Drug Repurposing: Phosphate Prodrugs of Anticancer and Antiviral FDA-approved Nucleosides as Novel Antimicrobials

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Objectives

Following a drug repurposing approach we aimed to investigate and compare the antibacterial and antibiofilm activities of different classes of phosphate prodrugs (HepDirect, cycloSal, SATE and mix-SATE) of antiviral and anticancer FDA-approved

nucleoside drugs (AZT, FUDR and GEM) against a variety of pathogenic Gram-positive and Gram-negative bacteria.

Methods

Ten prodrugs were synthesised and screened for antibacterial activity against 7 Gram-negative and 2 Gram-positive isolates fully susceptible to traditional antibiotics, alongside 6 Gram-negative and 5 Gram-positive isolates with resistance mechanisms. Their ability to prevent and eradicate biofilms of different bacterial pathogens in relation to the planktonic growth inhibition was evaluated as well, together with their effect on proliferation, viability and apoptosis of different eukaryotic cells.

Results

In general the prodrugs show decreased antibacterial activity compared to the parent nucleosides**.** CycloSal-GEM-MP prodrugs **20a** and **20b** were the most active agents against Gram-positive bacteria (*E. faecali* and *S. aureus*) with retention of their activity in case of antibiotic resistant isolates. cycloSal-FUDR MP **21a** partially retained good activity against the Gram-positives *E. faecalis E. faecium* and *S. aureus.* Most of the prodrugs tested displayed very potent preventative anti-biofilm specific activity, but not curative. In terms of cytoxicity, AZT prodrugs did not affect apoptosis or cell viability at the highest concentration tested, and only weak effects on apoptosis and/or cell viability were observed for GEM and FUDR prodrugs.

Conclusions

Among the different prodrug approaches, in general the cycloSal prodrugs appeared the most effective. In particular cycloSal (**17a**) and Mix SATE (**26**) AZT prodrugs combine the lowest cytotoxicity with high and broad antibacterial and anti-biofilm activity against Gram-negatives.

Introduction

The rise of antimicrobial resistance (AMR) and the concurrent reduction of the investment of the pharmaceutical industry in this area has been anticipated to cause 10,000,000 deaths per year by 2050 and an associated $1 trillion accumulative global healthcare cost,[1] if no concerted effort to discover and develop new drugs will be made by all countries.

In this scenario drug repurposing is becoming an increasingly attractive strategy to get novel and effective antimicrobials from FDA-approved drugs and compounds that failed in clinical trials. In this respect, numerous natural nucleosides and their synthetically modified analogues have been reported to have a moderate to good antibiotic activity towards different bacterial strains.[2] In particular, nucleoside analogues (NAs), developed as therapeutic drugs for the treatment of viral infections and/or cancer conditions have been shown to inhibit the synthesis of bacterial nucleic acids, which has been linked to their antibacterial and anti-biofilm formation activity.[2,3]

Antibacterial activities of FDA-approved NAs

Among several FDA approved nucleoside-based drugs 2',2'-difluoro-2'-deoxycytidine (**1**, GEM; gemcitabine), 3'-azido-3'-deoxythymidine (**2**, AZT; zidovudine), 5-fluoro-2'-deoxyruridine (**3**, FUDR; floxouridine) were found endowed with antimicrobial activity.[4, 5]

Specifically, AZT, an antiretroviral medication used to prevent and treat HIV infections, was found active against Gram-negatives (MIC values against *E. coli* were between 10 μM (2.67 mg/L) to 31.6 μM (8.4 mg/L), but inactive against Gram-positive bacteria. AZT antibacterial activity is due to its incorporation in DNA and subsequent inhibition of replication leading to increased double stranded breaks.[6]

GEM and FUDR nucleosides are well known for their anti-proliferative activity for which they have been granted FDA approval as chemotherapeutic agents. In particular, GEM was found very active against Gram-positive bacteria (e.g. MIC values against *S. aureus* and *S. pyogenes* of 0.01μM (2.63 x 10^{-3} mg/L) and 0.1 μM (2.63 x10^{-2} mg/L respectively), but inactive against Gram-negative bacteria.[5] GEM was also evaluated in an *in vivo* mouse model of *S. pyogenes* infection and demonstrated to reduce bacterial spread and systemic infection in the treated animal supporting its potential as antibiotic.[5] The bactericidal effect of GEM on several Gram-positive bacteria (among which *S. aureus*) has been suggested to be mediated by its incorporation into growing DNA and by inhibition of ribonucleotide reductase as in the case of its antitumoral effect.[7, 8] Most probably with the same mode of action GEM was found to strongly inhibit *Mycoplasma pneumoniae* with MIC_{90} value of 0.2 mg/L.[9]

FUDR was found active against both Gram-positive and Gram-negative bacteria with the lowest minimal inhibitory concentrations in the range of 0.003 μM to 1 μM (7.38 x 10^{-4} to 2.46 x 10^{-1} mg/L) and of 1 μM to 10 μM (2.46 x 10^{-1} to 2.48 mg/L), respectively, for the Gram-positive and the Gram-negative strains. Previous study has suggested that its bactericidal activity, against various Gram-positive and -negative bacteria, is mediated by incorporation into growing DNA and inhibition of thymidylate synthase.[8]

Requirements for NA antibacterial activity.

NAs used as antibacterial drugs need to cross the bacterial cell wall which, especially in the case of Gram-negatives, presents a stronger barrier for traditional small drug-like molecules as compared to the mammalian cell membrane. The bacterial cell wall is a stronger barrier due to both highly polar outer membranes and prolific efflux pumps that remove foreign compounds.

In addition, as is the case for the antiviral and anticancer analogues, antibacterial NAs need to be activated to their triphosphate forms before being incorporated in bacterial DNA and inducing termination of the replication process. The first step in this activation is conversion of the NA's to their monophosphate forms by bacterial deoxynucleoside kinases (dNKs).[4,5]

Since poor cell membrane permeation and lack of phosphorylation due to kinase resistance are known to be principal limitations associated with NAs used in antiviral and anticancer therapy,[10] these aspects need special attention when developing these compounds as antimicrobial agents.

Support for this notion is provided by Jordheim *et al*.[11] who showed that AZT develops stable, high level resistance after short-term exposure of *E.coli* to the drug *in vitro* at concentrations up to 100 MIC. The mechanism of resistance appears to involve the loss of thymidine kinase activity, which is required to convert AZT into its monophosphate form.[11] In a different study GEM bactericidal activity against *S. aureus* isolates was found not enduring with re-growth observed even at high concentration.[12] This was attributed to mutations in the nucleoside kinase gene SadAK indicating a role for this enzyme in GEM activity, consistent with previous observations in gemcitabine-resistant human cancer cells.[13]

Strategies to overcome NA limitations

More lipophilic AZT derivatives have been developed with the aim of improving the absorption and consequently their antiviral and bactericidal activity. Among them, the 5' leucine AZT prodrug **4** was reported to be the most effective with an MIC value of 0.125 mg/L against *Klebsiella pneumoniae*.[14]

To overcome resistance due to kinase deficiency and simultaneously poor transport

across cell membranes,[15] phosphate prodrug strategies, aimed to directly deliver the monophosphate nucleoside analogues inside the cell, were developed and are now widely employed to enhance the activity of the antiviral and anticancer NAs.[16]

Objectives

Interested in exploring the potential of phosphate prodrug approaches for the antibacterial field, the study presented here describes our investigation of different classes of phosphate prodrugs of AZT, GEM and FUDR as potential antibacterial drugs against Gram-positive and Gram-negative pathogens and as potential biofilm inhibitors. By potentially increasing NAs bacterial cell wall permeation and/or bypassing kinase phosphorylation, such prodrugs would enable to reach higher pharmacologically active concentrations of the drug in the bacterial cell and may thus help to reduce the dose-limiting cytotoxicity of the drug, an essential requisite for the antitumor agents GEM and FUDR to be used safely as antimicrobial drugs. As further advantage bacteria cannot become resistant to these compounds through first kinase mutation mechanism since this step would be bypassed. The mechanisms of prodrug activation vary significantly across the different classes of phosphate prodrug, encompassing chemical and/or enzymatic cleavages of the prodrug promoieties. Lacking specific information on which would be the most favorable activation mechanism inside a bacterial cell, we selected three classes of prodrugs with different cleavage pathways. Specifically, we investigated the cycloSal approach,[17] whose intracellular cleavage is based on an entirely pH-driven chemical hydrolysis. Then we explored bis *S*-acyl-2-thioethyl (SATE)- and mix SATE phosphotriester derivatives,[18, 19] which both release the nucleoside monophosphate upon an esterase-dependent activation process. Finally, we investigated cyclic 1,3-propanyl ester (HepDirect)

prodrugs, activated through oxidation reaction.[20]

Materials and methods

Chemical synthesis

Ten prodrugs were synthesized (Figure 2 and Table S1 in the Supplementary data, available at JAC Online). Their preparations as well as their characterization data are described in the Supplementary Data.

