A Trimethoprim Conjugate of Thiomaltose Has Enhanced Antibacterial Efficacy In Vivo

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Abstract

Trimethoprim is one of the most widely used antibiotics in the world. However, its efficacy is frequently limited by its poor water solubility and dose limiting toxicity. Prodrug strategies based on conjugation of oligosaccharides to trimethoprim have great potential for increasing the solubility of trimethoprim and lowering its toxicity, but they have been challenging to develop due to the sensitivity of trimethoprim to chemical modifications, and the rapid degradation of oligosaccharides in serum. In this report, we present a trimethoprim conjugate of maltodextrin termed TM-TMP, which increased the water solubility of trimethoprim by over 100 times, was stable to serum enzymes, and was active against urinary tract infections in mice. TM-TMP is composed of thiomaltose conjugated to trimethoprim, via a self-immolative disulfide linkage, and releases 4′-OH-trimethoprim (TMP-OH) after disulfide cleavage, which is a known metabolic product of trimethoprim and is as potent as trimethoprim. TM-TMP also contains a new

maltodextrin targeting ligand composed of thiomaltose, which is stable to hydrolysis by serum amylases and therefore has the metabolic stability needed for in vivo use. TM-TMP has the potential to significantly improve the treatment of a wide number of infections given its high water solubility and the widespread use of trimethoprim.

Graphical abstract
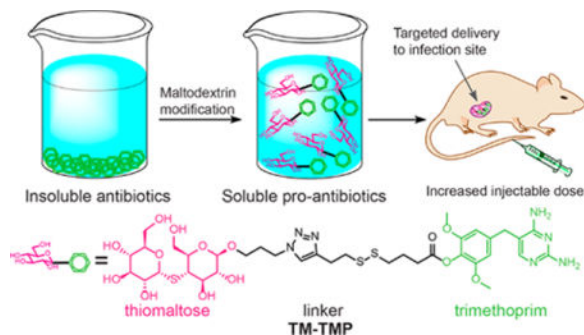
INTRODUCTION

Trimethoprim (TMP) is one of the most widely used antibiotics in the world,[1–4] and is used by millions of patients each year.[5] For example, TMP is used for the treatment of urinary tract infections (UTIs), Shigellosis, and Pneumocystis pneumonia, either alone or in combination with a sulfonamide.[6–10] However, despite its widespread use, TMP's efficacy is limited by its poor water solubility,[11] and drug delivery strategies that can improve the solubility of trimethoprim have the potential to impact multiple areas of medicine.[12–18] The poor water solubility of trimethoprim is a significant challenge for patients requiring intravenous dosing of trimethoprim, which is currently used for treating patients with Pneumocystis pneumonia, HIV patients, or patients with complicated UTIs. The current I.V. formulation for trimethoprim requires the use of organic solvents, which minimizes the dose of trimethoprim that can be given, and consequently, moderate levels of trimethoprim resistance cause significant medical problems.[8,10,19–21]

The conjugation of TMP to maltodextrins has great potential for improving the solubility and efficacy of TMP, due to the high water solubility of the maltodextrins and also their ability to target bacteria in vivo.[22,23] However, developing maltodextrin TMP conjugates has been challenging because TMP, like most antibiotics, is very sensitive to structural modifications and conjugation to an oligosaccharide will most likely destroy the efficacy of TMP.[24–26] In addition, maltodextrins are rapidly hydrolyzed in serum,[27–29] and this poses another challenge with using maltodextrins to increase the water solubility of TMP.

In this report we present a prodrug of trimethoprim termed TM-TMP, which is composed of thiomaltose conjugated to trimethoprim, via a self-immolative disulfide linkage. TM-TMP releases 4′-OH-trimethoprim (TMP-OH) after disulfide cleavage, which is a known metabolic product of trimethoprim and is as potent as trimethoprim (Figure 1).[30,31] TM-TMP contains a new maltodextrin targeting ligand composed of thiomaltose, which is stable to hydrolysis by serum amylases and therefore has the metabolic stability needed for in vivo

use. A patent has been filed by our laboratory for the use of thiomaltose for the diagnosis and treatment of bacteria infections.[32] A novel disulfide self-immolative linkage was also used to connect the thiomaltose to the trimethoprim, which is cleaved by thiols to release TMP-OH, via cyclization of the thiolate with the ester carbonyl (Figure 1B). TM-TMP should improve the efficacy of trimethoprim because it will enhance the water solubility of trimethoprim, and also concentrate trimethoprim to bacterially infected tissues, due to the exclusive expression of maltodextrin transporters on bacteria compared to mammalian cells. [33,34]

RESULTS AND DISCUSSION

Design of Thiomaltose-Trimethoprim (TM-TMP)

The synthesis of TM-TMP is shown in Figure 2. The key step in the synthesis of TM-TMP was the coupling of azidothiomaltose (**TM-N$_3$**) with the alkyne modified trimethoprim derivative (**2**). Briefly, trimethoprim was first selectively demethylated at its 4′ position and then coupled with a disulfide self-immolative linker through DCC/DMAP esterification. The synthesis of azido-thiomaltose is shown in Figure 3. The final product (**TM-TMP**) was synthesized via Cu-click chemistry and purified by reverse phase HPLC.

