synthesis. The use of β-lactam antibiotics is limited, however, as many bacterial species including *Mtb* can develop resistance to these drugs by secreting β-lactamase enzymes to hydrolyze the β-lactam ring, rending the antimicrobial agent ineffective. To release the drug dpMtx from the oligo(glutamic acid) (E_5) shielding element, a cephalosporin linker derived from 7-aminocephalosporanic acid was introduced in between the two components to yield the final compound, dpMtx-ceph-E_5 (Figure 1B). Additionally, the cephalosporin linker was flanked by alkyl spacers in order to facilitate peptide synthesis and allow for enzymatic access by β-lactamases.

To determine if the cephalosporin linker in this prodrug construct (P-dpMtx) was accessible and specifically cleaved by β-lactamases, a TAMRA-labeled version of the peptide delivery vector (TAMRA-$(F_xr)_3$-Ceph-E_5) was incubated with purified β-lactamase alone or along with two inhibitors of the enzyme, tazobactam and clavulanic acid, and the products were analyzed by HPLC (Figure 4A). Compared to TAMRA-$(F_xr)_3$-Ceph-E_5 alone, the addition of β-lactamase inhibitors decreased the relative cleavage and release of TAMRA-$(F_xr)_3$ by ~75 and ~25% by tazobactam (20 μg/mL) and clavulanic acid (40 μg/mL), respectively. Since charge shielding can impact the uptake of antimicrobial dpMtx by mycobacteria, the peptide uptake of TAMRA-labeled versions of dpMtx (TAMRA-$(F_xr)_3$), charge-modified versions of dpMtx (TAMRA-$(F_xr)_3$-K_5 and TAMRA-$(F_xr)_3$-E_5), and P-dpMtx (TAMRA-$(F_xr)_3$-Ceph-E_5) was measured in *M. smegmatis*. Modifying the compound's net charge from net positive to net negative completely abolished the uptake of the compound in *M. smegmatis*; however, the

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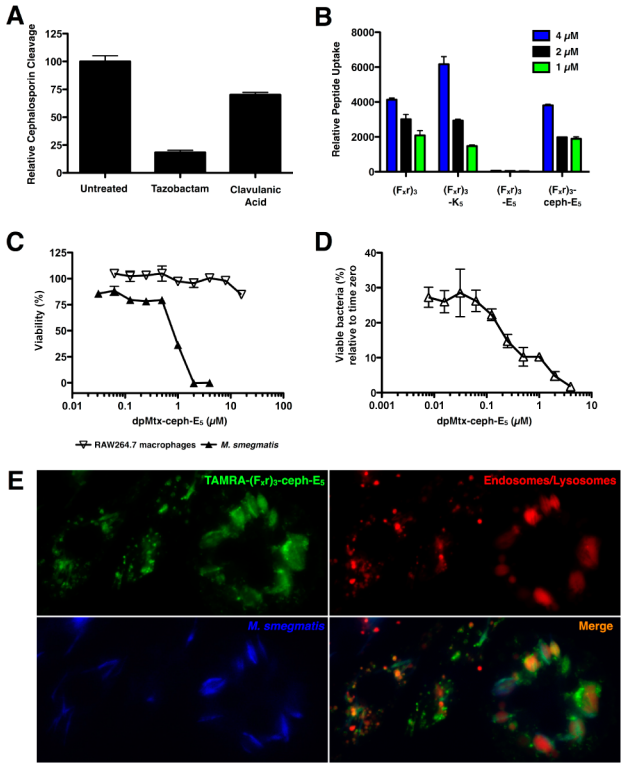