Bacterial strains and growth media

Susceptibility testing was performed on 7 fully susceptible (to all clinically relevant antimicrobials, see below)). Gram-negative isolates (*Escherichia coli* ATCC 25922, *Klebsiella pneumonia* clinical strain, *Proteus mirabilis* NCTC 10975, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enteritidis* clinical strain, *Acinetobacter baumannii* clinical strain and *Burkholderia cepacia* NCTC 10661), plus 2 fully susceptible (to clinically relevant antimicrobials: amoxicillin, co-amoxiclav, 1st and 3rd generation cephalosporins, ciprofloxacin, gentamicin, amikacin and carbapenems)) Gram-positive isolates *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212, alongside 6 Gram-negative and 5 Gram positive isolates with resistance mechanisms (Table 1). All isolates were cultured initially for pure growth on Columbia blood agar plates (with 5% horse blood) and incubated overnight at 37˚C in air. MICs of the clinical antimicrobial agents were determined by broth micro dilution (see method below) and interpreted using EUCAST.

The strains *Pseudomonas aeruginosa* PA14,[21] *Escherichia coli* TG1,[22] *Salmonella enterica* serovar Typhimurium ATCC14028[23] and *Staphylococcus aureus* SH1000[24,25] were used to evaluate biofilm prevention and eradication in this study and benchmark

against biofilm inhibitors that were reported and tested against the same strains before.[26] Overnight cultures were grown with aeration in LB broth at 37 °C.[22] Phosphate-buffered saline (PBS) was prepared by combining 8.8 g liter^{-1} NaCl, 1.24 g liter^{-1} K_2HPO_4, and 0.39 g liter^{-1} KH_2PO_4 (pH 7.4). RPMI 1640 medium with L-glutamine and without sodium bicarbonate was purchased from Sigma and buffered to pH 7.0 with MOPS (morpholinepropanesulfonic acid; Sigma, St. Louis, MO) (final concentration, 165 mM).

Microbroth dilution assay

MICs were determined using a microbroth dilution assay performed according to ISO 20776-1 standard using cation adjusted Mueller Hinton Broth (MHB). Each compound was tested in the range 0.008 – 128 mg/L (Log2) and 1nMol – 100μMol (Log10). Inocula were prepared using MHB and diluted to a final density of 1.5 x 10^5 cfu/mL when in the presence of the antimicrobial agent. Ciprofloxacin as comparator antimicrobial agent were tested alongside the compounds to assure quality control. Plates were incubated at 35±1˚C in air for 16-20 hours. MICs were determined as the first concentration without visible growth.

MBCs are a measure of bactericidal activity i.e. the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by ≥99.9%. MBCs were determined by culturing the first three wells directly after the MIC during microbroth dilution. Antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC.

Biofilm prevention assay

A static peg assay, described previously,[26-28] was used for bacterial biofilm formation. The Calgary Biofilm Device consists of a platform carrying 96 polystyrene pegs (Nunc no. 445497) that fits as a microtiter plate lid with one peg hanging into each microtiter plate well (Nunc no. 269789). Two-fold serial dilutions of the compounds (dissolved in 100% DMSO) in 100 µl liquid broth (TSB diluted 1/20) per well were prepared in the microtiter plate in duplicate (i.e. 2 technical repeats), with a maximum concentration of 1600 µM (2048 mg/L) and a minimum concentration of 0.8 µM (6.4 x 10^{-3} mg/L). Subsequently, an overnight culture of *S.* Typhimurium ATCC14028, *P. aeruginosa* PA14, *E. coli* TG1, or *S. aureus* SH1000 (all grown in LB) was diluted 1:100 into TSB 1/20 (or TSB for *S. aureus* SH1000) to obtain a density of 1x 10^6 cells/mL. Next, 100 µl was added to each well of the microtiter plate, resulting in a total volume of 200 µl medium per well (final concentration of compounds ranges from 800 µM (2% DMSO or ethanol) to 0.4 µM (0.001% DMSO or ethanol)). Untreated (only TSB 1/20 or TSB) and solvent controls (two-fold DMSO dilutions) were included at least in quadruplicate. The pegged lid was then placed on the microtiter plate and the plate was incubated without shaking for 24 h at 25 °C, except for *S. aureus* which was incubated for 48 h at 37 °C. In the latter condition the plates were placed in a sealed container with wet towels on the bottom to prevent evaporation of the growth medium. After incubation, the optical density at 600 nm (OD_{600}) was measured to determine the effect of the compounds on the planktonic cells in the microtiter plate using a Synergy MX multimode reader (Biotek, Winooski, VT). For quantification of biofilm formation, the pegs were washed once in 200 µl PBS and then the remaining attached bacteria were stained for 30 min with 200 µl 0.1% (w/v) crystal violet in an isopropanol/methanol/PBS solution (v/v 1:1:18). Excess stain was rinsed off by placing the pegs in a 96-well plate filled with 200 µl distilled water per well. After air-drying

the pegs (30 min), the dye bound to the adherent biofilm was extracted with 30% glacial acetic acid (200 µl per well of a 96-well plate). The optical densities at 570 nm (OD_{570}) of each well were measured using a Synergy MX multimode reader (Biotek, Winooski, VT). Three biologically independent repeats of the experiment were performed. The 50% biofilm inhibitory activity concentration (BIC_{50}) and 50% inhibitory activity concentration (IC_{50}) values for each compound were determined from the concentration gradient by using nonlinear curve fitting (GraphPad Prism 5; Graphpad Software, Inc., La Jolla, CA) on data of the three biologically independent repeats simultaneously (each being the average of the 2 technical repeats). OD_{570} values from treated wells were hereto compared to the mean OD_{570} value of the TSB 1/20 (or TSB for *S. aureus*) controls from each strain. BIC_{50} is defined as the concentration of compound needed to inhibit biofilm formation by 50%. IC_{50} is defined as the concentration of compound needed to inhibit planktonic growth by 50%. The activity is considered biofilm-specific if the BIC_{50} is at least two times lower than the IC_{50}. As reported before, compounds with BIC_{50} values ≤ 50 µM can be considered potent biofilm inhibitors and compounds with BIC_{50} values ≤ 10 µM can be considered very strong inhibitors in these assays. These benchmarks are based on previous *in house* screenings of anti-biofilm activity of reference compounds against the same bacterial strains as those included here.[28]

Biofilm eradication assay

Overnight cultures of *S.* Typhimurium ATCC14028, *E. coli* TG1, and *S. aureus* SH1000 were grown as described for the biofilm prevention assay and diluted 1/100 in TSB 1/20 or TSB (for *S. aureus*). 200 µl cell culture was then added to each well of the microtiter plate of the Calgary Biofilm Device (described above) and biofilms were grown for 24 or 48 h, as described above. The pegged lid was then transferred to a

microtiter plate containing two-fold serial dilutions (in duplicate) of test compound (dissolved in 100% DMSO) ranging from 800 µM (2% DMSO) to 0.4 µM (0.001 % DMSO) in TSB 1/20 or TSB. Untreated (only TSB 1/20 or TSB) and solvent controls (two-fold DMSO dilutions) were included at least in quadruplicate. Subsequently, the plates were incubated at 25 °C or 37 °C for 4 hours. Quantification of remaining biofilm on the pegs was performed as described in the biofilm prevention assay. Three biological repeats of the experiment were performed. The 50% biofilm eradication concentration (BEC_{50}) values for each compound were determined from the concentration gradient by using nonlinear curve fitting (GraphPad Prism 5; Graphpad Software, Inc., La Jolla, CA) on data of the three biologically repeats simultaneously (each being the average of the 2 technical repeats). OD_{570} values from treated wells were hereto compared to the mean OD_{570} value of the TSB 1/20 (or TSB for *S. aureus*) controls in each individual microtiter plate. BEC_{50} is defined as the concentration of compound needed to eradicate biofilms by 50%. The activity is considered biofilm-specific if the BEC_{50} is at least two times lower than the IC_{50}. Compounds with BEC_{50} values ≤ 50 µM can be considered potent biofilm inhibitors and compounds with BEC_{50} values ≤ 10 µM can be considered very strong inhibitors in these assays.[28]

Cytotoxicity determination

Cytotoxicity in tumor cell lines. Tumor cell lines were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA), except for the DND-41 cell line, which was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ Leibniz-Institut, Germany), and the Hap-1 cell line, which was ordered from Horizon Discovery (Horizon Discovery Group, UK). All cell lines were cultured as recommended by the suppliers. Media were purchased from Gibco Life

Technologies, USA, and supplemented with 10% fetal bovine serum (HyClone, GE Healthcare Life Sciences, USA). Adherent cell lines HCT-116, NCI-H460, Hap-1 and Capan-1 cells were seeded at a density between 500 and 1500 cells per well, in 384-well, black walled, clear-bottomed tissue culture plates (Greiner). After overnight incubation, cells were treated with the test compounds at seven different concentrations ranging from 20 to 1.28 x 10^{-3} µM (25.6 mg/L to 1.2 x 10^{-5} mg/L). Suspension cell lines HL-60, K-562 and DND-41 were seeded at densities ranging from 2500 to 5500 cells per well in 384-well, black walled, clear-bottomed tissue culture plates containing the test compounds at the same seven concentration points.

