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Repurposing zidovudine and 5-fluoro-2′-deoxyuridine as antibiotic drugs made possible by synergy with both trimethoprim and the mitochondrial toxicity–reducing agent uridine

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Objectives: The increasing frequency of antibiotic-resistant bacterial infections is a major public health challenge, and new antibiotic drugs are urgently needed. A rapid solution to the problem is to repurpose clinically approved compounds with antibacterial properties, such as the nucleoside analogues zidovudine (azidothymidine) or 5-fluoro-2′-deoxyuridine. Here we report the *in vitro* and *in vivo* antibacterial properties of double and triple combinations of azidothymidine or 5-fluoro-2′-deoxyuridine with uridine and/or trimethoprim.

Methods: We determined MICs of azidothymidine and 5-fluoro-2′-deoxyuridine, alone or combined with uridine and/or trimethoprim, against a selection of Gram-negative and Gram-positive bacteria. We also measured MICs of a selection of antibiotics of different classes as a function of uridine concentration. The efficacy of azidothymidine and 5-fluoro-2′-deoxyuridine with uridine and/or trimethoprim was measured in a murine peritonitis infection model.

Results: The addition of uridine enhanced the *in vitro* antibacterial activity of azidothymidine and 5-fluoro-2′-deoxyuridine, against Gram-negative and Gram-positive bacteria, respectively. Uridine also enhanced the *in vitro* antibacterial activity of azidothymidine/trimethoprim and 5-fluoro-2′-deoxyuridine/trimethoprim combinations. Triple combinations containing azidothymidine, trimethoprim and uridine, showed antibacterial synergy against Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) whereas the 5-fluoro-2′-deoxyuridine, trimethoprim and uridine combination showed synergy against the Gram-positive *Staphylococcus aureus*. The positive effect of uridine on the efficacy of azidothymidine/trimethoprim combination was also observed *in vivo* in a murine *E. coli* peritonitis model.

Conclusions: Triple combinations of these clinically approved compounds warrant further investigations as therapies to combat antibiotic-resistant infections.

Introduction

The increasing prevalence of MDR pathogenic bacteria poses a growing threat to effective and safe human and animal healthcare. The WHO and CDC have compiled lists of priority pathogens, including both Gram-negative and Gram-positive bacteria, where effective antibiotic therapies already are severely limited and for which new antibacterial therapies are urgently required.[1,2] Despite this, the clinical development pipeline for new antibiotics is small compared with other therapeutic areas, and very few novel compounds are reaching the market.[3] The pipeline problem is mainly caused by the high cost of discovery and development of novel drugs, combined with the expected low pricing of antibiotic drugs, which make these products commercially non-profitable.[4] One approach to mitigate the costs and risks associated with drug development is drug repurposing, i.e. to investigate the

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use of drugs, already approved in human medicine, against other therapeutic indications.

Nucleoside analogues constitute a broad group of drugs and prodrugs that have found use in e.g. antiviral and anticancer therapies,[5] as exemplified by zidovudine (azidothymidine) and floxuridine (5-fluoro-2′-deoxyuridine, or 5-FdUrd). Azidothymidine is approved for the treatment of infections caused by HIV where its triphosphate effectively blocks viral replication, whereas 5-fluoro-2′-deoxyuridine is used in anticancer treatments, in particular against colorectal cancer but also against kidney and stomach cancer. Interestingly, azidothymidine and 5-fluoro-2′-deoxyuridine have also been reported to have antibacterial activity against Gram-negative and Gram-positive human pathogens, alone[6–14] and in combination with other antibiotics.[15–18] Thus, both azidothymidine and 5-fluoro-2′-deoxyuridine could potentially be repurposed for future use as antibiotic drugs, but these nucleoside analogues are associated with significant problems, including mitochondrial toxicity for azidothymidine[19–21] and bone marrow depression for 5-fluoro-2′-deoxyuridine,[22] which may discourage broadening their use as antibacterial treatments. Co-administration with uridine has been found to reduce known side effects, such as the mitochondrial toxicity, in HIV patients treated with azidothymidine.[23–25] Uridine has also been shown to reduce the toxicity of 5-fluorouracil, a 5-fluoro-2′-deoxyuridine metabolite, in cancer patients.[26]

Both azidothymidine and 5-fluoro-2′-deoxyuridine have effects on nucleic acid synthesis in prokaryotic and eukaryotic cells.[6,27–29] Phosphorylation of azidothymidine by a thymidine kinase results in azidothymidine-5′-triphosphate (AZTTP), which is a DNA chain terminator. Additionally, azidothymidine may competitively inhibit the thymidine kinase–catalysed phosphorylation of 2′-deoxythymidine (dT) to 2′-deoxythymidine-5′-monophosphate (dTMP). Hydrolysis of 5-fluoro-2′-deoxyuridine results in 5-fluorouracil, which is an inhibitor of thymidylate synthase, which catalyses the methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to dTMP. Thus, both azidothymidine and 5-fluoro-2′-deoxyuridine contribute to lowering the cellular levels of dTMP. An approved clinical antibiotic also affecting DNA synthesis by lowering the cellular levels of dTMP is trimethoprim (TMP). Trimethoprim is a dihydrofolate reductase (DHFR) inhibitor that stops the synthesis of tetrahydrofolic acid (THF), a precursor to N^5,N^{10}-methylene tetrahydrofolic acid (5,10-CH_2-THF), which in turn is a cofactor necessary for methylation of dUMP to dTMP by thymidylate synthase.[30]