The solubility of trimethoprim in water is less than 1 mg/mL and therefore cannot be delivered to patients at high doses. We hypothesized that the thiomaltose motif in TM-TMP would greatly enhance the water solubility of trimethoprim and also prevent its unfavorable and nonspecific diffusion into mammalian cells. Therefore, we measured the octanol/water partition coefficient (LogP) and water solubility of TM-TMP and compared it to trimethoprim. The LogP of TM-TMP was determined to be −1.42, whereas the logP of TMP was 0.89. In addition, the solubility of TM-TMP in water was determined to be greater than 200 mg/mL; in contrast, the solubility of TMP in water was only 0.4 mg/mL. Therefore, TM-TMP increased the water solubility of TMP by over 250-fold and will enable much higher doses of trimethoprim to be given in vivo. In addition, the low LogP of TM-TMP and its large size should prevent TM-TMP from diffusing into mammalian cells, and this will further increase the dose that TM-TMP can be administered to patients.

Stability of Thiomaltose

A key challenge with using maltodextrins for bacterial targeting is their premature degradation in vivo, as there are numerous enzymes in human tissues, such as amylases and maltases, which are designed to cleave oligosaccharides. Therefore, we investigated the stability of thiomaltose against maltase, the enzyme in the kidney that is responsible for cleaving maltose in humans, and compared its stability to maltose. Thiomaltose or maltose were incubated with 10 units of maltase and the hydrolysis kinetics was determined by quantifying the glucose released. Figure 4 demonstrates that maltose is rapidly hydrolyzed under these conditions, and has a half-life of less than 30 min, whereas thiomaltose had under 1% hydrolysis after 3 h treatment. The thioacetal linkage of thiomaltose therefore significantly extends the stability of maltose to enzymatic hydrolysis and TM-TMP should have the enzymatic stability needed for in vivo use.

Cleavage Kinetics of TM-TMP

TM-TMP is designed to circulate in tissue, target bacteria, and then release free trimethoprim due to exogenous thiol mediated cleavage. We investigated the cleavage kinetics of TM-TMP in the presence of glutathione. TM-TMP was mixed with a 10 mM concentration of glutathione (GSH), in PBS, at 37 °C, and then analyzed via HPLC at 10 and 30 min. Figure 5A demonstrates that glutathione can cleave TM-TMP and catalyze the release of free TMP-OH. For example, TM-TMP was completely hydrolyzed after 10 min of incubation with glutathione, and under these conditions, it generated an equimolar concentration of free TMP-OH. In contrast, in the absence of GSH, TM-TMP was stable in PBS for at least 2 days without any observable degradation.

In addition, we also tested the stability of TM-TMP in serum. TM-TMP was mixed with fetal bovine serum (FBS), and the released TM-TMP was assayed via HPLC. Figure 5B demonstrates that TM-TMP has a half-life of approximately 1 h in serum, presumably due to the presence of thiols in the serum (around 500 μM).[35,36] TM-TMP is a low-molecular-weight hydrophilic compound that should rapidly diffuse through tissue, and therefore its 1 h serum stability half-life should be sufficient for it to target bacteria in vivo.

Uptake of Thiomaltose Conjugates

Thiomaltose contains a thioacetal linkage and the tolerance of oligosaccharide transporters to the thioacetal linkage has never been investigated. We therefore synthesized a fluorophore labeled thiomaltose conjugate, thiomaltose-perylene (TM-P, Figure 6A), to examine the uptake of TM-P and its specificity for bacteria. A 500 μL suspension of *E. coli* (O.D. = 0.5) was incubated with 20 μM TM-P for 2 h. The bacterial cells were washed with PBS and lysed. The fluorescence intensity of the sample was measured and normalized to the protein content. Similarly, 10^5 macrophage cells were incubated with 20 μM TM-P for 2 h and the cells were lysed. The specificity of TM-P for bacteria was determined by comparing the fluorescence intensity in bacteria versus macrophages, normalized to intracellular protein content. Figures 6B demonstrates that TM-P has high specificity for bacteria, as the uptake of TM-P was 98-fold higher in bacteria when compared to mammalian cells. Thus, the thioacetal linkage is well tolerated by oligosaccharide transporters, and thiomaltose has similar specificity for bacteria over mammalian cells as endogenous oligosaccharide targeting ligands, such as maltose and maltohexaose. We further tested the specificity of TM-P with lamB mutant *E. coli*, which is a mutant strain that lacks the outer membrane maltodextrin transporter lamB and cannot internalize maltodextrins.[37,38] As shown in Figure 6C, the mutant strain had a 2.5-fold decrease in uptake compared to wild-type *E. coli*. A fluorescent image of TM-P treated *E. coli* was also obtained to further validate the ability of *E. coli* to internalize TM-P (see Supporting Information, Figure S11).