The plates were incubated and monitored at 37 °C for 72h in an IncuCyte® (Essen BioScience Inc., Ann Arbor, MI, USA) for real-time imaging of cell proliferation. Images were taken every 3 h, with one field imaged per well under 10x magnification. Area under the curve (AUC) values were calculated and used to determine the IC_{50} values.

Cytotoxicity in non-tumorous cells was evaluated in two different assays: apoptosis induction assay with peripheral blood mononuclear cells (PBMC) and normal cell viability assay with normal human lung fibroblasts (HEL299 cells). Cytotoxicity of GEM, AZT, FUDR and their prodrugs was compared to five FDA approved control drugs: Docetaxel (chemotherapeutic agent), BTZ043 racemate (anti-mycobacterial agent), Mebendazole (anti-helminthic agent) and Hygromycin B (selective antibiotic). The highly cytotoxic Staurosporine (a phase II terminated drug) was included as well.[29]

For the apoptosis induction assay PBMC were isolated from buffy coat preparations of healthy donors (obtained from the Blood Transfusion Center (Leuven, Belgium)). PBMC isolation was executed by density gradient centrifugation over Lymphoprep (d=1.077 g/ml) (Nycomed, Oslo, Norway) and cultured in cell culture medium

(DMEM/F12, Gibco Life Technologies, USA) containing 8% FBS. PBMC were seeded at 28000 cells per well in 384-well, black-walled, clear-bottomed tissue culture plates containing the test compounds at six different concentrations ranging from 20 (1.6 mg/L) to 6.4 x 10^{-3} µM (5.12 x 10^{-4} mg/L).

Propidium Iodide was added at a final concentration of 1 µg/mL, and IncuCyte® Caspase 3/7 Green Reagent was added as recommended by the supplier. The plates were incubated and monitored at 37°C for 72 hours in the IncuCyte®. Images were taken as in the cytotoxicity assay of tumor cells described above. Quantification of the fluorescent signal after 72 h in both channels using the IncuCyte® image analysis software, allowed to calculate the percentage of live, dead and apoptotic cells. The percentage of live cells in the treated wells containing the test compounds was normalized to the percentage of live cells in the untreated control. All compounds were tested in two independent experiments (2 biological repeats).

For the normal cell viability assay, normal human lung fibroblasts (HEL299 cells ordered from ATCC) were seeded in standard 384-well tissue culture plates and grown for 3 days until confluence was reached. The test compounds were added at six different concentrations ranging from 20 to 6.4 x 10^{-3} µM (1.6 mg/L - 5.12 x10^{-4} mg/L), and the plates were incubated at 37 °C for 72 h. Cell viability was measured colorimetrically by treating with the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay reagent (Promega) according to the manufacturer's instructions. Absorbance of the samples was measured at 490 nm using a SpectraMax Plus 384 microplate reader (Molecular Devices). All compounds were tested in duplicate (two technical repeats), in three independent experiments (three biological repeats).

Results

Structures and chemical synthesis

The structures of the novel compounds used for this study are reported in Figure 2. The synthetic procedures for all the prodrugs synthesized are reported in Schemes S1 and S2 in the supplementary data. A summary of all the analogues prepared for this study, including parent nucleoside, substitution pattern and class of prodrugs is reported in Table S1.

Bacteriostatic and bactericidal activities

The prodrugs synthesized, along with the parent drugs, were tested for their antibacterial activity against a panel of Gram-positive and Gram-negative fully susceptible to classical antibiotics (see material and method section for details) and resistant isolates, which are of clinical relevance and for which new antimicrobials are needed. Although it is unlikely that the resistance mechanisms (except for the Trimethoprim one),[30] that confer resistance to the selected strains, would induce them to become resistant to our prodrugs, we wanted to evaluate if the compounds would be effective to treat these pathogens, which are relevant for a clinical setting.

Neither GEM nor its prodrugs showed antibacterial (i.e. bacteriostatic or bactericidal) activity against Gram-negative bacterial strains either fully susceptible or resistant. (Tables 2 and 3). Against Gram-positive bacteria, cycloSal derivatives gave the best antibacterial activities among the GEM prodrugs, although they were less potent than GEM itself, with compound **20a** being the most active agent. For both fully susceptible *E. faecalis* and *S. aureus* strains, Log2 MICs of 1 mg/L and Log10 MICs of 10 μM were observed (Table 2). MBCs for **20a** against these same strains were 8 mg/L and 1 mg/L (Log2) and 100 μM and 10 μM (Log10), respectively, indicating bactericidal activity (Table 2). Compound **20a**, when tested on the strains of *S. aureus* resistant to

flucloxacillin (methicillin, *mecA*), erythromycin (MLSB), glycopeptides (hVISA) and tetracycline (not genetically confirmed), and a strain of vancomycin resistant *E. faecium* (vanA) exhibited Log2 MICs of 1 mg/L, 1 mg/L, 1 mg/L, 2 mg/L and 1 mg/L respectively (Table 2). Log10 MICs were all 10 μM (Table 2). In addition, MBCs for compound **20a** were similar to MICs against both sensitive and resistant strains. A similar trend was observed also with the cycloSal derivative **20b** (Table 2). The other types of GEM prodrugs, SATE **24** and mix SATE **28** compounds were moderately active against fully susceptible *S. aureus* strains (Log2 MICs of 16 mg/L and Log10 MICs of 100 μM for both compounds) (Table 3). Moreover, MICs were similar (16 mg/L and 100 μM) for *S. aureus* with resistance mechanisms. These compounds were highly active against fully susceptible *E. faecium* (Log2 MICs 1 mg/L and 0.5 mg/L respectively, Log10 MICs 10 μM and 10 μM respectively). Bactericidal activity was borderline according to MBC values (Table 3). HepDirect-GEM-MP prodrug **14** was almost inactive against all the different bacterial strains tested (Table 3).

In agreement with literature, parental AZT mainly showed activity against Gram-negative and not Gram-positive bacterial strains. With regard to AZT prodrugs, mix SATE **26** was the only prodrug that retained some activity, albeit very moderate, against most Gram-negative strains, with a Log2 MIC of 100 μM (Table 4). Interestingly, the mix SATE prodrug approach seems to confer some activity versus *A. baumannii* and *B. cepacia* (Log 2 MIC values of 64 and 32 mg/L respectively), not observed with unmodified AZT. Prodrugs **17a** and **11** proved to be completely inactive with MIC_{50} values always higher than 100 μM (Table 4).

Although the FUDR parent nucleoside displayed excellent antibacterial activity against numerous Gram-negative and Gram-positive bacteria, cycloSal prodrug **21a** was the only compound that displayed a partial retention of activity, whilst **15** exhibited a strong

decrease in the antibacterial activity compared to the respective parent nucleoside (Table 4).

Biofilm prevention

Next the capacity of the compounds to prevent biofilm formation was assayed (preventative activity) against major pathogens involved in biofilm-associated disease. The results are summarised in Table 5.