Figure 4. Incorporation of a cephalosporin linker rescues the activity of dpMtx-E_5 and demonstrates selective toxicity against *M. smegmatis*. (A) The cephalosporin linker is cleaved by β-lactamases, as evidenced by the cotreatment of purified enzyme with TAMRA-$(F_xr)_3$-Ceph-E_5 alone or with β-lactamase inhibitors tazobactam (20 μg/mL) and clavulanic acid (40 μg/mL). (B) Uptake of TAMRA-$(F_xr)_3$ in *M. smegmatis* after 72 h of incubation is affected by the overall net charge but is rescued with the incorporation of a cephalosporin linker. (C) Toxicity of dpMtx-Ceph-E_5 in RAW264.7 macrophages and *M. smegmatis* after 72 h of treatment. (D) Clearance of intracellular *M. smegmatis* in RAW264.7 macrophages after 40 h of treatment. Percent viability represents the viability of the internalized bacteria compared to bacterial numbers used for macrophage infection. Untreated control wells showed 33% viability compared to time zero; reduction in percent viability for control wells represents macrophage clearance of internalized *M. smegmatis*. (E) Wide-field fluorescence microscopy of TAMRA-$(F_xr)_3$-Ceph-E_5 (6 μM) with endosomes/lysosomes (dextran, Alexa Fluor 647; 10 kDa MW) and *M. smegmatis* (labeled with Marina Blue-NHS) after 24 h of incubation in RAW264.7 macrophages.

introduction of the cephalosporin linker was able to rescue the uptake of the net-negative compound, as measured by flow cytometry (Figure 4B). Furthermore, the introduction of the cephalosporin linker maintained the therapeutic index of dpMtx since P-dpMtx was minimally toxic to macrophages while still maintaining its antimicrobial activity against *M. smegmatis* (IC_{50} = 0.8 μM) (Figure 4C). P-dpMtx was also able to effectively clear intracellular *M. smegmatis* from infected macrophages with greater than 50% clearance at 250 nM (Figure 4D). To determine if the final peptide vector $(F_xr)_3$-ceph-E_5 was still able to colocalize with *M. smegmatis* within endo/lysosomal compartments, the peptide vector was labeled with TAMRA and visualized in infected macrophages using fluorescence microscopy (Figure 4E). Colocalization between Alexa Fluor 647 dextran, the TAMRA-labeled peptide vector, and Marina blue-labeled *M. smegmatis* verified the intracellular localization of the peptide vector to its intended target within the phagolysosomes. Premature release of the delivery peptide prior to endocytosis or phagolysosome accumulation would have resulted in a mitochondrial subcellular distribution as seen with the TAMRA-$(F_xr)_3$ compound (Figure S3). Thus, these data indicate that the cephalosporin linker can effectively release an active dpMtx to kill intracellular mycobacteria within phagolysosomes.

We have designed and tested P-dpMtx, a mycobacterial prodrug that can overcome several challenges to *Mtb* drug design. P-dpMtx can (1) display antimicrobial activity against mycobacteria, (2) traffic to phagolysosomes within macrophages where intracellular mycobacteria reside, (3) be activated by mycobacteria-specific enzymes, (4) effectively penetrate the mycobacterial cell wall, and (5) be synergistically used with other antimicrobials. Thus, these data suggest that using a prodrug approach to releasing active dpMtx can effectively clear β-lactam-resistant intracellular mycobacteria infections by targeting antimicrobials within phagosomes where latent mycobacteria reside.

METHODS

General Cell Culture and Bacterial Growth Conditions. RAW 264.7 macrophage cells were cultured in RPMI (Life Technologies, Carlsbads, CA) supplemented with 4% (v/v) FBS at 37 °C with 5% CO_2. *M. smegmatis* and *M. tuberculosis* H37Ra were grown in Middlebrook 7H9 liquid broth media, supplemented with 1× ADN (5 g/L BSA, 2 g/L dextrose, 0.85 g/L NaCl). Sterile-filtered Tween-80 was added at 0.05% to overnight liquid cultures to prevent clumping. Tween-80 was not added to media during imaging or drug sucesptibility testing.