Thiomaltose should be internalized by a wide variety of oligosaccharide transporters, such as the maltodextrin transporters (lamB and others) and other general pores (OmpF, OmpC, and others).[37] We compared the uptake of TM-P against maltohexaose conjugated to perylene (MH-P),[22] as it was previously reported that longer oligosaccharides were less effectively internalized by *E. coli*.[39] As shown in Figure 6D, the uptake of TM-P is about 4–5-fold higher than MH-P, which is consistent with previous studies demonstrating that

maltose is internalized more efficiently than maltohexaose.[39,40] In addition, TM-P was stable in serum, and showed no detectable degradation in serum after 24 h incubation with fetal bovine serum (FBS), whereas MH-P was rapidly degraded in FBS (see Supporting Information, Figure S8). Finally, HPLC analysis of the bacteria lysate from *E. coli* (BL21(DE3)) treated with TM-P demonstrated that it was stable in the intracellular environment of the bacteria, and remained as a single peak HPLC, even after internalization by bacteria (see Supporting Information, Figure S9). A similar thiomaltose conjugate, TM-IR780, was also synthesized to further prove the stability of thiomaltose conjugates (see Supporting Information, Figure S10).

In Vitro Activity of TMP-OH and TM-TMP

TM-TMP releases TMP-OH after reduction by thiols, which binds *E. coli* dihydrofolate reductase (DHFR) as tightly as TMP, and had a similar minimum inhibitory concentration (MIC) value against bacteria as TMP.[41] We observed that TMP-OH had a similar activity to TMP on a lab strain of *E. coli* (BL21(DE3)), and both TMP and TMP-OH had an MIC around 1 μM against BL21(DE3) (see Supporting Information, Figure S12). We therefore further tested the activity of TMP and TM-TMP on a clinical *E. coli* strain (*E. coli* 209) As expected, addition of 500 μM of GSH into the incubation broth significantly increased the efficacy of the TM-TMP, and the MIC of TM-TMP with GSH approached the MIC of free TMP, which was about 0.86 μM (Figure 7).

In Vivo Activity of TM-TMP in a UTI Mouse Model

We selected urinary tract infection (UTI) caused by uropathogenic *E. coli* as an initial testbed for TM-TMP. There is an urgent need for new UTI treatment strategies because of the increasing frequency of UTIs caused by drug-resistant *E. coli*. Furthermore, UTIs are the most common community-acquired bacterial infection in the world, and affect more than 250 million people globally.[42] Trimethoprim is one of the drugs used for treating UTIs, but it is frequently ineffective because of dose limiting toxicity and low water solubility, and therefore, strategies that can lower TMP's toxicity and increase its water solubility have great potential.

Here, mice were transurethrally infected with a 50 μL suspension containing $(1–2) \times 10^7$ of a clinical *E. coli* strain (*E. coli* 209), and the infection was allowed to develop for 2 days. The mice were then treated with either TMP or TM-TMP, injected via tail vein, at a dose of 10 mg/kg equivalent of TMP per mouse per day for 3 days. At day 5 of infection, the mice were sacrificed, and both kidneys and bladders were collected for enumeration of *E. coli* colony forming units (CFU).

Figure 8 demonstrates that TM-TMP is active in treating UTIs in a mouse model. For example, after 3 days of treatment, most of the bladders (7/9) from TM-TMP treated mice were cured and had no detectable bacteria, while in TMP treated mice, 5 mice had evidence of infection. Statistical analysis with the Mann–Whitney U test showed that the TM-TMP group was significantly different from the control group, while the TMP group was not. However, no statistical difference was observed between TM-TMP group and TMP group, due to the large errors in both groups. In the kidneys, TM-TMP treated mice had a lower

number of bacteria (777 CFUs/mL, geometric mean), while the TMP treated group had an average of 925 CFUs/mL and the untreated group's average was even higher at 5169 CFUs/mL, though no statistical significance was observed. The greater effect of TM-TMP on the bladder than the kidney infection may be due to higher concentrations of TM-TMP in the bladder than in the tissue of the kidneys.