Specifically our microbial test panel included *S. aureus* (Gram-positive cocci), which can colonize different types of implantable devices,[31] chronic wounds[32] and catheters;[33,34] *E. coli* (Gram-negative γ-proteobacteria), known to form biofilms on *i.e.* urinary catheters,[32] plant material,[35] and food (contact) surfaces,[36] *S.* Typhimurium, one of the most important causes of foodborne infections worldwide and a notorious biofilm former both inside and outside the host,[37] and *P. aeruginosa* (Gram-negative γ-proteobacteria), a Gram-negative opportunistic pathogen that can infect immunocompromised people such as cystic fibrosis patients and cause life-threatening chronic lung infections.[38]

GEM and its cycloSal prodrug **20a** showed very high preventative activity against biofilms of *S. aureus*, with BIC_{50} values of 0.22 µM and 0.32 µM respectively, whereas prodrugs **14** and **24** were instead only moderately active (BIC_{50} values between 72 – 226 µM). Differently from the MIC test, only very weak activity for GEM and **20a** against planktonic cells of *S. aureus* was observed in this assay, possibly because a different strain of *S. aureus* was used. This indicates that the observed activity of GEM against *S. aureus* was biofilm-specific. In addition, GEM and all the prodrugs were very active in preventing biofilms of *E. coli* (BIC_{50} values < 1.5 µM). GEM itself, but not the prodrugs, was also preventative against biofilms of *S.* Typhimurium with BIC_{50} of

13.91 µM. No activity against planktonic growth of Gram-negatives (consistent with MIC tests and literature) was observed for GEM and its prodrugs, therefore indicating that the anti-biofilm activity is biofilm-specific. Against *P. aeruginosa,* no activity was observed.

AZT itself showed strong preventative activity against biofilms of S. Typhimurium and *E.coli* with BIC_{50} values < 0.4 µM, but there was no curative activity observed. The activity against *S.* Typhimurium was biofilm-specific, whereas strong inhibition of planktonic growth of *E. coli*, consistent with MIC tests, suggests non-biofilm specific activity for this nucleoside against this strain. Although AZT prodrugs showed complete loss of activity against planktonic cells, the preventative activity against biofilms of *S.* Typhimurium and *E. coli* was largely retained (BIC_{50} values between < 0.4 µM – 100 µM) rendering these compounds anti-biofilm-specific. No activity against Gram-positive *S. aureus* nor Gram-negative *P. aeruginosa* was observed for both parent nucleosides and prodrugs. FUDR presented very strong preventative anti-biofilm activity against both *S. aureus* and the Gram-negatives *S.* Typhimurium and *E. coli* with BIC_{50} values < 0.4 µM. Since also the planktonic growth was inhibited, the anti-biofilm activities of FUDR are not biofilm-specific. In contrast to the observed loss of activity against planktonic cells, HepDirectFUDR-MP **15** (BIC_{50} = < 0.4 µM – 225.3 µM) and CycloSal-FUDR-MP **21a** (BIC_{50} = <0.4 µM – 10.25 µM) largely retained preventative anti-biofilm activity against the three species. No activity was observed against *P. aeruginosa* either for the parent nucleoside or its prodrugs.

Biofilm eradication

Once the preventive activity was established the ability to disrupt existing biofilms (curative activity) was investigated against the same pathogens and the results are summarized in Table 5.

Where GEM had a potent curative activity against *S. aureus* (BEC_{50} value of 20.13 µM), its prodrug did not show curative activity. For either AZT or its prodrugs, no curative activity was observed. FUDR showed potent curative anti-biofilm activity against *S. aureus* and *S.* Typhimurium (BEC_{50} values of 17.29 µM and 1.815 µM respectively), but not against *E. coli*. Moderate curative anti-biofilm activity was observed for CycloSal-FUDR-MP **21a** against *S. aureus* and S. Typhimurium (BEC_{50} values of 93.52 µM and 59.90 µM respectively) (Table 5).

Cytotoxicity

In order to compare the anti-proliferative activity of the NA derivatives with their parental nucleosides, the *in vitro* cytotoxicity of the parent nucleosides and their prodrugs was assayed on seven different tumor cell lines: Capan-1 (pancreatic adenocarcinoma), Hap-1 (chronic myeloid leukemia), HCT-116 (Colorectal carcinoma), NCI-H460 (Lung Carcinoma), DND-41 (acute lymphoblastic leukemia), HL-60 (acute myeloid leukemia), K-562 cell lines (chronic myeloid leukemia) (Table 6).

As expected GEM, FUDR and the majority of their derivatives have high anti-proliferative activity against the tumor cell lines with IC_{50} in the nanomolar range. AZT is less cytotoxic with IC_{50} values ranging from 3.9 to 13 µM. Among all the prodrugs AZT cycloSal **17a** shows the lowest anti-proliferative activity compared to the parent compounds IC_{50} values higher than > 20 µM.

Concerned about the toxicity of our prodrugs, which might limit their future development as antimicrobials, we decided to also investigate on their effect against non-tumorous cells. Hereto, an apoptosis induction assay with PBMC (Peripheral Blood Mononuclear Cells) and a normal cell viability assay with HEL299 cells (normal

human lung fibroblasts) were performed. Cytotoxicity of our compounds on these normal cells was compared to four FDA-approved control drugs: docetaxel (chemotherapeutic agent), BTZ043 racemate (anti-mycobacterial agent), mebendazole (anti-helminthic agent) and hygromycin B (selective antibiotic). The highly cytotoxic staurosporine (a phase II terminated drug) was included as a positive control.[29]

As displayed in Figure 3, GEM and its derivatives caused a weak induction of apoptosis in PBMC, but only at the highest concentration (20 µM). Derivatives cycloSal-GEM-MP **20a** and mix SATE-GEM-MP **28** showed a small decrease in cytotoxicity compared to the parent nucleoside GEM (respectively, 10.1 % and 14.5 % decrease of apoptosis induction in PBMC at 20 µM of compound). FUDR, AZT and their derivatives did not exhibit a significant induction of apoptosis.

In HEL299 cells (Figure 4) we measured no decrease in viability of cells treated with AZT and its derivatives. The cytotoxicity profile of the cycloSal **17a** and mix SATE **26** prodrugs even showed a small increase of cell viability compared to AZT (respectively, 13.3 % and 8.7 % increase of viable HEL299 cells at 20 µM of compounds). FUDR and its derivatives exhibited a limited impact on cell viability at concentrations ranging from 0.8 µM to 20 µM. HepDirect-FUDR-MP and cycloSal-FUDR-MP did not have an improved cytotoxicity profile compared to their parent nucleoside. GEM and its prodrugs showed a limited decrease in viability even at the lowest concentrations (6.4 x 10^{-3} µM – 3.2 x 10^{-2} µM). At 20 µM all GEM prodrugs and GEM itself exhibit a decrease of around 40% in cell viability. However, two out of four FDA approved control drugs showed a similar decrease. There is no improvement in the cytotoxicity profile of GEM derivatives compared to their parent nucleoside.

Discussion

Nucleoside analogues constitute an important class of small molecules, which have played a pivotal role in the treatment of viral infections and in cancer care. Only few reports directly describe the NAs antimicrobial activity and their potential mechanisms, although their antibacterial actions can be anticipated. Monophosphate prodrug approaches have been applied to anticancer and antiviral NAs to overcome their limitations. In this study, first we investigated if phosphate approaches would be able to improve the antibacterial properties of three FDA-approved nucleoside-based drugs (GEM, AZT and FUDR) with known antimicrobial activities,[4,5] by potentially increasing their bacterial cell wall permeation and/or bypassing kinase phosphorylation. Next, we investigated the ability of these prodrugs to eradicate and/or prevent the formation of biofilms of major pathogens involved in biofilm-associated disease.[39]

Antibacterial Activity

From the antibacterial assays the different prodrug approaches in general were not able to improve the antimicrobial activity of the parent drugs. The MIC values are generally higher for the novel derivatives and in some cases the activity spectrum has become narrower. Across the different prodrugs cycloSal-GEM-MP **20a**, cycloSal-FUDR-MP **21a** and Bis and Mix(SATE)-GEM-MP (**24** and **28**) show moderate to good antimicrobial activity against Gram-positives, whereas FUDR and AZT antibacterial activity against Gram-negatives is either completely lost or significantly decreased in their cycloSal derivatives as well as in the other classes of prodrugs. These results may indicate that permeation and/or activation for the prodrugs can occur only in Gram-positive bacteria, in accordance with the difficulty to cross the cell wall of Gram-negative bacteria. The fact that AZT and FUDR nucleosides are active against Gram-negatives could suggest that they might use nucleoside-specific membrane

transporters,[40] for which the prodrugs are not substrates.