When used together with trimethoprim, uridine was even found to increase the antibacterial effect when added to diverse laboratory media for sensitivity testing.[31–33] In summary, the antibacterial effects of azidothymidine, 5-fluoro-2′-deoxyuridine and trimethoprim, which all, at least in part, act by lowering the cellular levels of dTMP, along with the protective and potentiating effects of uridine, motivate investigating these compounds as prospective novel combination antibiotic drugs.

Thus, in the present study, we investigated the possibilities of repurposing the antiviral drug azidothymidine and the anticancer drug 5-fluoro-2′-deoxyuridine as antibiotic drugs in combination with trimethoprim and uridine. We present *in vitro* data showing strong synergistic effects of azidothymidine and 5-fluoro-2′-deoxyuridine, against Gram-negative and Gram-positive bacteria, respectively, when combined with trimethoprim or uridine, or with both trimethoprim and uridine. We further present data showing the same synergistic effects in a mouse infection model.

Material and methods

Bacterial pathogens and maintenance

For *in vitro* experiments, strains of *Acinetobacter baumannii* LMG 1041^T, *Escherichia coli* LMG 15862 and ATCC 25922, *Enterobacter cloacae* LMG 2783^T, *Klebsiella pneumoniae* LMG 20218, *Pseudomonas aeruginosa* LMG 6395 and *Staphylococcus aureus* LMG 15975 were purchased from Belgian Coordinated Collection of Microorganisms (BCCM/LMG, Gent, Belgium) whereas the strain of *Bacillus cereus* CCUG 7414^T was obtained from the Culture Collection of the University of Gothenburg (CCUG, Gothenburg, Sweden). Strains were maintained as advised by the respective culture collections or according to standard maintenance procedures and used in tests performed at SLU, Uppsala, Sweden (Tables 1 and 2; and Tables S1 and S2, available as Supplementary data at *JAC* Online). For *in vivo* experiments, *E. coli* ATCC 25922 was used as the infectious agent in the murine peritonitis model (see below).

Antimicrobial agents and maintenance

Nucleosides and antimicrobial agents used were: zidovudine (azidothymidine), 5-fluoro-2′-deoxyuridine, trimethoprim, aditoprim, ampicillin, aztreonam, brodimoprim, chloramphenicol, chlortetracycline, ciprofloxacin (CIP), erythromycin, kanamycin, meropenem, methicillin, penicillin G, polymyxin B, vancomycin and uridine (U), all purchased from Sigma Aldrich (Darmstadt, Germany). Rifampicin was purchased from Duchefa Biochemie (Haarlem, The Netherlands). Substances were stored and maintained as advised by manufacturers. For bioassay purposes substance stocks of 200 to 50 000 mg/L were prepared in deionized H_2O or in methanol (MeOH) depending on their solubility and stored at −20°C. Before MIC analyses fresh stocks of between 0.01 and 2000 mg/L in MeOH were prepared before application into wells of microtitre plates.

MIC determination

The MICs of all compounds/compound combinations were measured according to CLSI guidelines (M07-2012), with a few modifications, by a broth microdilution method in 96-well microtitre plates. Thymidine counteracts the antibiotic effect of DHFR inhibitors such as trimethoprim and therefore Mueller–Hinton broth, which contains beef extracts, was avoided to minimize the thymidine concentrations in the test media. The test media were either the defined medium AM3 Broth (BD Difco Ltd) mixed with PBS (Amresco LLC, Solon, USA) in a 1:1 ratio (*S. aureus*, *B. cereus*) or in AM3 mixed with PBS in a 1:4 ratio (all other organisms). Prior to MIC tests, the cell concentration of all tested pathogens was adjusted to between 1 and 5×10^5 cells/mL by dilution of deep-frozen stock cultures, previously subjected to viable cell counting. Viable cell counting was done on Vegetable Peptone Broth (VPB, Oxoid Ltd) agar plates (VPA-plates, 10 g VPB, 15 g agar in 1000 mL deionized H_2O), which were incubated at 37°C in the darkness after inoculation,

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Table 1. MICs of azidothymidine (AZT), 5-fluoro-5-deoxyuridine (5-FdUrd) and trimethoprim (TMP), against Gram-negative and Gram-positive strains, as a function of uridine concentration

