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Maximizing the Therapeutic Window of an Antimicrobial Drug by Imparting Mitochondrial Sequestration in Human Cells

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Supporting Information

ABSTRACT: The number of antimicrobial agents available for use in humans is limited by the difficulty of discovering chemical agents with selective toxicity to bacterial targets. Numerous small molecule inhibitors have potential as antimicrobial agents, yet their use has been prevented by high levels of toxic cross-reactivity in human cells. For example, methotrexate (Mtx) is an effective antimetabolite that exerts its effects by inhibiting DHFR. It is a potent antibacterial when accumulated intracellularly, but toxicity in human cells limits clinical utility in infectious disease treatment. Here, we describe peptide conjugates of Mtx that are sequestered into the mitochondria of human cells (mt-Mtx). This alteration in localization of Mtx, which directs it away from its enzyme target, decreases its toxicity in human cells by a factor of 10^3. Mt-Mtx, however, maintains activity against a variety of pathogenic Gram-positive organisms, including methicillin-resistant *Staphylococcus aureus* (MRSA). The results from this proof-of-principle study describe a novel methodology for augmenting the antibacterial efficacy of drugs amenable to peptide conjugation while simultaneously decreasing their toxicity to the host organism.

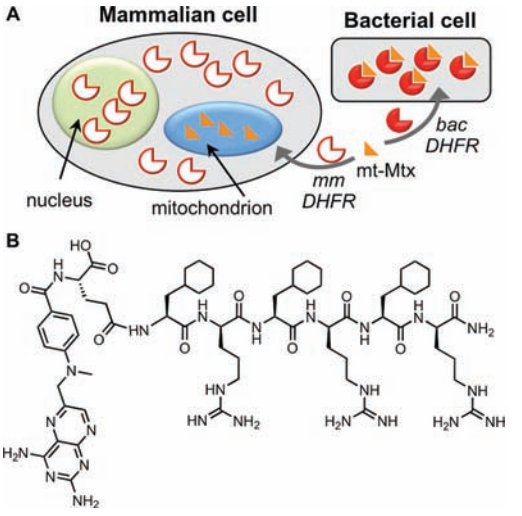

Figure 1. Design of Mtx conjugates that would facilitate sequestration of methotrexate in the mitochondria. (A) Conjugation to a mitochondria-penetrating peptide allows uptake of small molecule inhibitors into bacteria that can then reach a protein target. In human cells, however, these molecules are effectively sequestered within mitochondria away from the human target, reducing toxicity. (B) Structure of mt-Mtx. The potent DHFR inhibitor methotrexate (Mtx) was coupled to a mitochondria-penetrating peptide.

Antimicrobial agents that exhibit high levels of specificity for bacterial targets while maintaining low levels of toxicity against human cells are in short supply.[1,2] Considerable effort has been put toward the discovery of small molecule inhibitors with potent and selective bacterial toxicity. Classically, antimicrobial discovery either excludes bacterial targets with human orthologs from drug discovery, or attempts to introduce specificity for the bacterial enzyme through extensive structure−activity relationship studies.

The enzyme dihydrofolate reductase (DHFR) is an example of a protein target where drug development has yielded clinically used compounds with varying specificities for certain cell types.[3,4] Methotrexate (Mtx) is an example of a drug that inhibits bacterial DHFRs, but also inhibits human DHFR and therefore is too toxic to be used as an antibacterial. The cytotoxic effects of Mtx against human cells permits its use as an antineoplastic agent.

DHFR catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) and is essential for the synthesis of thymidine, purines, and certain amino acids.[3] Although the product of the DHFR-mediated reaction is required in the mitochondria of human cells, DHFR is solely found in the cytoplasm and the nucleus.[5] We hypothesized that by using a targeting vector to redirect Mtx to the mitochondria, where there is no DHFR present, we might render it nontoxic to human cells while not affecting its activity as an antibacterial given the lack of cellular compartments in prokaryotes (Figure 1A). While not all drugs might be amenable to conjugation with a targeting vector, Mtx is an excellent candidate to test this approach. This drug is typically present as poly glutamyl conjugate within the cell,[6] suggesting the modified site could be used for conjugation to a mitochondrial-targeting vehicle. Indeed, previous studies have shown that Mtx is amenable to peptide modification on the gamma carboxylic acid[7] while retaining its activity as an inhibitor of the DHFR enzyme.