Preventative anti-biofilm activities

All the prodrugs and NAs showed high preventative anti-biofilm activity with broader spectra compared to the antibacterial activities against planktonic cells. This suggests that the anti-biofilm target/targets are possibly located outside the cell wall, and therefore these compounds do not need to cross the bacterial cell wall to be effective, and/or that these compounds do not need to be activated to interact with the anti-biofilm target. Although cycloSal-GEM-MP **20a** does not show a clear benefit over GEM, being both very strong and specific inhibitors for the formation of *S. aureus* and *E. coli* biofilm, this prodrug could show advantages with regard to evolutionary robustness, stability and cellular uptake, which need to be further evaluated. On the contrary all AZT and FUDR prodrugs show to be very strong and specific preventative inhibitors of biofilm formation respectively of *S.* Typhimurium and *E. coli*, and *S. aureus* and *E. coli* while AZT and FUDR themselves are not specific at all. The lack of antibacterial activity observed for these derivatives can be considered an advantage, despite no improved preventive anti-biofilm activity over their parent NA's, since it can reduce the selective pressure thus decreasing the risk of the development of antimicrobial-resistant organisms.[41,42]

Curative anti-biofilm activities

For some compounds, curative anti-biofilm activity was observed. GEM and FUDR were potent against biofilms of *S. aureus* and FUDR even showed strong curative activity against *S.* Typhimurium. Moderate anti-biofilm activity was measured for cycloSal-FUDR-MP **21a** against *S. aureus* and *S.* Typhimurium. However, the curative

activity of FUDR and its **21a** derivative is probably not due to a specific anti-biofilm action since antibacterial activity is high as well. In *E. coli,* it is remarkable that high IC_{50} and preventative BIC_{50} values for AZT, FUDR and their derivatives did not translate into curative anti-biofilm activity. However, the fact that no cells were removed from the biofilm during curative treatment as measured after crystal violet staining, does not necessarily indicate that no cells were killed.

Cytotoxicty

Cytotoxicity assays indicated that AZT does not induce apoptosis in PBMC and shows no effect on the viability of non-cancerous cells at the highest concentration tested (20 µM), and neither do the AZT derivatives. Also FUDR does not induce apoptosis in PBMCs, and it only weakly affects the viability of HEL299 cells. The FUDR prodrugs have a similar effect. GEM on the other hand does induce apoptosis in PBMC, causing a decrease in the number of viable cells, but only at the highest concentration tested. Metabolic activity of HEL299 cells was also affected by GEM, even at lower concentrations (0.032 µM). Interestingly, the negative impact on PBMC is less pronounced for the cyclosal and Mix SATE prodrugs of GEM. It should be noted that these effects on apoptosis and cell viability are generally moderate as compared to the positive control staurosporine and lay in the same range as the effects the FDA approved drugs mebendazol and docetaxel have on cell viability of normal cells (at concentrations higher than 0.16 and 0.0064 µM resp.) and apoptosis induction in PBMC (at 20 µM for mebendazol).

Conclusion

In conclusion, the parent nucleosides have shown good antibacterial activity against Gram-negative and/or Gram-positive strains in accordance with the data reported in the

literature. In general all the prodrugs, tested in the present work, showed on average a decrease of the antibacterial activity compared to the parent nucleosides. Among all the compounds CycloSal-GEM-MP **20a** and CycloSal-FUDR-MP **21a** showed moderate antibacterial activity against selectively Gram-positive bacteria. No significant antibacterial effect was found for these prodrugs against Gram-negative bacteria indicating that they most probably are not able to cross the bacterial cell wall of these pathogens, or cannot be activated. However the performance of the prodrug in an *in vivo* model could be different due to improved pharmacokinetic properties of these derivatives versus their parent NAs.

As far as the biofilm inhibition is concerned, the results clearly indicated that the prodrugs investigated have the potential to be developed as preventive anti-biofilm agents rather than to eradicate pre-formed biofilms. GEM, FUDR and AZT exhibited widened broad-spectrum of high preventive anti-biofilm activity, but this activity was biofilm-specific only for GEM. Although the majority of prodrugs lost their activity against planktonic cells, when compared to their parent nucleoside, they did largely retain their activity against biofilms showing the potential for their development as specific anti-biofilm agents.

However, further studies to unravel their full potential for the biofilm prevention and /or its eradication will be essential, including evaluation of their ability to inhibit biofilm formation of clinically isolated bacterial strains and determination of the targets and involvement of the prodrugs activation mechanisms. In terms of cytotoxicity, AZT and its prodrugs do not show any effects on apoptosis or cell viability at the highest concentration tested. Only a weak effect on apoptosis was observed for GEM and its prodrugs and weak effects on cell viability for GEM and FUDR and their prodrugs.

These effects however lay in the same range as those for the FDA-approved drugs mebendazol and docetaxel.

In conclusion our results indicate that the nucleoside prodrugs are promising for the development as antimicrobials. In particular the AZT cycloSal (**17a)** and Mix SATE (**26)** prodrugs combine the lowest cytotoxicity with high and broad antimicrobial activity.

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Transparency declarations

None to declare

Supplementary data

Supplementary data are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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Table 1

Isolate ID	Strain ID	Resistant to:	Mechanism
Escherichia coli	ATCC 25922	Sensitive strain	-
Klebsiella pneumonia,	Clinical strain	Sensitive strain	-
Proteus mirabilis	NCTC 10975	Sensitive strain	-
Pseudomonas aeruginosa	ATCC 27853	Sensitive strain	-
Salmonella enteritidis	Wild strain	Sensitive strain	-
Acinetobacter baumannii	Wild strain	Sensitive strain	-
Burkholderia cepacia	NCTC 10661	Sensitive strain	-
Staphylococcus aureus	ATCC 29213	Sensitive strain	-
Enterococcus faecalis	ATCC 29212	Sensitive strain	-
E. coli	ATCC 35218	Ampicillin	β-lactamase
E. coli	clinical strain	Carbapenems	NDM
E. coli	NCTC 13353	third-generation cephalosporin	CTX-M
E. coli	clinical strain	Nitrofurantoin, Trimethoprim	Multiple
K. pneumoniae	ATCC 700603	fourth-generation cephalosporin	SHV-18
K. pneumoniae	NCTC 13442	Carbapenems	OXA-48
S. aureus	NCTC 12493	Flucloxacillin	MecA
S. aureus	ATCC BAA-977	Erythromycin/ Clindamycin	MLSB
S. aureus	ATCC 700698	Vancomycin	hVISA
S. aureus	Clinical strain	Tetracycline	-
E. faecium	Clinical strain	Vancomycin	VanA

Table 2. *In vitro* antimicrobial activities of HepDirect GEM-MP (**14**) and Cyclosal GEM-MP prodrugs (**20a-b**) against a panel of sensitive and resistant Gram positive (+) and Gram negative (-) bacterial strains.

Bacterial Strain			Resistance	GEM (1)				HepDirect GEM-MP (14)				CycloSal GEM-MP					
												(20a)				(20b)	
				Log2 MIC[1]	Log10 MIC[2]	Log2 MBC[1]	Log10 MBC[2]	Log2 MIC[1]	Log10 MIC[2]	Log2 MBC[1]	Log10 MBC[2]	Log2 MIC[1]	Log10 MIC[2]	Log2 MBC[1]	Log10 MBC[2]	Log 2 MIC[1]	Log10 MIC[2]
-	***E. coli***	ATCC 25922	Sensitive strain	>128	>100	-	-	>128	>100	-	-	>128	>100	-	-	>128	>100
-	***K. pneumoniae***	Wild strain	Sensitive strain	-	-	-	-	>128	>100	-	-	>128	>100	-	-	>128	>100
-	***P. mirabilis***	NCTC 10975	Sensitive strain	-	-	-	-	>128	>100	-	-	>128	>100	-	-	>128	>100
-	***P. aeruginosa***	ATCC 27853	Sensitive strain	>128	>100	-	-	>128	>100	-	-	>128	>100	-	-	>128	>100
-	***S. enteritidis***	Wild strain	Sensitive strain	-	-	-	-	>128	>100	-	-	>128	>100	-	-	>128	>100
-	***A. baumannii***	Wild strain	Sensitive strain	-	-	-	-	>128	>100	-	-	>128	>100	-	-	>128	>100
-	***B. cepacia***	NCTC 10661	Sensitive strain	-	-	-	-	>128	>100	-	-	>128	>100	-	-	>128	>100
+	***S. aureus***	ATCC 29213	Sensitive strain	0.008	0.1	0.008	1	>128	>100	-	-	1	10	1	10	2	>100
+	***E. faecalis***	ATCC 29212	Sensitive strain	0.03	0.1	0.125	1	64	>100	128	-	1	10	8	100	16	10
-	***E. coli***	ATCC 35218	Ampicillin	-	-	-	-	>128	>100	-	-	>128	>100	-	-	>128	>100
-	***E. coli***	Wild strain	Carbapenems	-	-	-	-	>128	>100	-	-	>128	>100	-	-	>128	>100
-	***E. coli***	NCTC 13353	3 GEN CEPH[3]	-	-	-	-	>128	>100	-	-	>128	>100	-	-	>128	>100
-	***E. coli***	Wild strain	NIT, TRM[4]	-	-	-	-	>128	>100	-	-	>128	>100	-	-	>128	>100
-	***K. pneumoniae***	ATCC 700603	4 GEN CEPH[5]					>128	>100	-	-	>128	>100	-	-	>128	>100
-	***K. pneumoniae***	NCTC 13442	Carbapenems	-	-	-	-	>128	>100	-	-	>128	>100	-	-	>128	>100
+	***S. aureus***	NCTC 12493	Flucloxacillin	-	-	-	-	>128	100	-	-	1	10	1	10	2	10
+	***S. aureus***	ATCC BAA-977	ERY/CLIND[6]	-	-	-	-	>128	100	-	-	1	10	1	10	4	10
+	***S. aureus***	ATCC 700698	Vancomycin	0.03	0.1	0.06	0.1	>128	100	-	-	1	10	1	10	2	100
+	***S. aureus***	Wild strain	Tetracycline	-	-	-	-	>128	100	-	-	2	10	2	10	4	10
+	***E. faecium***	Wild strain	Vancomycin	-	-	-	-	32	100	64	>100	1	10	4	100	1	1