		MIC of tested antibiotic drugs, mg/L)			
		Uridine, mg/L			
Antibiotic drug	Pathogen	0	100	300	500
AZT	*E. coli* LMG 15862	32	16	8	8
	S. aureus LMG 15975	>32	>32	>32	>32
	E. coli ATCC 25922	16	8	4	4
	E. cloacae LMG 2783^T	16	8	8	8
	K. pneumoniae LMG 20218	32	16	16	8
	A. baumannii LMG 1041^T	>32	>32	>32	>32
	P. aeruginosa LMG 6395	>32	>32	>32	>32
	B. cereus CCUG 7414^T	>32	>32	>32	>32
5-FdUrd	*E. coli* LMG 15862	>32	>32	>32	32
	S. aureus LMG 15975	16	4	0.12	0.12
	E. coli ATCC 25922	>32	>32	>32	32
	E. cloacae LMG 2783^T	>32	>32	>32	32
	K. pneumoniae LMG 20218	>32	>32	>32	32
	A. baumannii LMG 1041^T	>32	>32	>32	>32
	P. aeruginosa LMG 6395	>32	>32	>32	>32
	B. cereus CCUG 7414^T	>32	>32	16	16
TMP	*E. coli* LMG 15862	16	16	1	1
	S. aureus LMG 15975	>32	>32	0.25	0.25
	E. coli ATCC 25922	32	32	4	2
	E. cloacae LMG 2783^T	32	32	2	2
	K. pneumoniae LMG 20218	>32	>32	16	16
	A. baumannii LMG 1041^T	8	8	8	8
	P. aeruginosa LMG 6395	>32	>32	>32	>32
	B. cereus CCUG 7414^T	>32	>32	>32	>32

and viable colonies counted after 16 to 24 h. Cell concentrations were always confirmed by viable cell counting directly after MIC tests. In order to estimate MICs, the appropriate sample volumes of tested compounds and/or their combinations out of the stock solutions of between 0.01 and 2000 mg/L in MeOH were dispensed into the wells of flat-bottomed microtitre plate(s) and the solvent was evaporated in a fume-hood to avoid any antibacterial effects from MeOH. For synergy MIC determinations, the compounds to be tested were pipetted into wells of 96-well microtitre plates separately, and the solvent (MeOH) was evaporated between each pipetting step. Afterwards, suspensions of pathogen cells in the appropriate medium were dispensed to the wells of microtitre plates and the growth of pathogens was monitored after 16 to 20 h of incubation at 37°C in the darkness. Positive controls comprised respective pathogen cells suspended in untreated sterile appropriate media without addition of compounds(s), and negative controls comprised the medium only. The MIC was defined as the lowest concentration of each compound and/or compound combination with no visible growth of pathogen. All MIC tests were performed in duplicate and repeated at least twice.

The impact of thymidine on the MIC of mixtures of azidothymidine, trimethoprim and uridine, against *E. coli* ATCC 25922, was measured in a separate experiment. The MIC for azidothymidine/trimethoprim mixtures (1:1 ratio) in the presence of 50 mg/L uridine and 0 mg/L or 2 mg/L thymidine, was determined according to CLSI guidelines. Compounds, in water or DMSO, were diluted in Mueller–Hinton broth, and were added to microtitre plates. Fresh overnight colonies of *E. coli* ATCC 25922 from a 5% horse blood agar plate were suspended to a turbidity of 0.5 McFarland and further diluted to 1×10^6 cfu/mL in Mueller–Hinton broth. A total of 100 µL diluted bacterial suspensions were added to wells containing 100 µL of test solutions, and the plates were incubated at 35°C for 16–20 h.

Efficacy in a murine peritonitis infection model

The *in vivo* effect of azidothymidine, trimethoprim and their combinations with uridine was tested in two experiments (1 and 2 below) in a murine peritonitis infection model at a contract laboratory, performed in agreement with the policies and ethical regulations applying to animal experiments. Thirty-eight and 50 outbred NMRI female mice (4 mice per treatment, 26–30 g, Taconic) were used in experiments 1 and 2, respectively. *E. coli* ATCC 25922 was used as infectious agent in both experiments.

Fresh overnight colonies of *E. coli* ATCC 25922 from a 5% horse blood agar plate were suspended and diluted in sterile saline with 10% mucin to approximately 1×10^6 cfu/mL with final

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Table 2. MICs of azidothymidine (AZT) against *E. coli* LMG 15862, *E. coli* ATCC 25922 and *K. pneumoniae* LMG 20218, and MICs of 5-fluoro-2′-deoxyuridine (5-FdUrd) against *S. aureus* LMG 15975, as a function of trimethoprim (TMP) concentration (rows) and uridine concentration (columns)