To impart mitochondrial (mt) localization to Mtx, we relied on our prior discovery of mitochondria-penetrating peptides, a class of short cationic peptides that efficiently localize to the mitochondria of cells and can deliver covalently linked small molecule cargoes.[8,9]

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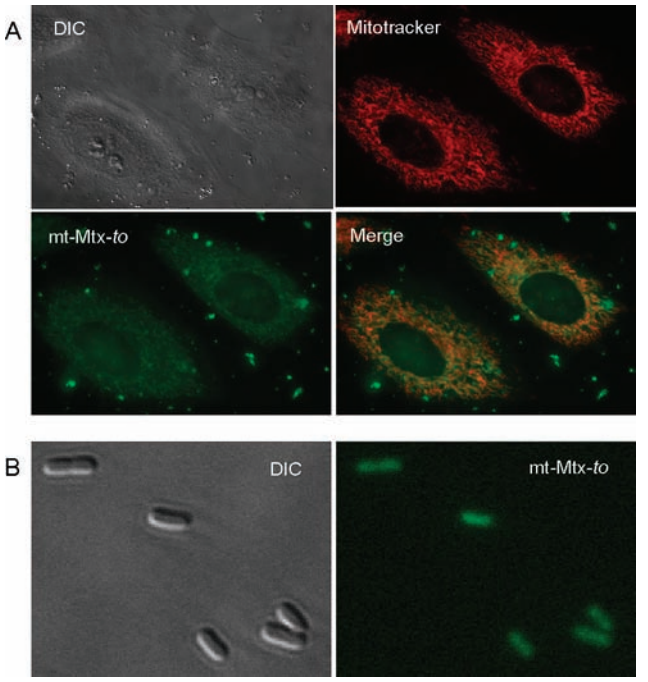

Figure 2. (A) Fluorescently labeled mt-Mtx localizes to the mitochondria of HeLa cells. Signal from mt-Mtx-to shows intracellular localization in HeLa cells characteristic of mitochondrial accumulation. Mitochondrial staining with commercially available Mitotracker Deep Red 633 shows a similar staining pattern. (B) mt-Mtx-to accumulates within the cytoplasm of *E. coli* JW5503.

A mitochondria-specific version of Mtx (mt-Mtx) was generated by coupling the drug to the N-terminus of a peptide consisting of 3 repeating units of cyclohexylalanine and *d*-arginine (Figure 1B). This peptide gives the conjugate the required physicochemical properties necessary to promote efficient and specific mitochondrial accumulation.[8]

To assess intracellular localization of mt-Mtx, the fluorophore thiazole orange (*to*)[10] was attached to the C-terminus of the peptide (Figure S1). Treatment of HeLa cells with mt-Mtx-*to* produced a distinct mitochondrial staining pattern (Figure 2A). Overlay with a commercially available mitochondrial stain, Mitotracker, suggests specific mitochondrial accumulation for the peptide conjugate.

In order to assess bacterial localization and characterize the activity of mt-Mtx, we used as a model a strain of *Escherichia coli* (JW5503) that lacks the *tolC* gene that can cause drug efflux. The use of this strain presented the opportunity to observe where the drug conjugate would localize once inside a bacterial cell in the absence of competing efflux. When introduced into *E. coli* JW5503, the drug exhibited a distinct cytoplasmic localization (Figure 2B).

Analysis of the toxicity of mt-Mtx indicated that the molecule was selectively toxic to bacteria. Mt-Mtx exhibits potent inhibition of *E. coli* JW5503 (*tolC* null) with a 1 μM MIC (LD_{50} = 0.40 μM) while simultaneous addition of the peptide and Mtx or each isolated component exhibit no effects below 10 μM (Figure 3A).

While the activity of mt-Mtx was significantly increased in *E. coli*, it was dramatically attenuated in human cells (Figure 3B). While treatment with unmodified Mtx yielded a LD_{50} of 14.1 nM in a mammalian cell line, the LD_{50} value of mt-Mtx was greater than 32 μM. This represents more than a 2200-fold attenuation of Mtx activity in a human cell line.