[1]Concentration values are expressed in mg/L; [2]Concentration values are expressed in μM;[3]Third generation cephalosporin; [4]Nitrofurantoin, Trimethoprim; [5]Fourth generation cephalosporin; [6]Erythromycin, Clindamycin.
Each compound was tested in the range 0.008 – 128 mg/L (Log 2) and 1nMol – 100μMol (Log10); - not tested

Table 3. *In vitro* antimicrobial activities of SATE GEM-MP (**24**) and Mix-SATE GEM-MP (**28**) against a panel of sensitive and resistant Gram positive (+) and Gram negative (-) bacterial strains.

Bacterial Strain			Resistance	GEM (1)				Bis(SATE) GEM-MP (24)				Mix SATE GEM- MP(28)			
				Log2 MIC[1]	Log10 MIC[2]	Log2 MBC[1]	Log10 MBC[2]	Log2 MIC[1]	Log10 MIC[2]	Log2 MBC[1]	Log10 MBC[2]	Log2 MIC[1]	Log10 MIC[2]	Log2 MBC[1]	Log10 MBC[2]
-	***E. coli***	ATCC 25922	Sensitive strain	>128	>100	-	-	>128	>100	-	-	>128	>100	-	-
-	***K. pneumoniae***	Wild strain	Sensitive strain	-	-	-	-	>128	>100	-	-	>128	>100	-	-
-	***P. mirabilis***	NCTC 10975	Sensitive strain	-	-	-	-	>128	>100	-	-	>128	>100	-	-
-	***P. aeruginosa***	ATCC 27853	Sensitive strain	>128	>110			>128	>100	-	-	>128	>100	-	-
-	***S. enteritidis***	Wild strain	Sensitive strain	-	-	-	-	>128	>100	-	-	>128	>100	-	-
-	***A. baumannii***	Wild strain	Sensitive strain	-	-	-	-	>128	>100	-	-	>128	>100	-	-
-	***B. cepacia***	NCTC 10661	Sensitive strain	-	-	-	-	>128	>100	-	-	>128	>100	-	-
+	***S. aureus***	ATCC 29213	Sensitive strain	0.008	0.1	0.008	1	16	100	16	100	16	100	16	100
+	***E. faecalis***	ATCC 29212	Sensitive strain	0.03	0.1	0.125	1	8	10	32	100	8	10	128	>100
-	***E. coli***	ATCC 35218	Ampicillin	-	-	-	-	>128	>100	-	-	>128	>100	-	-
-	***E. coli***	Wild strain	Carbapenems	-	-	-	-	>128	>100	-	-	>128	>100	-	-
-	***E. coli***	NCTC 13353	3 GEN CEPH	-	-	-	-	>128	>100	-	-	>128	>100	-	-
-	***E. coli***	Wild strain	NIT, TRM	-	-	-	-	>128	>100	-	-	>128	>100	-	-
-	***K. pneumoniae***	ATCC 700603	4 GEN CEPH	-	-	-	-	>128	>100	-	-	>128	>100	-	-
-	***K. pneumoniae***	NCTC 13442	Carbapenems	-	-	-	-	>128	>100	-	-	>128	>100	-	-
+	***S. aureus***	NCTC 12493	Flucloaxacillin	-	-	-	-	32	100	32	100	16	100	32	>100
+	***S. aureus***	ATCC BAA-977	ERY/CLIND	-	-	-	-	16	100	16	100	16	100	16	100
+	***S. aureus***	ATCC 700698	Vancomycin	0.03	0.1	0.06	0.1	8	100	16	100	32	100	32	100
+	***S. aureus***	Wild strain	Tetracycline	-	-	-	-	16	100	16	100	16	100	64	>100
+	***E. faecium***	Wild strain	Vancomycin	-	-	-	-	1	10	4	100	0.5	10	4	100

[1]Concentration values are expressed in mg/L; [2]Concentration values are expressed in μM. [3]Third generation cephalosporin; [4]Nitrofurantoin, Trimethoprim; [5]Fourth generation cephalosporin; [6]Erythromycin, Clindamycin. Each compound was tested in the range 0.008 – 128 mg/L (Log 2) and 1nMol – 100μMol (Log10); - not tested.

Table 4. *In vitro* antimicrobial activities of AZT (**1**) and its Hepdirect (**11**) and cycloSal (**17a**) prodrugs; and FUDR (**2**) and its Hepdirect (**15**) and cycloSal (**21a**) prodrugs, against a panel of sensitive and resistant Gram-positive (+) and Gram-negative (-) bacterial strains.

ID		Strain ID	Resistance	AZT (2)		HepDirect-AZT-MP (11)		cycloSal-AZT- MP (17a)		Mix SATE-AZT-MP (26)		FUDR (3)		HepDirect-FUDR- MP (15)		cycloSal-FUDR-MP(21a)	
				Log2 MIC[1]	Log10 MIC	Log2 MIC[1]	Log10 MIC[2]	Log 2 MIC[1]	Log2 MIC[1]	Log10 MIC[2]	Log2 MIC[1]	Log2 MIC[1]	Log10 MIC	Log2 MIC[1]	Log10 MIC[2]	Log2 MIC[1]	Log10 MIC[2]
-	***E. coli***	ATCC 25922	Sensitive strain	1	1	128	>100	>128	>100	64	100	0.25	1	>128	>100	32	32
-	***K. pneumoniae***	Wild strain	Sensitive strain	1	10	>128	>100	>128	>100	64	100	0.25	10	>128	>100	32	32
-	***P. aeruginosa***	ATCC 27853	Sensitive strain	>128	>100	>128	>100	>128	>100	64	100	>128	>100	>128	>100	>128	>128
-	***S. enteritidis***	Wild strain	Sensitive strain	0.5	1	128	>100	64	>100	64	100	2	10	>128	>100	>128	>128
-	***A. baumannii***	Wild strain	Sensitive strain	>128	>100	>128	>100	>128	>100	32	100	128	>100	>128	>100	>128	>128
-	***B. cepacia***	NCTC 10661	Sensitive strain	>128	>100	>128	>100	>128	>100	64	100	>128	>100	>128	>100	>128	>128
+	***S. aureus***	ATCC 29213	Sensitive strain	>128	>100	>128	>100	>128	>100	64	>100	0.008	0.1	64	100	0.06	0.06
+	***E. faecalis***	ATCC 29212	Sensitive strain	>128	>100	128	>100	>128	>100	128	100	0.015	0.1	32	100	0.25	0.25
-	***E. coli***	ATCC 35218	Ampicillin	1	10	>128	>100	>128	>100	64	100	1	10	>128	>100	64	64
-	***E. coli***	Wild strain	Carbapenems	4	10	>128	>100	>128	>100	64	100	0.5	1	>128	>100	32	32
-	***E. coli***	NCTC 13353	3 GEN CEPH[3]	0.5	10	>128	>100	>128	>100	64	100	1	10	>128	>100	128	128
-	***E.coli***	Wild strain	NIT, TRM[4]	0.5	10	>128	>100	>128	>100	64	100	2	10	>128	>100	128	128
-	***K. pneumoniae***	ATCC 700603	4 GEN CEPH[5]	2	10	>128	>100	128	>100	64	100	1	10	>128	>100	64	64
-	***K. pneumoniae***	NCTC 13442	Carbapenems	>128	10	>128	>100	>128	>100	64	100	2	10	>128	>100	>128	>128
+	***S. aureus***	NCTC 12493	Flucloxacillin	>128	>100	128	>100	>128	>100	32	100	0.008	0.01	32	100	0.03	0.03
+	***S. aureus***	ATCC BAA-977	ERY/CLIND[6]	>128	>100	128	>100	>128	>100	128	>100	0.008	0.1	32	100	0.03	0.03
+	***S. aureus***	ATCC 700698	Vancomycin	>128	>100	>128	>100	>128	>100	128	>100	0.008	0.1	64	>100	0.06	0.06
+	***S. aureus***	Wild strain	Tetracycline	>128	>100	>128	>100	>128	>100	128	>100	0.008	0.1	64	100	0.06	1
+	***E. faecium***	Wild strain	Vancomycin	>128	>100	64	>100	>128	>100	32	100	0.008	0.1	32	100	0.5	1