Comb. with AZT or 5-FdUrd	Pathogen	MIC (mg/L) for AZT or 5-FdUrd combined with TMP and uridine				
		TMP (mg/L)	uridine (mg/L)			
			0	100	300	500
A) AZT	*E. coli* LMG 15862 β-Lactamase producer MIC (TMP): 16 mg/L	0	32	16	8	8
		0.03	16	16	2	1
		0.06	4	2	1	0.5
		0.12	1	1	0.5	0.25
		0.25	0.25	0.25	0.06	0.03
	E. coli ATCC 25922 MIC (TMP): 32 mg/L	0	16	8	4	4
		0.03	16	2	1	2
		0.06	4	1	1	1
		0.12	1	0.25	0.5	0.5
		0.25	0.5	0.25	0.25	0.06
	K. pneumoniae LMG 20218 β-Lactamase producer MIC (TMP): >32 mg/L	0	32	16	16	8
		0.06	32	32	4	4
		0.12	8	8	2	1
		0.25	8	2	2	1
		0.5	1	0.25	0.25	0.03
		1	0.25	0.25	0.06	0.03
B) 5-FdUrd	*S. aureus* LMG 15975 Erythromycin resistant MIC (TMP): >32 mg/L	0	16	4	0.12	0.12
		0.015	8	2	0.12	0.12
		0.03	8	2	0.12	0.12
		0.06	8	2	0.12	0.12
		0.12	8	2	0.06	0.06
		0.25	8	2	0.03	0.03

Explanatory arrows: AZT/5-FdUrd MIC changes caused by increased (a) TMP concentrations, (b) uridine concentrations, (c) TMP and uridine concentrations.

concentration of 5% mucin. Approximately 1 h before inoculation, mice were treated orally with 45 µL Nurofen (20 mg ibuprofen/mL corresponding to approximately 30 mg/kg) as a pain relief. Inoculation was performed by intraperitoneal injection of 0.5 mL of the above *E. coli* ATCC 25922 suspension. Afterwards, the mice were treated subcutaneously in the neck region with a 0.2 mL single dose of compound at 1 h post infection. The dosing was based on a mean weight of 28 g. The mice were sacrificed after 1 h or 5 h post infection, and 2 mL sterile saline was injected intraperitoneally and the abdomen gently massaged before it was opened and fluid sampled with a pipette. Each sample was diluted 10-fold in saline, and 20 µL spots were applied and spread on agar plates. All agar plates were incubated for 18–22 h at 35°C in ambient air, and colonies were counted to establish cfu/mL. The boxplot was generated in R using package ggplot2. Statistical analysis, to find significant differences between treatments, was by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test using R. Multi-way ANOVA (in R) was used to analyse which of the categorical variables (concentrations of azidothymidine, trimethoprim, uridine and ciprofloxacin, respectively) significantly affected the cfu readings. Mice were monitored for clinical signs of toxicity after treatment. As positive control, treatment with ciprofloxacin was used (14 mg/kg in experiment 1 and 21 mg/kg in experiment 2), and as negative control treatment vehicle only was

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used. Ciprofloxacin was chosen as the reference compound because it is a potent inhibitor of *E. coli* ATCC 25922 and works well in the peritonitis infection model with this mouse breed (it is not a benchmark for the experimental compounds). Clinical symptoms in mice were scored according to a 0–6 score scale based on their behaviour and clinical signs where score 0 = healthy; score 1 = minor clinical signs of infection (slower movements, light piloerection in the skin); score 2 = moderate signs of infection (lack of curiosity or changed activity, piloerection in the skin, changed body position); score 3 = severe signs of infection (reduced movements, piloerection in the skin, slightly pinched eyes, tucked up belly, changed body position); score 4 = severe signs of infection (stiff movements, piloerection in the skin, pinched eyes, cold, pain); score 5 = the mouse does not move, is cold, lying on its side; and score 6 = the mouse is dead.

Results and discussion

The effect of uridine on the MIC of azidothymidine, 5-fluoro-2′-deoxyuridine, trimethoprim and selected other antibiotics against E. coli *and* S. aureus

The antibacterial properties of nucleoside analogues such as azidothymidine and 5-fluoro-2′-deoxyuridine are well documented[7,11,12,15] as also is the effect of uridine and its derivatives on the toxicity of azidothymidine and 5-fluoro-2′-deoxyuridine.[24,26] The application of these nucleosides together with uridine thus seems reasonable as it could minimize the toxicity of the nucleosides and increase the possibility of their repurposing as treatment against resistant human pathogens. An obvious prerequisite for combining azidothymidine or 5-fluoro-2′-deoxyuridine with uridine in an antibiotic combination drug, is that uridine does not antagonize the antibacterial effect of these compounds. Accordingly, to investigate the impact of uridine on the antibacterial effect of azidothymidine and 5-fluoro-2′-deoxyuridine, these nucleoside analogues were tested against the Gram-negative *E. coli* LMG 15862 (penicillin-resistant strain) and the Gram-positive *S. aureus* LMG 15975 (MRSA strain) as a function of uridine concentration (Table 1). Additionally, several commercial antibiotics of different classes, including trimethoprim, were tested in parallel (Table 1 and Table S1).