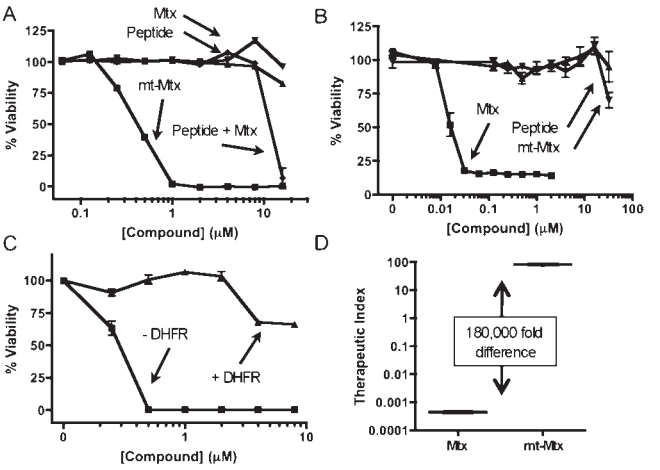

Figure 3. Therapeutic activity of mt-Mtx (A) Antimicrobial activity of methotrexate-peptide conjugates in *E. coli* JW5503. Addition of a mitochondria-penetrating peptide to methotrexate decreased the MIC of the compound from >16 μM to 1 μM. (B) Toxicity of mt-Mtx in a human (HeLa) cell line. Methotrexate shows a potent inhibition of cellular viability with a LD_{50} of 14.1 nM. Both the parent peptide and mt-Mtx do not show appreciable toxicity up to the maximum concentration tested (32 μM). (C) Overexpression of DHFR (FolA) on the pCA24N plasmid resulted in a greater than 16-fold increase in the MIC of mt-Mtx. (D) The therapeutic index of Mtx and mt-Mtx was calculated for each compound using LD_{50} values from *E. coli* JW5503 and HeLa cells. The antibacterial activity of mt-Mtx was observed to be 180,000-fold better than Mtx.

A therapeutic index (TI) for Mtx versus mt-Mtx can be calculated from the ratio of the HeLa LD_{50} to the *E. coli* JW5503 LD_{50} (Figure 3D). The higher the therapeutic index, the greater the desired effect opposed to undesired cellular toxicity. Adding a mt-specific peptide to Mtx increased its therapeutic potential as an antimicrobial over 180 000-fold (Mtx TI = 4.41×10^{-4}, mt-Mtx TI = 79.5). Directly comparing the therapeutic index of Mtx and mt-Mtx as an antimicrobial shows that mitochondrial targeting of Mtx changes the activity of Mtx to that of an antimicrobial. When wild-type *E. coli* were tested (Figure S2), the potency of mt-Mtx was decreased due to efficient Mtx efflux (LD50 =13 μM, MIC = 32 μM), but a 6000-fold improvement in the therapeutic index of Mtx was still observed.

We further assessed the impact of mt-Mtx on mitochondrial function and health. Decreases in mitochondrial membrane potential and decreases in cellular ATP levels are phenotypes associated with mitochondrial toxicity.[11] Treatment of HeLa cells with mt-Mtx did not affect mitochondrial membrane potential or cellular ATP levels (Figure S4). Moreover, upon removal of compound from the cell media, intracellular levels of the compound decreased in a time-dependent manner (Figure S4), with >50% of the compound cleared from the cell in 24 h.

To assess whether the cellular target of mt-Mtx was indeed still DHFR, we sought to observe the effect of DHFR overexpression on growth inhibition of *E. coli*. Multicopy suppression has previously been shown to identify inhibitors of the DHFR enzyme.[12] Indeed, overexpression of DHFR in JW5503 suppressed the antibacterial effects of trimethoprim, a known DHFR inhibitor (Figure S3). In the case of mt-Mtx, increased expression of DHFR resulted in over a 16-fold increase in the MIC (Figure 3C). These data suggest that mt-Mtx is inhibiting bacterial growth due to a DHFR-specific mechanism.