Compound Synthesis. *Cephalosporin Linker (C1) Synthesis.* 7-Aminocephalosporanic acid (350 mg, Sigma-Aldrich) was dissolved in 10 mL of DMF and 700 μL of triethylamine (TEA) at 60 °C for 10 min. Boc-Gly-OH (0.525 g, 3 mmol), TEA (1.4 mL, 5 mmol), and *O*-(benzotriazol-l-yl)-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (HBTU) (0.94 g, 2.5 mmol) were prereacted in 5 mL of DMF for 5 min, added to 7-ACA, and reacted for 4 h. The reaction mixture was dissolved in a 10× volume of 0.5 M HCl and extracted with EtOAc. EtOAc was extracted twice more with 0.5 M HCl. EtOAc was rotoevaporated to dryness, resuspended in minimal MeOH, and precipitated in cold ether. Boc-Gly-cephalosporanic acid (18.5 μmol) was reacted with 3-mercaptoproprionic acid (100 μmol, Sigma-Aldrich) to displace the 3′-acetate according to a previously reported method.[24] The reaction was carried out at 60 °C overnight in 500 μL of H_2O with the addition of a 1 M solution of $NaHCO_3$ dropwise until a pH of 5 to 6 was achieved. A lower pH was used in this protocol to prevent ring opening of the cephalospoin. The compound was purified by RP-HPLC on a C_{18} column with a H_2O/MeCN gradient in 0.1% TFA and lyophilized to dryness. Compound structure is depicted in Figure S5. The compound identity was confirmed by electrospray ionization mass spectroscopy: expected m/z = 475.11, found = 498.1 ($M + Na^+$) and 514.1 ($M + K^+$).

Peptide (TAMRA and Methotrexate Conjugate) Synthesis. Solid-phase synthesis was performed on a Rink amide MBHA resin (0.7−1 mmol/g, 100−200 mesh) (NovaBiochem) using a Prelude Protein Technologies peptide synthesizer. Peptides were synthesized on a 25 μmol or 50 μmol scale. Methotrexate and TAMRA were coupled onto the peptide N-terminus as previously described.[10,20] Briefly, Fmoc-L-glutamic acid-α-

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tertbutyl ester was coupled to resin-bound peptide using 4 equiv of HBTU and 8 equiv of *N*,*N*-diisopropylethylamine (DIPEA) in *N*,*N*-dimethylformamide (DMF) for 2 h. APA (4 equiv) was coupled using *N*-hydroxybenzotriazole (HOBt, 2 equiv), HBTU (4 equiv), and DIPEA (8 equiv) for 1 h at room temperature. Fluorescently labeled peptides were synthesized by N-terminal coupling of 5(6)-carboxytetramethylrhodamine (TAMRA, Anaspec, Fremont, CA) to the resin-bound peptide with HBTU (4 equiv) and DIPEA (8 equiv) in 1 mL of DMF for 2 h at room temperature. Peptides were deprotected and cleaved from the resin using TFA/triisopropylsilane (TIS)/H_2O (95:2.5:2.5) and precipitated in cold diethyl ether. Peptides were purified to >95% purity by RP-HPLC on a C_{18} column with a H_2O/MeCN gradient in 0.1% TFA, and the identity was confirmed by electrospray ionization mass spectrometry. Methotrexate-conjugated peptides were quantified at 302 nm in 0.1 N NaOH using a methotrexate extinction coefficient of 22 700 M^{-1} cm^{-1}. TAMRA-conjugated peptides were quantified at 553 nm using an extinction coefficient of 40 000 M^{-1} cm^{-1}. dpMtx sequence Mtx-F_x-r-F_x-r-F_x-r-$CONH_2$; expected m/z = 1380.84, found = 1380.8. F_x = cyclohexylalanine, r = D-arginine, e = D-aspartic acid, k = D-lysine, Mtx = methotrexate. TAMRA-$(F_xr)_3$ sequence TAMRA-F_x-r-F_x-r-F_x-r-$CONH_2$; expected m/z = 1356.81, found = 1356.8. TAMRA-$(F_xr)_3$-E_5 sequence TAMRA-F_x-r-F_x-r-F_x-r-e-e-e-e-e-$CONH_2$; expected m/z = 2002.03, found = 2002.06. TAMRA-$(F_xr)_3$-K_5 sequence TAMRA-F_x-r-F_x-r-F_x-r-k-k-k-k-k-$CONH_2$; expected m/z = 1998.3, found = 1998.3. Mtx-$(F_xr)_3$-E_5 sequence Mtx-F_x-r-F_x-r-F_x-r-e-e-e-e-e-$CONH_2$; expected m/z = 2026.05, found = 2026.05. Mtx-$(F_xr)_3$-K_5 sequence Mtx-F_x-r-F_x-r-F_x-r-k-k-k-k-k-$CONH_2$; expected m/z = 2021.31, found = 2022.3.