Several of the 18 tested antibiotics showed a strong MIC dependence on the presence of uridine, with a MIC reduction of at least two 2-fold dilutions (Table 1 and Table S1). Uridine reduced the MIC of the DHFR inhibitors trimethoprim, aditoprim and brodimoprim against both tested pathogens, exemplified by the MIC of trimethoprim dropping from 16 to 1 mg/L against *E. coli* LMG 15862 and from >32 to 0.25 mg/L against *S. aureus* LMG 15975, when the uridine concentration was increased from 0 to 300 mg/L uridine or more. In a previous study, MICs of trimethoprim were determined for strains of *E. coli*, *Aerobacter cloacae* (=*Enterobacter cloacae*) and *P. aeruginosa* in increasing concentrations of uridine.[32] For the *E. coli* strain, the MIC decreased from 10 mg/L to 1.25 mg/L, when uridine was increased from 10 mg/L to 100 mg/L, whereas for *E. cloacae*, the corresponding decrease was from 80 mg/L to 1.25 mg/L. These MIC reductions, due to increasing uridine concentrations, were larger than the reductions observed in the present study.

The two nucleoside analogues azidothymidine and 5-fluoro-2′-deoxyuridine showed different MIC responses in the presence of uridine. For azidothymidine, the *E. coli* MIC dropped from 32 to 8 mg/L when the uridine concentration was increased from 0 to 300 mg/L, whereas uridine had no effect on *S. aureus* azidothymidine MIC (Tables 1 and S1). For 5-fluoro-2′-deoxyuridine, the results were the opposite, i.e. no MIC effect was observed against *E. coli*, whereas a pronounced MIC reduction was observed against *S. aureus*, with MIC changing from 16 to 0.12 mg/L, in the presence of 300 mg/L uridine or more (Tables 1 and S1). Importantly, the addition of uridine did not have a general effect on MICs across all classes of antibiotics but was specific for DHFR inhibitors (Table S1).

Similar results were obtained when the MICs of azidothymidine, 5-fluoro-2′-deoxyuridine and trimethoprim as a function of increasing concentrations of uridine were measured against an extended panel of microorganisms (Table 1). The MICs of azidothymidine against the Gram-negative strains *E. coli* ATCC 25922, *K. pneumoniae* LMG 20218 and *E. cloacae* LMG 2783^T were reduced in the presence of uridine, whereas the MIC of 5-fluoro-2′-deoxyuridine was reduced against the Gram-positive strain *B. cereus* CCUG 7414^T (Table 1). The tested isolate of *A. baumannii* was one exception and the uridine concentration did not affect the MIC against azidothymidine, 5-fluoro-2′-deoxyuridine or trimethoprim (Table 1). The same observation was made for the isolate of *P. aeruginosa* in this study (Table 1), but also in a previous study when a *P. aeruginosa* isolate was tested against trimethoprim in increasing concentrations of uridine resulting in no observable effect of the uridine.[32]

The effects of triple combinations of azidothymidine or 5-fluoro-2′-deoxyuridine with trimethoprim and uridine on the MICs for selected bacterial pathogens

The observation that the addition of uridine reduced the MICs of azidothymidine, 5-fluoro-2′-deoxyuridine and trimethoprim, prompted us to test the effects on MICs of triple combinations of azidothymidine or 5-fluoro-2′-deoxyuridine, with trimethoprim and uridine, combining compounds targeting different components of bacterial dT metabolism. Azidothymidine in combination with trimethoprim and uridine was tested against two strains of *E. coli* and one strain of *K. pneumoniae*. 5-Fluoro-2′-deoxyuridine in combination with trimethoprim and uridine was tested against one strain of *S. aureus*. For each strain the MIC of azidothymidine or 5-fluoro-2′-deoxyuridine was measured at several fixed concentrations of trimethoprim, and for each trimethoprim concentration the uridine concentrations were varied between 0 and 500 mg/L (Table 2). For azidothymidine against *E. coli* and *K. pneumoniae*, without added uridine, there was synergy with trimethoprim. The azidothymidine MICs dropped from >32, 16 and 32 mg/L, respectively, down to 0.25, 0.5 and 0.25 mg/L at the trimethoprim concentrations 0.25, 0.25 and 1 mg/L, respectively (Table 2, part A, uridine ‘0’ column). The azidothymidine MIC improvements were thus in the range 32-fold to >128-fold. The trimethoprim MICs for these isolates were 16, 32 and >32 mg/L, respectively. With the addition of uridine at 500 mg/L, the azidothymidine

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MICs dropped further, to 0.03, 0.06 and 0.03 mg/L at the trimethoprim concentrations 0.25, 0.25 and 1 mg/L, respectively (Table 2, part A, uridine '500' column). This corresponds to a further 8-fold improvement of the azidothymidine MIC by the addition of uridine. The total azidothymidine fold-MIC improvements associated with the addition of trimethoprim and uridine, in the tested concentration ranges, were between 270-fold and >1070-fold.

For 5-fluoro-2′-deoxyuridine against *S. aureus*, without added uridine, the MIC improvement by trimethoprim addition was less pronounced. 5-Fluoro-2′-deoxyuridine MIC was reduced from 16 mg/L to 8 mg/L at the trimethoprim concentration 0.25 (Table 2, part B, uridine '0' column). The trimethoprim MIC for this isolate was >32 mg/L. However, with uridine present at 500 mg/L, the synergy was more pronounced with the 5-fluoro-2′-deoxyuridine MIC decreasing from 0.12 mg/L without trimethoprim, to 0.03 mg/L at 0.25 mg/L trimethoprim, i.e. a 4-fold MIC improvement. The total MIC lowering for 5-fluoro-2′-deoxyuridine by the addition of trimethoprim and uridine was approximately 530-fold.