[1]Concentration values are expressed in mg/L; [2]Concentration values are expressed in μM. [3]Third generation cephalosporin; [4]Nitrofurantoin, Trimethoprim; [5]Fourth generation cephalosporin; [6]Erythromycin, Clindamycin.
Each compound was tested in the range 0.008 – 128 mg/L (Log 2) and 1nMol – 100μMol (Log10) - not tested

Table 5. Antimicrobial susceptibilities of planktonic and biofilm *S. aureus (+), S.* Typhimurium (-), *P. aeruginosa (-) and E. coli (-)* in the presence of parent nucleosides (**1-3**) and their HepDirect (**11**, **14** and **15**), cycloSal (**17a**, **20a** and **21a**) bis (SATE) (**24**) and mix-SATE (**26** and **28**) prodrugs.

Comp.	*S. aureus (+)* SH1000			*S. Typhimurium* (-) ATCC14028			*P. aeruginosa (-)* PA14			*E. coli (-)*TG1		
	Anti-Biofilm Activity		Antibacterial Plank. Activity	Anti-Biofilm Activity		Antibacterial Plank. Activity	Anti-Biofilm Activity		Antibacterial Plank. Activity	Anti-Biofilm Activity		Antibacterial Plank. Activity
	Preventative	Curative		Preventative	Curative		Preventative	Curative		Preventative	Curative	
	BIC_{50}[1]	BEC_{50}[1]	IC_{50}[1]	BIC_{50}[1]	BEC_{50}[1]	IC_{50}[1]	BIC_{50}[1]	BEC_{50}[1]	IC_{50}[1]	BIC_{50}[1]	BEC_{50}[1]	IC_{50}[1]
GEM (1)	0.22	20.13	589.3	13.91	>800	577.3	>800	-	>800	<0.4	>800	1268
Bis (SATE)-GEM-MP (24)	72.50	-	277.5	>800	-	>800	>800	-	>800	<0.4	>800	1596
HepDirect -GEM-MP (14)	226.7	-	>800	>800	-	>800	>800	-	>800	1.59	>800	>800
cycloSal-GEM-MP (20a)	0.32	-	~800	>800	-	>800	>800	-	>800	<0.4	-	>800
Mix SATE-GEM-MP (28)	140.4	-	659.8	>800	-	>800	>800	-	>800	<0.4	>800	>800
AZT (2)	586.8	>800	>800	<0.4	>800	233.6	>800	-	>800	<0.4	>800	<0.4
cycloSal-AZT-MP (17a)	>800	>800	>800	11.15	>800	>800	>800	-	>800	<0.4	>800	>800
HepDirect-AZT-MP (11)	>800	>800	>800	6.31	>800	>800	>800	-	>800	<0.4	>800	150.4
Mix SATE-AZT-MP (26)	415.4	>800	>800	100-200	>800	>800	>800	-	>800	<0.4	>800	>800
FUDR (3)	<0.4	17.29	14.43	0.4	1.815	0.649	>800	-	434.5	<0.4	>800	0.52
HepDirect-FUDR-MP (15)	13.8	-	>800	225.3	-	>800	>800	-	>800	<0.4	>800	370.4
cycloSal-FUDR-MP(21a)	<0.4	93.52	11.39	10.25	59.90	41.76	>400	-	>400	<0.4	>800	23.38

[1]Concentration values are expressed in μM

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Table 6. Cytotoxicity data for parent nucleosides (**1-3**) and their HepDirect (**14-15**), cycloSal (**17a, 20a** and **21a**) bis (SATE) (**24**) and mix-SATE (**26** and **28**) prodrugs performed on Capan-1 (pancreatic adenocarcinoma), Hap-1 (chronic myeloid leukemia), HCT-116 (Colorectal carcinoma), NCI-H460 (Lung Carcinoma), DND-41 (acute lymphoblastic leukemia), HL-60 (acute myeloid leukemia), K-562 cell lines (chronic myeloid leukemia).

Comp.	Capan-1	Hap-1	HCT-116	NCI-H460	DND-41	HL-60	K-562
	IC_{50}[1]	IC_{50}[1]	IC_{50}[1]	IC_{50}[1]	IC_{50}[1]	IC_{50}[1]	IC_{50}[1]
GEM (1)	0.002	0.003	0.004	0.004	0.004	0.003	0.008
Bis (SATE)-GEM-MP (24)	0.002	0.004	0.004	0.004	0.002	0.001	0.05
HepDirect-GEM-MP (14)	0.001	0.003	0.004	0.004	0.001	0.003	0.07
CycloSal-GEM-MP (20a)	0.002	0.004	0.003	0.008	0.02	0.003	0.05
Mix SATE-GEM-MP (28)	0.004	0.005	0.008	0.06	0.002	0.001	0.006
AZT (2)	8.7	12.6	10.8	8.9	3.9	4.1	13.0
cycloSal-AZT- MP(17a)	>20	>20	>20	>20	>20	>20	>20
Mix SATE-AZT-MP (26)	3.2	3.9	12.3	9.3	8.8	7.0	13.9
FUDR (3)	0.003	0.004	10.4	0.2	0.02	0.5	0.2
HepDirect-FUDR-MP (15)	0.004	0.007	4.6	0.4	0.02	0.6	0.3
cycloSal-FUDR-MP (21a)	0.003	0.1	0.9	4.7	0.02	0.1	0.1
Doxetacel	5.3	8.9	5.1	3.6	0.8	1.2	1.6
Staurosporine	22.2	8.2	19.7	41.1	17.7	57.2	37.3

23 [1]Concentration values are expressed in μM

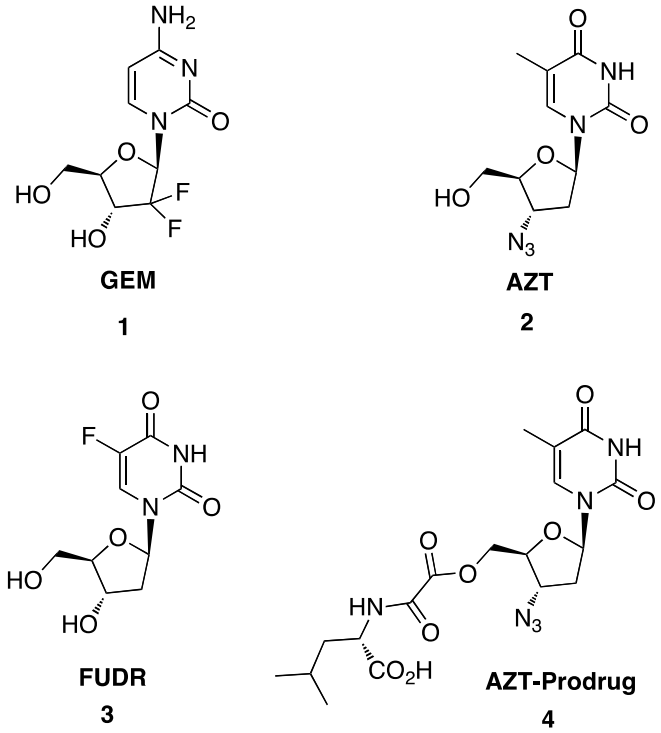

Figure 1. Structures of nucleic acid inhibitors: GEM (**1**), AZT (**2**), FUDR (**3**), AZT-prodrug (**4**).