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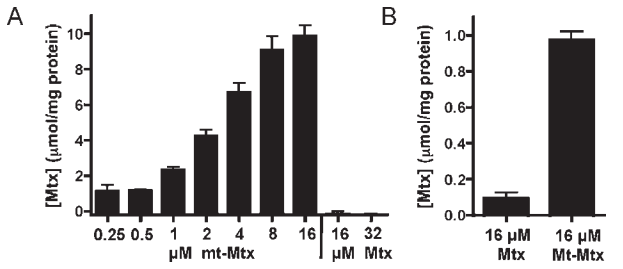

Figure 4. (A) Relative intracellular concentration in *E. coli* JW5503 and dose-dependent increases in intracellular drug concentrations. With Mtx treatment alone, observable accumulation of drug was not detectable. (B) Mt-Mtx accumulates at significantly higher levels within HeLa cells compared to unfunctionalized Mtx.

We evaluated the levels of Mtx in *E. coli* and mammalian cells to confirm that mt-Mtx accumulates in both cell types. Incubation of mt-Mtx with *E. coli* showed a marked dose-dependent increase in peptide concentration. With Mtx, however, no significant accumulation was detectable even at the highest concentrations tested (Figure 4A), showing that the enhanced potency of the drug in this bacterial strain is derived from peptide-mediated uptake. Similarly, in the mammalian cell line, mitochondrial targeting of Mtx with a peptide resulted in a significant increase in intracellular levels of Mtx (Figure 4B) when compared to Mtx alone. The bacterial and mammalian cell uptake cannot be directly compared because of differing protein levels, but it is clear that toxicity does not result in mammalian cells even when significant levels of mt-Mtx are present.

With proof-of-principle that mitochondrial sequestration of mt-Mtx could produce a significant therapeutic window for a model organism, we moved on to study a series of bacterial species, many of which are clinically relevant. We tested a panel of Gram-positive strains, and observed activity across a variety of organisms. In *Enterococcus faecalis*, mt-Mtx exhibited comparable activity to the parent compound, but given the attenuated activity in human cells, its therapeutic index was >10. This represents a >3000-fold improvement over the parent drug (Figure 5A). In *Bacillus subtilis*, mt-Mtx was significantly more toxic than unmodified Mtx, which produced >10 000 fold improvement in the therapeutic index of the DHFR inhibitor (Figure 5B). In *Streptococcus pneumoniae*, Mtx is very active, but the MIC is still in the range where significant toxicity occurs in human cells, leading to a therapeutic index <1. For mt-Mtx, the MIC is higher, but the fact that much lower toxicity is observed with this compound in human cells still gives the drug a high therapeutic index in *S. pneumoniae* (Figure 5C). In *Staphylococcus aureus*, improved potency is again observed, and the therapeutic index of the drug is improved over 7000-fold (Figure 5D). Interestingly, even higher potency is observed for methicillin-resistant *S. aureus*, and the therapeutic index approaches 10 (Figure 5E).

While the peptide appendage did not improve the toxicity of Mtx in all of the bacterial strains tested, it was effective at improving the potency in *E. faecalis*, *B. subtilis*, and *S. aureus*. Given that higher levels of uptake were observed in *E. coli*, we wondered if uptake was increased in other types of bacteria. Levels of Mtx and mt-Mtx were studied in *E. faecalis* and *S. aureus* (Figure S5), and increased uptake for the peptide conjugate over unmodified Mtx was observed in both cases. In addition, the MIC values correlated with the concentration ranges where large increases in uptake occurred, indicating that levels of toxicity