Charge-Modifying Peptide NHS-Suberic Acid-E_5-Gly (C2) Synthesis. Solid-phase synthesis was performed on a glycine precharged Trt resin using a Prelude Protein Technologies peptide synthesizer. A peptide consisting of five units of Boc-protected D-glutamic acid was synthesized on resin. NHS-suberic acid was conjugated to peptide on resin (1 equiv) using suberic acid bis(*N*-hydroxysuccinimide) (5 equiv) with DIPEA (3 equiv) in DMF. Peptide was cleaved from resin in 1% TFA in DCM (5 × 1 min). The cleavage reaction was terminated with pyridine, and peptide was precipitated in cold ether and dried. The compound structure is depicted in Figure S6, and the compound identity was confirmed by electrospray ionization mass spectroscopy. Expected m/z = 973.3, found = 973.3.

dpMtx-ceph-E_5 and TAMRA-$(F_xr)_3$-ceph-E_5 Synthesis. dpMtx or TAMRA-$(F_xr)_3$ peptides were synthesized on a Rink amide MBHA resin with an Mtt-protected lysine at the C-terminus. Mtt was deprotected on resin by the addition of 3% TFA in DCM. Cephalosporin linker C1 (1 equiv) was conjugated to the on-resin peptide (1 equiv) in the presence of DIPEA (4 equiv) and HBTU (1 equiv) in DMF in a 3 h reaction. The Boc group from the C1 molecule was subsequently removed in the presence of 20% TFA in DCM (3 × 10 min), and resin was washed with DMF, MeOH, and DCM. Charge-modifying peptide C2 was then added to the resin-bound peptide in DMF (5 eq of C2, 3 eq of DIPEA) in an overnight reaction. Peptide was cleaved from the resin using TFA/TIS/H_2O (95:2.5:2.5) and precipitated in cold ether. Peptides were purified to >90% purity by RP-HPLC on a C_{18} column with a H_2O/MeCN gradient in 0.1% TFA, and the identity was confirmed by electrospray ionization mass spectroscopy. TAMRA-$(F_xr)_3$-ceph-E_5: expected m/z = 2700.27, found = 2700.2. Mtx-$(F_xr)_3$-ceph-E_5: expected m/z = 2724.29, found = 2724.26.

Analysis of Toxicity (Macrophages). Macrophage cells were cultured as described above. Cells were seeded in 96-well flat-bottomed tissue culture plates (Starsted, NC) at a density of 1500 cells per well. The culture medium was removed, and cells were washed with RPMI. Concentrated peptide stocks were diluted in RPMI + 10% dialyzed FBS and incubated with cells for 72−96 h. The cellular viability was analyzed after the indicated times using the CCK-8 viability dye (Dojindo, Rockville, MD) at an absorbance of 450 nm.

Analysis of Toxicity (Bacteria). Minimum inhibitory concentration (MIC) determinations were performed in a 96-well format. Briefly, overnight cultures were subcultured at 1:100 and grown to an OD_{600} of 0.5. Cells were diluted 1:2000 in fresh media with test compounds and were incubated for 72 h at 37 °C for *M. smegmatis* and for 2 weeks for *M. tuberculosis*. Growth was monitored at OD_{600}. Combination experiments with *M. smegmatis* were accomplished by treating bacteria with an array consisting of 2-fold dilutions of both compounds. The FIC index was calculated according to the formula FIC index = FIC_{comp1} + FIC_{comp2}. FIC = [X]/MicX, in which [X] is the lowest inhibitory concentration in the presence of a codrug.