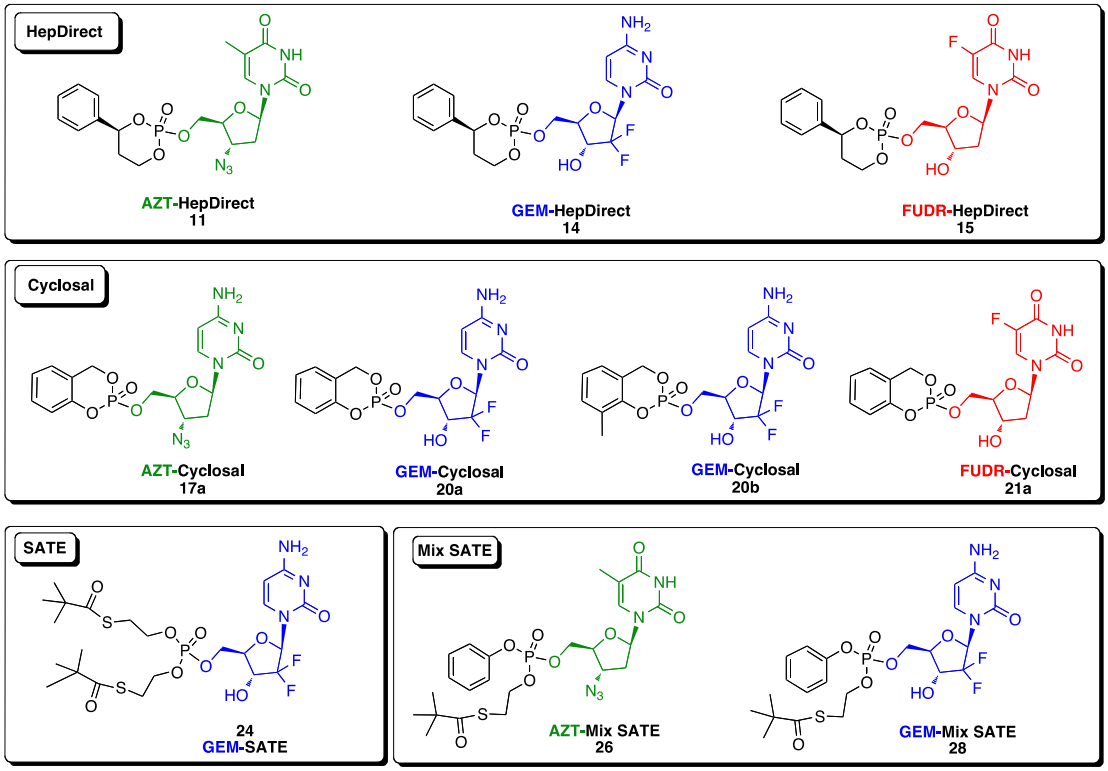

Figure 2. Chemical structures of the prodrugs used for these study.

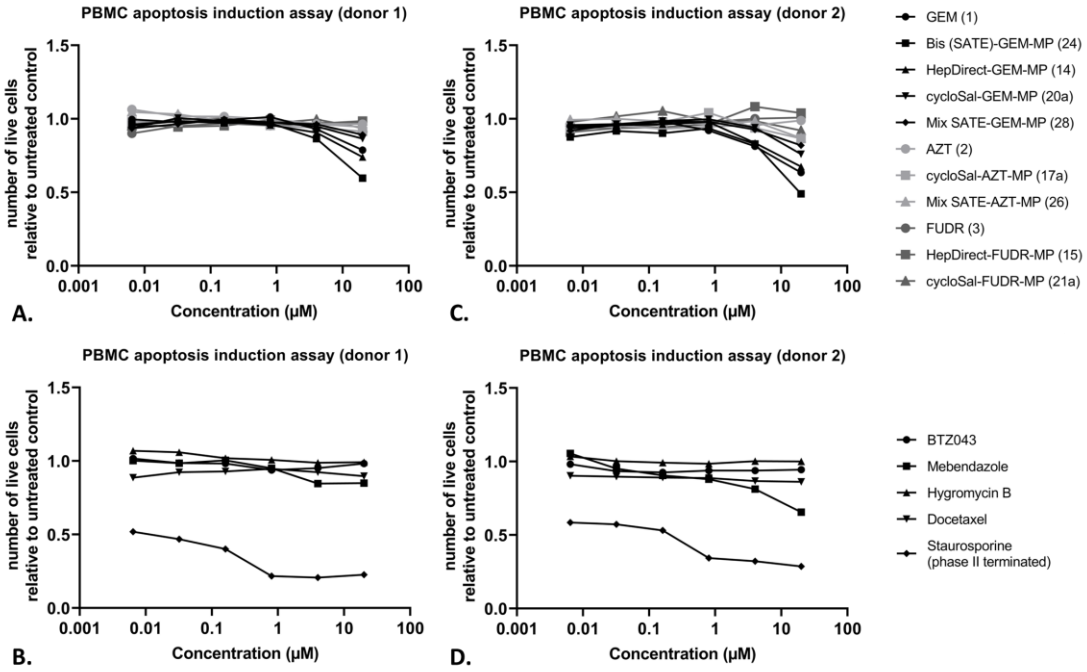

Figure 3. Apoptosis induction in PBMC. PBMCs of two donors were treated with parent nucleosides (**1-3**) and their HepDirect (**14-15**), cycloSal (**17a**, **20a** and **21a**) bis (SATE) (**24**) and mix-SATE (**26** and **28**) prodrugs (A and C) and with reference antimicrobials and anticancer drugs (B and D).

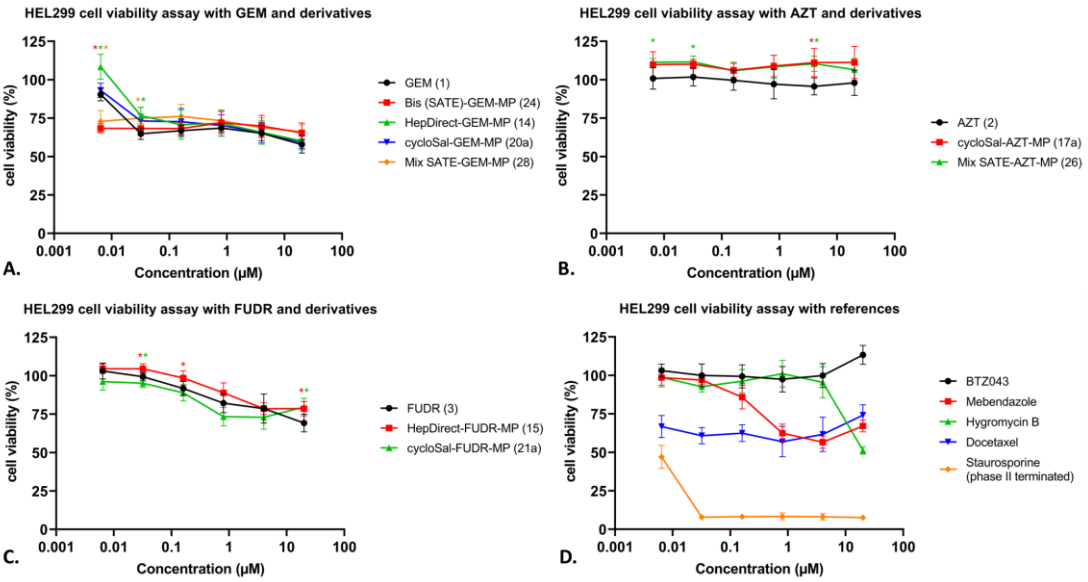

Figure 4. HEL299 cell viability assay. HEL299 cells were treated with parent nucleosides (**1-3**) (A-C) and their HepDirect (**14-15**), cycloSal (**17a**, **20a** and **21a**) bis (SATE) (**24**) and mix-SATE (**26** and **28**) prodrugs (A), and with reference antimicrobials and anticancer drugs (D). Cell viability (%) is displayed in function of

compound concentration (µM) after 72h of incubation. Statistical difference between each parent nucleoside and its derivatives was determined with two-way ANOVA-tests with multiple comparisons. Stars (colour coded according to legend) indicate a significant deviation of the corresponding prodrug from the parent nucleoside with $p < 0.05$.