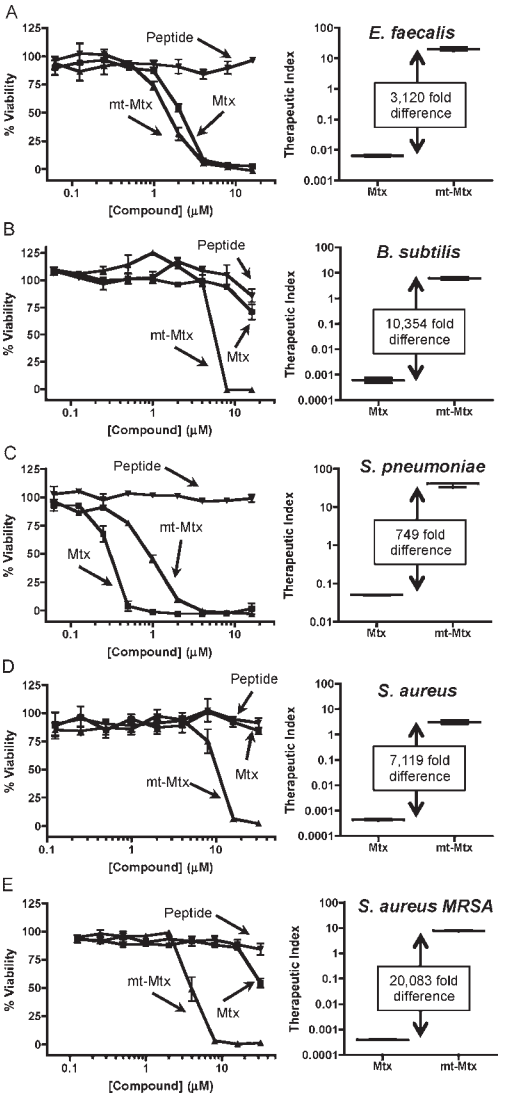

Figure 5. Toxicities and therapeutic index calculations for mt-Mtx and Mtx for Gram-positive pathogens. (A) Activity of mt-Mtx and Mtx against *E. faecalis* (ATCC 29212). Peptide MIC > 16 μM, mt-Mtx MIC = 4 μM, Mtx MIC = 4 μM. (B) Activity of mt-Mtx and Mtx against *B. subtilis*. Peptide MIC > 16 μM, mt-Mtx MIC = 8 μM, Mtx MIC > 16 μM. (C) Activity of mt-Mtx and Mtx against *S. pneumoniae* (ATCC 49619). Peptide MIC > 16 μM, mt-Mtx MIC = 2 μM, Mtx MIC = 0.5 μM. (D) Activity of mt-Mtx and Mtx against *S. aureus* (ATCC 29213). Peptide MIC > 32 μM, mt-Mtx MIC = 16 μM, Mtx MIC > 32 μM (E) Activity of mt-Mtx and Mtx against MRSA (ATCC BAA-1720). Peptide MIC > 32 μM, mt-Mtx MIC = 8 μM, Mtx MIC > 32 μM. The therapeutic index of Mtx and mt-Mtx were calculated for each organism. The fold differences in the calculated therapeutic indices are indicated.

are correlated with levels of uptake that may differ among different types of bacteria.

While very high levels of specificity and therapeutic indices were therefore observed in a variety of Gram-positive bacteria, it is noteworthy that no appreciable activity was observed for the Gram-negative bacteria *Pseudomonas aeruginosa* or *Neisseria meningitidis* when concentrations as high as 16 μM were tested (data not shown). It appears that the cell envelope of these pathogens does not permit the compound to enter.

In summary, by engineering mitochondrial localization into an antibacterial agent, we were able to render it nontoxic to

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mammalian cells. Mitochondrial localization was imparted to methotrexate by attaching a mitochondria-penetrating peptide, which sequestered the drug from its enzymatic target. Mtx remained active when conjugated to a mitochondrial targeting vector, and exhibited high levels of activity in the presence of clinically relevant Gram-positive pathogens. The combination of the antibacterial activity with lowered mammalian cell toxicity produced large improvements in the therapeutic indices for the DHFR inhibitor.

Clinically used antibacterial drugs target only a few processes (e.g., cell wall biosynthesis, DNA synthesis, and protein synthesis). This is largely due to the significant differences observed between these systems in prokaryotic and eukaryotic organisms. Using a mitochondrial targeting system to sequester small molecules allows for the inhibition of targets that have orthologs only found within the cytoplasm and nucleus of cells, diversifying the pathways and targets accessible to antimicrobial development. It is noteworthy that only drugs amenable to conjugations would be candidates for this approach, but this subset of compounds should provide a rich resource to explore the clinical utility of mitochondrial antimicrobial sequestration.

■ ASSOCIATED CONTENT

Supporting Information. Materials and Methods, supporting figures, and bacterial strain table are included in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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