Possible rationale for the synergistic effect on MICs of azidothymidine or 5-fluoro-2′-deoxyuridine in combination with trimethoprim and uridine

Azidothymidine, 5-fluoro-2′-deoxyuridine, trimethoprim and uridine each affect bacterial dT metabolism (Figure 1). Azidothymidine competes with dT for phosphorylation (Figure 1, step 1), resulting in a lowered level of dT available for stepwise phosphorylation to 2′-deoxythymidine-5′-triphosphate (dTTP) and subsequent incorporation into DNA, but also AZTTP, which is a DNA polymerization terminator (Figure 1, step 2). Trimethoprim is a DHFR inhibitor that blocks the synthesis of THF (Figure 1, step 3), which is necessary for the production of 5,10-CH_2-THF, which in turn is the precursor for methylation of dUMP to dTMP by the enzyme thymidylate synthase. Uridine has been shown to inhibit the enzyme thymidine phosphorylase,[34] which catalyses the formation of dT from thymine, thus also lowering the levels of dT available for phosphorylation to dTTP (Figure 1, step 4). Thus, for the combination azidothymidine, trimethoprim and uridine, there are three different established mechanisms all leading to lowering of the dTTP levels in the bacterial cells, and in addition the DNA polymerization termination caused by AZTTP, leading to the strong synergy observed. 5-Fluoro-2′-deoxyuridine will be phosphorylated to 5-fluoro-2′-deoxyuridine-5′-monophosphate, which is an inhibitor of thymidylate synthase (Figure 1, step 5), also resulting in lowered levels of dTTP for DNA synthesis. Thus, when 5-fluoro-2′-deoxyuridine is combined with trimethoprim and uridine, there are also three different mechanisms that lead to inhibited DNA synthesis due to lack of dTTP, resulting in strong synergy between the components. Another possible effect of uridine, which would further strengthen the synergy between the components, is competitive inhibition of dT uptake by the bacterial cell (Figure 1, step 6), assuming that dT and uridine enter the bacterial cell using the same machinery.

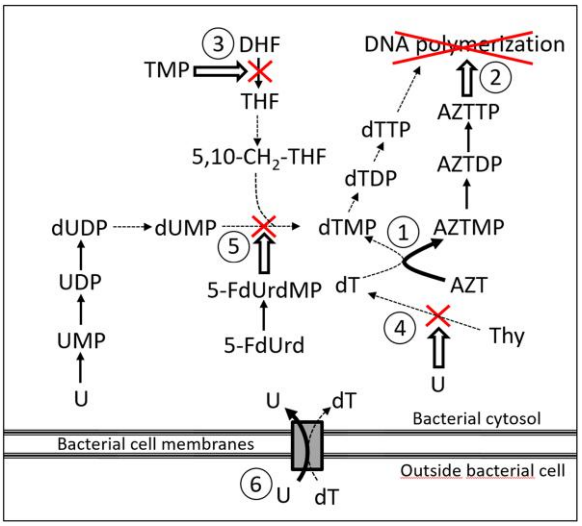

Figure 1. Proposed mode of action for triple combination of azidothymidine (AZT) or 5-fluoro-2′-deoxyuridine (5-FdUrd) with trimethoprim (TMP) and uridine (U). (1) By competition, AZT reduces the amount of 2′-deoxythymidine-5′-monophosphate (dTMP) available for DNA synthesis. (2) AZT is phosphorylated to AZT-5′-triphosphate (AZTTP), which is a DNA polymerase inhibitor. (3) TMP inhibits the formation of tetrahydrofolic acid (THF) from dihydrofolic acid (DHF). THF is transformed into 5,10-methylenetetrahydrofolate (5,10-CH_2-THF), which is used for the formation of dTMP from 2′-deoxyuridine-5′-monophosphate (dUMP). (4) U inhibits the formation of 2′-deoxythymidine (dT) from thymine (Thy). (5) 5-FdUrd is phosphorylated to 5-FdUrd-5′-monophosphate (5-FdUrdMP), which inhibits the methylation of dUMP to dTMP. (6) U may via competition reduce the bacterial uptake of dT.