The analysis of in cellulo *Mycobacteria* clearance was determined as described below. RAW 264.7 cells were seeded on a 12-well plate at 100 000 cells per well 1 day before infection with bacteria. Saturated *M. smegmatis* cultures were subcultured at 1:100 and grown for approximately 16 h to an OD_{600} of 0.5−0.7. Macrophages were infected with *M. smegmatis* at a multiplicity of infection (MOI) of 10 for 3 h at 37 °C. Infected cells were washed with RPMI (no FBS) and treated with gentamycin (50 μg/mL) for 2 h. Infected macrophages were subsequently washed and treated with the compound of interest for 40 h. Media and treatment compounds were replenished after the first 20 h. RAW cells were then washed in D-PBS and lysed with 400 μL of sterile water and vigorous pipetting. Samples were then serially diluted, spotted on fresh Middlebrook + ADN plates, and incubated at 37 °C for 48 h. Viable intracellular bacteria were determined by counting individual colonies.

Analysis of Compound Cleavage by β-Lactamase. Reaction components consisted of 2 μg/mL enzyme (β-lactamase from *Enterobacter cloacae*, a group 1 cephalosporinase, Sigma-Aldrich, P4524), 25 mM Hepes pH 7, 5% glycerol, and 12 μM TAMRA-$(F_xr)_3$-ceph-E_5. Reactions were started with the addition of compound and proceeded at room temperature for 10 min. Reactions were terminated by the addition of HPLC buffer B and loaded onto an Agilent 1100 HPLC for analysis. Compounds were separated by paired-ion chromatography (PIC) on a C_{18} reverse-phase column. Chromatography conditions were a linear gradient from 0 to 100% buffer B over 10 min. Buffer A: 10 mM TBAHS (tetrabutyl ammonium hydrogen sulfate), 25 mM PO_4 pH 6; buffer B: 4 mM TBAHS, 10 mM PO_4 pH 5, 60% acetonitrile.

Microscopy. Cells were seeded in 8-well μ-slides (iBidi, Germany) at a density of 50 000 cells per well 1 day prior to experiments in the RPMI medium supplemented with 10% FBS. For intracellular *M. smegmatis* imaging, a 1.0 OD_{600} culture was washed once in D-PBS and was fluorescently labeled with 3 μM Marina Blue-SE (Life Technologies, Carlsbads, CA) in sterile PBS for 30 min at 37 °C. *M. smegmatis* was washed twice in Middlebrook 7H9 and used to

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infect macrophages with an MOI of 40 for 3 h in RPMI at 37 °C. Cells were then washed once with media and treated with 50 μg/mL gentamycin in RPMI for 2 h to kill extracellular bacteria. Dextran Alexa Fluor 647 (10 000 MW, Anionic, Life Technologies, Carlsbad, CA) was added to media at 10 μg/mL along with TAMRA-labeled peptides $(F_xr)_3$, $(F_xr)_3K_5$, $(F_xr)_3E_5$, and TAMRA-$(F_xr)_3$-ceph-E_5. Cells were incubated for 16−24 h, washed twice in RPMI, and imaged using an inverted Zeiss Observer Z1 microscope. Mitochondria of cells were stained using Mitotracker Deep Red (Life Technologies, Carlsbads, CA) at 50 nM for 20 min.

Flow Cytometry. Saturated *M. smegmatis* cultures were subcultured at 1:100 and grown for approximately 18 h to an OD_{600} of 1.0. Bacteria were washed and incubated with TAMRA-labeled compounds to final concentrations of 4, 2, and 1 μM in 7H9 Middlebrook + ADN media for 30 min at 37 °C while shaking. Bacteria were washed twice with PBS by centrifugation at 9000*g* for 3 min. Compound uptake was analyzed on a BD FACSCanto flow cytometer (BD Biosciences) with excitation at 488 nm and emission wavelengths of 564 to 606 nm collected. Median relative fluorescence units are reported. Data was collected at approximately 300 events per second with a minimum of 10 000 events collected per sample.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00099.

Additional figures describing the compound activity (S1 and S2), intracellular localization of fluorescently labeled compounds (S3 and S4), and structures of intermediates (S5−S7) (PDF)

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Author Contributions

M.P.P. and S.O.K. conceived the experiments. M.P.P. and J.S. performed the experiments. The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Mtb, *Mycobacterium tuberculosis*; Mtx, methotrexate; TAMRA, tetramethylrhodamine

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