CONCLUSIONS

In summary, we have developed a new trimethoprim prodrug composed of trimethoprim conjugated to thiomaltose. A disulfide self-immolative linker was developed to connect the thiomaltose with the trimethoprim. TM-TMP is stable under normal buffered conditions, has a half-life of about 1 h in complete serum, but is rapidly cleaved under high concentrations of glutathione, to release the free trimethoprim–OH. Thiomaltose is also fully resistant to enzymatic hydrolysis and is metabolically stable. TM-TMP has over 200 times better water solubility than unmodified trimethoprim, and has an MIC of around 1 μM on *E. coli* in the presence of 500 μM of glutathione. TM-TMP was shown to be at least as effective as TMP alone in the treatment of UTI in a mouse model, which validates the design of the prodrug. Thiomaltose conjugation via a self-immolative linkage provides a convenient method for increasing the solubility of antibiotics and improving their efficacy, and has the potential to be adapted to a wide variety of hydrophobic antibiotics.

EXPERIMENTAL PROCEDURES

General Methods

^{1}H NMR spectra were recorded in CDCl$_3$, CD$_3$OD, or D$_2$O on a Bruker AVB-400 spectrometer at 298 K. TMS (δ (ppm)$_H$ = 0.00) was used as the internal reference. ^{13}C NMR spectra were recorded in either CDCl$_3$, CD$_3$OD, and D$_2$O at a 100 MHz on a Bruker AVB-400 spectrometer, using the central resonances of CDCl$_3$ (δ (ppm)$_C$ = 77.0) and methanol (δ (ppm)$_C$ = 50.4) as the internal references. Chemical shifts are reported in ppm and multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), and m (multiplet). Coupling constants, *J*, are reported in hertz (Hz). High-resolution mass spectra (HRMS) were obtained on a AB SCIEX TOF/TOF 5800 system and are reported as *m/z* (relative intensity). Chemicals and solvents were purchased from Aldrich or VWR and used without further purification. All reactions were performed under anhydrous conditions under N$_2$ or argon and monitored by TLC on MD Millipore Silica Gel 60 F254 Glass Plates. Detection was accomplished by examination under UV light (254 nm) and by charring with 20% sulfuric acid in methanol.

Synthetic compounds were purified with either flash chromatography using silica gel (230–400 mesh) or reverse phase HPLC. The HPLC was performed on a Shimadzu system with a CBM-20A Prominence communication bus module, a SPD-M20A Prominence Diode Array detector, a DGU-20A5 Prominence degasser, and two LC-6AD pumps. The column used for the HPLC purification was an XBridge BEH C8 OBD prep column (5 μm, 19 × 150 mm).

Compound Synthesis

The synthesis and the characterization of new compounds are shown in detail in the Supporting Information.

LogP Analysis

To a solution of TM-TMP (200 μL of 100 μM in DI water) was added 200 μL of 1-octanol. The mixture was vortexed for 2 min and then left to stand until the two phases separated clearly. Both the aqueous phase and the organic phase were then analyzed with an analytical HPLC, monitoring at 254 nm. The column used for the HPLC analysis was an XBridge BEH C8 column (5 μm, 4.6 × 150 mm), and the elution was performed with 0.1% TFA in H$_2$O (A) and 0.1% TFA in acetonitrile (B). The gradient used was: 0–4 min, 5% B; 4–23 min, 5% to 100% B; 23–25 min, 100% B. The peak representing TM-TMP was integrated and the LogP was calculated as Log10(area$_{octanol}$/area$_{water}$).

Maltase Hydrolysis

To 100 μL of 20 μM of thiomaltose or maltose solution was added 100 μL of 20 unit/mL maltase (Sigma, α-Glucosidase from *Saccharomyces cerevisiae*) in PBS. The solution was incubated at room temperature, and the glucose concentration was determined using a glucose assay kit (Sigma) at 30, 60, 120, and 180 min, following the method described in the kit manual. This was done in triplicate and the average was reported.

Solubility of TM-TMP

To a 100 mg of lyophilized TM-TMP powder, 500 μL of water was added, which yielded a clear viscous solution.