Effects of azidothymidine, trimethoprim and uridine on peritoneal E. coli *infection in mice*

Inspired by the promising *in vitro* results from combining azidothymidine, trimethoprim and uridine against *E. coli*, we decided to have this combination tested for efficacy against *E. coli* ATCC 25922 in a murine peritonitis infection model in two different studies. The first study investigated the effects of increasing azidothymidine and trimethoprim doses while keeping uridine at a fixed concentration (Figure 2, part 1). Compared with single doses of azidothymidine or trimethoprim at 30 mg/kg, the 10/10/50 mg/kg treatment with AZT/TMP/uridine gave approximately two orders of magnitude lower cfu ($P<0.05$ for both comparisons), clearly demonstrating the potency of these triple combinations compared with treatment with only azidothymidine or trimethoprim at higher concentrations. It was apparent that increasing the AZT/TMP/uridine dosing, from 1/1/50 mg/kg, respectively, up to 10/10/50 or 30/30/50 mg/kg, increased the antibacterial effect of the treatment by around two orders of magnitude ($P<0.01$ and $P<0.05$, respectively). In the second study, azidothymidine and trimethoprim doses were kept constant, while uridine doses were increased (Figure 2, part 2). The combination AZT/TMP/uridine at 3/3/300 mg/kg gave around 3.8 orders of magnitude lower cfu ($P<0.01$) than single-compound treatment with trimethoprim, azidothymidine or uridine at 3, 3 or 300 mg/kg, respectively, again showing the potency of the triple combination. The data for the triple combinations, as presented in Figure 2 (part 2), suggest a dose–response behaviour for uridine, but the cfu reductions (1.0 and 1.7 orders of magnitude, respectively) caused by increasing the uridine dose

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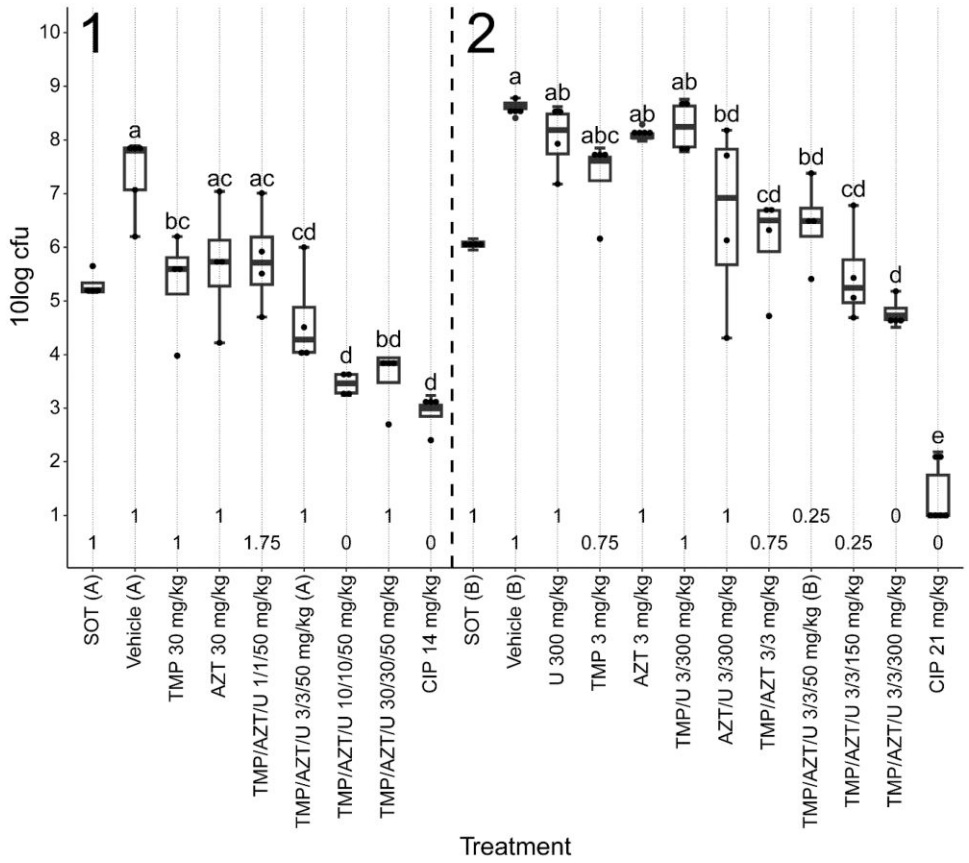

Figure 2. Efficacy of treatments with azidothymidine (AZT), trimethoprim (TMP) and uridine (U), as single compounds or in double or triple combinations, on *E. coli* ATCC 25922 in a murine peritonitis model compared with vehicle (negative control) and ciprofloxacin (CIP, positive control). Part 1 presents data from an AZT and TMP dose dependence study. Part 2 presents data from a uridine dose dependence study. Boxes represent first and third quartiles. Whiskers show minimum and maximum values. Black bars represent median values. Letters a–e indicate significant differences ($P < 0.05$), with treatments sharing letters not being significantly different. Numbers at the bottom show mean clinical scores at termination. SOT, start of treatment.

from 50 to 150 and 300 mg/kg were not significant. However, when the data were analysed by multi-way ANOVA to estimate how the cfu values depend on the concentrations of trimethoprim, azidothymidine, uridine and ciprofloxacin as categorical variables, this linear model predicts uridine and ciprofloxacin to significantly influence the cfu readings ($P < 0.01$ and $P << 0.001$, respectively). Similar results ($P < 0.01$ and $P << 0.001$ for uridine and ciprofloxacin, respectively) were obtained when the azidothymidine and trimethoprim dose dependence experiment (Figure 2, part 1) was analysed using multi-way ANOVA. These *in vivo* results support the synergies observed *in vitro*, and highlight the effect of uridine in these triple combinations. It should be noted that the previously reported alleviation of azidothymidine toxicity by uridine[23–25] was not evaluated in the current study, but only the antibacterial effect resulting from adding uridine to azidothymidine and/or trimethoprim.

High levels of thymidine have been reported to cause underestimation of the *in vitro* efficacy of trimethoprim and related DHFR inhibitor antibiotics.[35] Mice have been reported to have high tissue concentrations of thymidine,[36–38] which may lead to an underestimation of the efficacy of the combinations of trimethoprim with azidothymidine and uridine in the murine infection model (Figure 2). To test if thymidine causes an underestimation of the potency of the triple combination AZT/TMP/uridine, the MIC (fixed ratio 1:1 between azidothymidine and trimethoprim, and uridine fixed at 50 mg/L) was determined against *E. coli* ATCC 25922, in the absence or presence of thymidine at 2 mg/L. The MIC value for *E. coli* ATCC 25922 was 0.06/0.06/50 mg/L without thymidine and 0.5/0.5/50 mg/L in the presence of 2 mg/L thymidine, i.e. ~8-fold higher in the presence of thymidine. The MICs for single compounds azidothymidine and trimethoprim were 0.5 and 0.5 mg/L, respectively, without thymidine, and >2 and 1 mg/L, respectively, in the presence of 2 mg/L thymidine. These results clearly show that the presence of thymidine will underestimate the potency of these triple combinations, just as previously shown for trimethoprim,[35] and also for other DHFR inhibitors. Murine models are standard for initial drug testing, but these results suggest that murine models, with high thymidine levels, are suboptimal for evaluation of these triple combinations, just as previously observed for single treatment with trimethoprim.[39] Another possible problem with this murine model is the use of ciprofloxacin as positive control.

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Recently, thymine was shown to potentiate ciprofloxacin killing of *E. coli* ATCC 25922 in an insect infection model.[40] If the elevated thymidine level in mice is accompanied by a high concentration of thymine, this might boost the activity of ciprofloxacin in this infection model, leading to underestimation of the antibiotic activity of the tested compounds.

The serum or plasma concentrations of azidothymidine, trimethoprim or uridine obtained in the mice of this study were not measured. However, human serum or plasma concentrations corresponding to the MICs against the tested *E. coli* strains for the AZT/TMP/uridine combination (Table 2), i.e. much lower than 1 mg/L for azidothymidine and trimethoprim, can easily be obtained by oral or IV administration,[27,30] and 300–500 mg/L for uridine can be obtained IV[26] or by oral intake of the prodrug uridine triacetate.[41] The cfu reductions obtained in the murine infection model, at azidothymidine, trimethoprim and uridine dosing considered safe for humans,[23,27,30] can be expected to be further enhanced if tested in animal models with lower tissue thymidine levels such as dogs and pigs, or in humans.

Conclusions

The nucleoside analogues azidothymidine and 5-fluoro-2′-deoxyuridine have well-known antibiotic properties, but their innate toxicity limits their possible use as antibiotics. Uridine has previously been shown to counteract the toxicity of azidothymidine and 5-fluoro-2′-deoxyuridine,[23–26] and is used clinically for this purpose. We show that combinations of azidothymidine and 5-fluoro-2′-deoxyuridine with uridine also increase the antibacterial potency of each of the drugs. Azidothymidine/uridine and 5-fluoro-2′-deoxyuridine/uridine combinations have several-fold lower MICs for Gram-negative and Gram-positive bacteria, respectively, compared with azidothymidine and 5-fluoro-2′-deoxyuridine alone. Addition of the DHFR inhibitor trimethoprim to the azidothymidine/uridine and 5-fluoro-2′-deoxyuridine/uridine combinations, improved the MICs substantially further. The same trends for the double and triple combinations were valid also in a murine peritonitis infection model. A detailed understanding of the mechanisms behind the observed synergy, and in particular the differences between Gram-positive and Gram-negative synergistic relationships, may be very important for the optimization of these drug combinations towards possible clinical use, but this was outside the scope of the current study. Azidothymidine, 5-fluoro-2′-deoxyuridine and uridine are all clinically approved compounds, and therefore, these triple combinations should have a short way to the clinic, which warrants further investigations as therapies to combat antibiotic-resistant infections.

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Transparency declarations

J.J.L., J.B., B.G., C.N., A.B. and B.Ö. are shareholders of Ultupharma AB, which holds the intellectual property rights to the published findings. J.J.L. and J.B. were employed by Ultupharma AB during the end of this study, and B.Ö. is CEO of Ultupharma AB. Ultupharma AB approved the publication of the data. Nothing to declare for S.C. and D.H.

Supplementary data

Tables S1 and S2 are available as Supplementary data at *JAC* Online.

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