Cleavage Kinetics of TM-TMP

To 90 μL of PBS or 10 mM GSH or FBS was added 10 μL of 1 mM TM-TMP in PBS. The solution was incubated at 37 °C and analyzed with HPLC using the same conditions used for the LogP analysis. For the TM-TMP cleavage in FBS, peaks representing TM-TMP and TMP-OH were integrated, and % TMP-OH released was calculated as the area$_{TMP-OH}$/(area$_{TM-TMP}$ + area$_{TMP-OH}$). Over the analytical time frame, the sum of two peak areas was constant, indicating 1:1 conversion of TM-TMP into TMP-OH.

Uptake of TM-P

To a 500 μL suspension of *E. coli* (BL21(DE3), O.D600 = 0.5) was added 10 μL of 1 mM TM-P or MH-P. The bacterial cells were incubated at 37 °C for 2 h and then centrifuged at 10 000 rpm for 5 min. The supernatant was discarded and the cell pellet was washed with 0.5 mL of PBS for 3 times, via centrifugation. The cell pellet was then lysed with 0.5 mL of bacterial lysis buffer (Thermo Scientific B-PER Bacterial Protein Extraction Reagents). The fluorescence intensity of the sample was measured and the protein concentration was determined using a Pierce BCA protein assay kit.

Similarly, 10^5 macrophage cells were incubated with 20 μM TM-P for 2 h and the cells were lysed with 0.5 mL of mammalian cell lysis buffer.

For the lamB mutant study, to a 1 mL suspension of *E. coli* (10^8 CFUs, control strain (ATCC 33456), or lamB mutant strain (JW3992-1, Yale CGSC)) was added 5 μL of 1 mM TM-P. The bacterial cells were incubated at 37 °C for 1 h and then centrifuged at 10 000 rpm for 5 min. The supernatant was discarded and the cell pellet was washed with 1 mL of PBS for 3 times, via centrifugation. The cell pellet was then suspended into 100 μL of PBS, and the fluorescent intensity was evaluated by using the plate readers.

In Vitro Antibacterial Activity

To a 96-well plate well containing 200 μL of LB medium (with or without 0.5 mM GSH) was added 1 μL of *E. coli* (OD600 = 0.5) and 2 μL of stock drug solution (TM-TMP in PBS and TMP-OH in DMSO). The plate was incubated at 37 °C, shaken at 190 rpm for 24 h, and the OD600 was measured using a Tecan Infinite 200 plate reader.

UTI Infection Model

Seven-week-old female C3H/HeOuJ (Jackson laboratories) were transurethrally infected with a 50 μL suspension containing $(1–2) \times 10^7$ CFU of *E. coli* under 3% isoflurane. Mice were deprived of water for 1 h and had urine expelled from their bladders immediately before inoculation. The animals were allowed to recover from anesthesia and were given water 1 h later. The experiment was divided into control and experimental groups, each group containing 9 mice. Control groups included mice untreated with any drug, and mice treated with trimethoprim (Chem-Impex International Inc. Wood Dale, IL), while the experimental group was treated with thiomaltose-trimethoprim (TM-TMP). The drugs were injected every 12 h for 3 days at concentration of 10 mg/kg, intravenously. Extraintestinal pathogenic *E. coli* 209 were used in this experiment and were isolated from a patient with a bloodstream infection. To prepare the inoculum, bacteria were grown under static conditions in Luria broth at 37 °C to induce the expression of type 1 pili as previously described.[43] *E. coli* 209 is trimethoprim sensitive.

Mice were euthanized at day 5 (after the end of treatment). The bladder and both kidneys of each animal were removed and homogenized in sterile PBS after which the homogenates were serially diluted and plated onto LB agar (Difco Laboratories) in triplicate. The number of *E. coli* colonies on each plate was counted after overnight incubation at 37 °C and was used to calculate the total number of bacteria in each bladder or pair of kidneys.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

ABBREVIATIONS

TM   thiomaltose

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TMP	trimethoprim
TM-TMP	thiomaltose trimethoprim conjugate
TM-P	thiomaltose perylene conjugate
TMP-OH	4′-OH-trimethoprim
MH-P	maltohexaose perylene conjugate
LogP	octanol/water partition coefficient
UTI	urinary tract infection
TM-N$_3$	azidothiomaltose
FBS	fetal bovine serum
GSH	glutathione
CFU	colony forming unit

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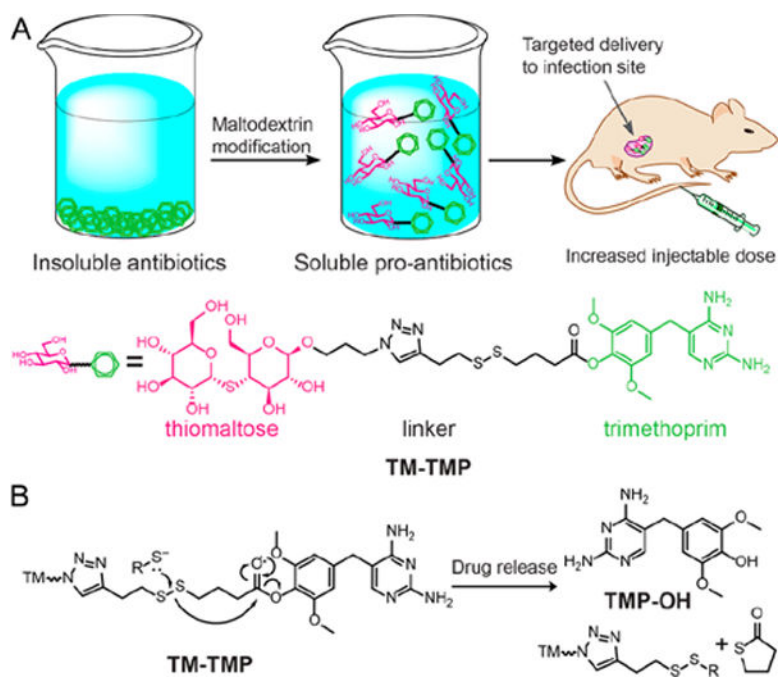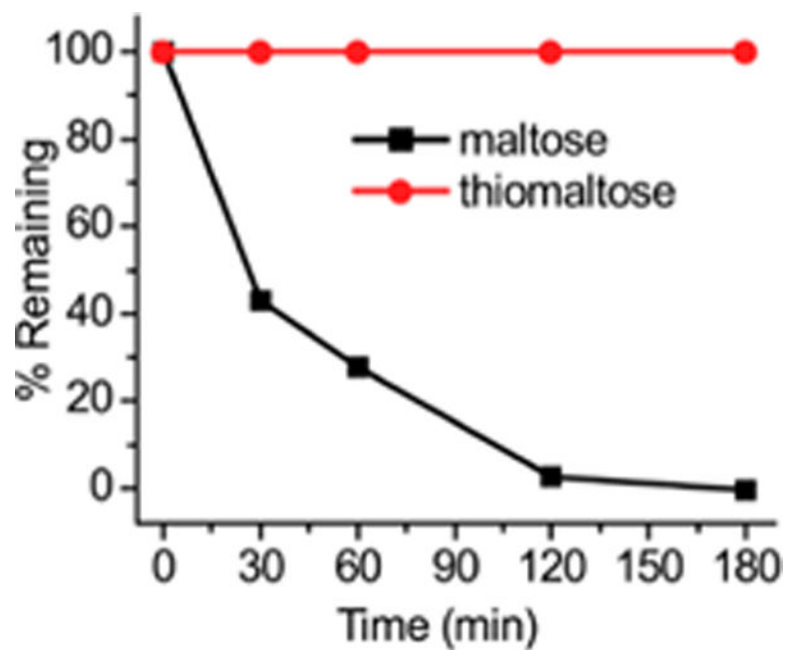
Figure 1.
(A) Thiomaltose trimethoprim (TM-TMP): A new trimethoprim prodrug with enhanced water solubility that targets bacteria. (B) Proposed cleavage of TM-TMP in the presence of exogenous thiols.
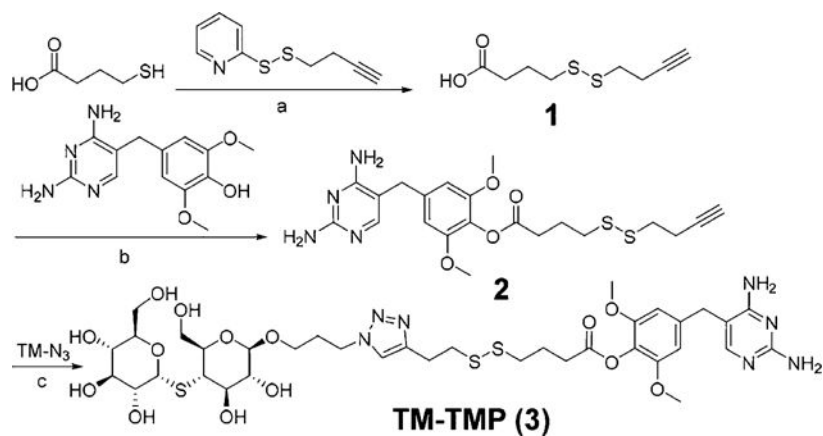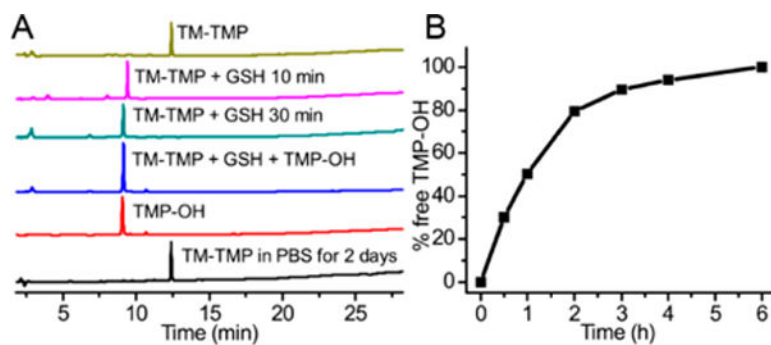
Figure 2.
Synthesis of thiomaltose-trimethoprim (**TM-TMP**). (a) DCM, TEA, rt, 55%. (b) DCC, DMAP, TEA, DMF, rt, 29%. (c) CuI, DIPEA, DMSO, rt, 65%.
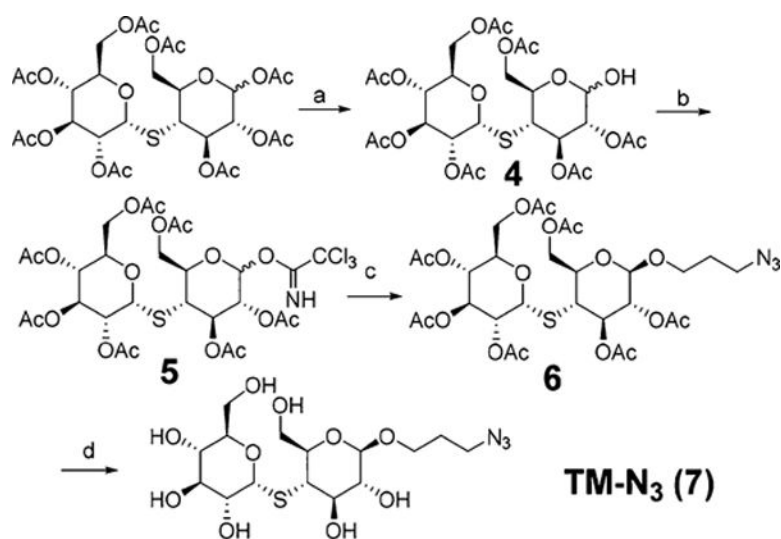
Figure 3.
Synthesis of azido-thiomaltose (**TM-N$_3$**). (a) NH$_2$NH$_2$·HOAc, DMF, rt, 81%. (b) Trichloroacetonitrile, DBU, DCM, rt, 97%. (c) Azidopropanol, TMSOTf, DCM, −20 °C, 45%. (d) MeONa, MeOH, quantitative.
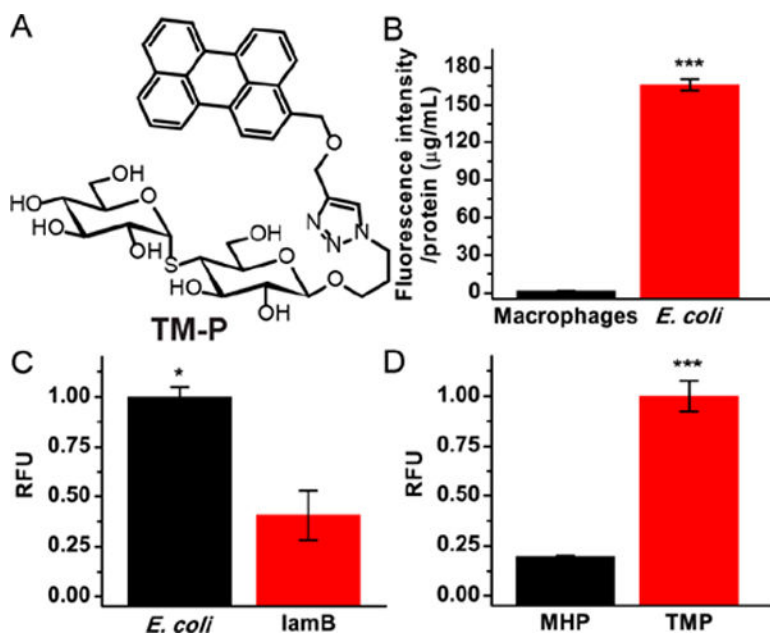
Figure 4.
Thiomaltose is stable to maltase hydrolysis. When incubated with maltase, thiomaltose (red) was less than 1% hydrolyzed, whereas maltose (black) was hydrolyzed completely in 3 h. Experiments were done in triplicate and error bars were too small to be visible.
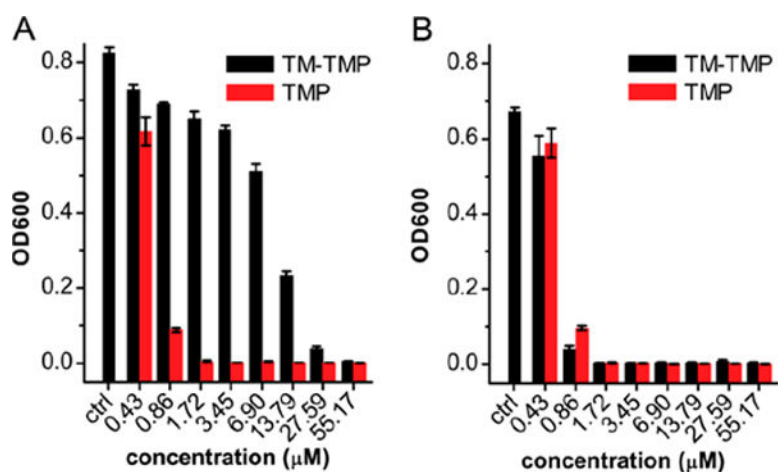
Figure 5.
Release kinetics of TM-TMP. (A) Chromatograms monitored at 254 nm of TM-TMP in water (yellow), TM-TMP (100 μM) incubated with 10 mM GSH for 10 min (purple) and 30 min (green), TM-TMP incubated with GSH mixed with TMP-OH (blue), TMP-OH in water (red), TM-TMP in PBS at 37 after 2 days (black). (B) Cleavage kinetics of TM-TMP (100 μM) in FBS.
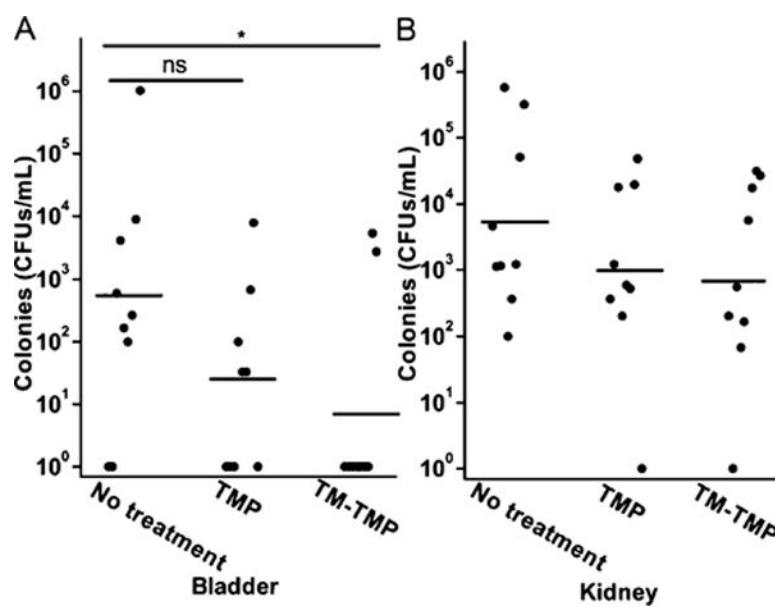
Figure 6.
Uptake studies using TM-P. (A) Structure of TM-P. (B) Uptake of thiomaltose-perylene in bacteria was 98-fold higher than in macrophages. (C) Uptake of thiomaltose-perylene in wild-type *E. coli* was 2.5-fold higher than that in lamB mutant. (D) Uptake of thiomaltose-perylene in bacteria was 6-fold higher than that of maltohexaose-perylene. The statistical significances were determined using a two-sample Student's *t*-test (*p 0.05, ***p 0.001).

Figure 7.
TM-TMP is active against *E. coli* and has similar efficacy to TMP in the presence of GSH.
In vitro activity of TM-TMP against *E. coli* 209. (A) OD600 of *E. coli* after 24 h incubation
with various concentrations of TM-TMP (black) and TMP (red), in the absence of GSH. (B)
OD600 of *E. coli* after 24 h incubation with various concentration of TM-TMP (black) and
TMP (red), in the presence of 500 μM of GSH.

Figure 8.
TM-TMP is active at treating UTIs in a mouse model. In vivo activity of TM-TMP against *E. coli* 209 in the UTI mouse model. (A) CFU counts of bladder samples. (B) CFU counts of kidney. Solid circles represent experimental results, and horizontal lines indicate the geometric mean. $*P < 0.05$; ns, not significant, one-tailed Mann–Whitney U test.