Dual-Activity Fluoroquinolone-Transportan 10 Conjugates offer alternative Leukemia therapy during Hematopoietic Cell Transplantation

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List of nonstandard abbreviations

AL - Acute Leukemia
ALL - Acute Lymphoid Leukemia
AML - Acute Myeloid Leukemia
B_{SI} - Bacteriostatic Selectivity Index
CML - Chronic Myeloid Leukemia
CIP - Ciprofloxacin
CPP- Cell-Penetrating Peptide
EC - Effective Concentration
IC - Inhibitory Concentration
HC_{50} - Hemolytic Concentration
HCT - Hematopoietic Cell Transplantation
H_{SI} - Hemolytic Selectivity Index
M_{SI} - Malignancy Selectivity Index
LVX - Levofloxacin
ROS - Reactive Oxygen Species
TP10 - Transportan 10
ABSTRACT

Hematopoietic cell transplantation (HCT) is often considered a last resort leukemia treatment, fraught with limited success due to microbial infections, a leading cause of mortality in leukemia patients. To address this critical issue, we explored a novel approach by synthesizing antileukemic agents containing antibacterial substances. This innovative strategy involves conjugating fluoroquinolone antibiotics, such as ciprofloxacin (CIP) or levofloxacin (LVX), with the cell-penetrating peptide (CPP) transportan 10 (TP10). Here, we demonstrate that the resultant compounds display promising biological activities in preclinical studies. These novel conjugates not only exhibit potent antimicrobial effects but are also selective against leukemia cells. The cytotoxic mechanism involves rapid disruption of cell membrane asymmetry leading to membrane damage. Importantly, these conjugates penetrated mammalian cells, accumulating within the nuclear membrane without significant effect on cellular architecture or mitochondrial function. Molecular simulations elucidated the aggregation tendencies of TP10 conjugates within lipid bilayers, resulting in membrane disruption and permeabilization. Moreover, mass spectrometry analysis confirmed efficient reduction of disulfide bonds within TP10 conjugates, facilitating release and activation of the fluoroquinolone derivatives. Intriguingly, these compounds inhibited human topoisomerases, setting them apart from traditional fluoroquinolones. Remarkably, TP10 conjugates generated lower intracellular levels of reactive oxygen species (ROS) compared to CIP and LVX. The combination of antibacterial and antileukemic properties, coupled with selective cytostatic effects and minimal toxicity towards healthy cells, positions TP10 derivatives as promising candidates for innovative therapeutic approaches in the context of antileukemic HCT. This study highlights their potential in search of more effective leukemia treatments.

SIGNIFICANCE STATEMENT

Fluoroquinolones are commonly used antibiotics, while TP10 is a CPP with anticancer properties. In HCT, microbial infections are the primary cause of illness and death.Combining TP10 with fluoroquinolones enhanced their effects on different cell types. The dual pharmacological action of these conjugates offers a promising proof-of-concept solution for leukemic patients undergoing HCT. Strategically designed therapeutics, incorporating CPPs with antibacterial properties, have the potential to reduce microbial infections in the treatment of malignancies.

INTRODUCTION

Microbial infections, varying by the type of hematologic malignancy, can contribute to approximately 7.1% of deaths in leukemia treatment (Chen et al., 2022). They are a noticeable concern
in children with acute lymphoid leukemia (ALL) (O'Connor et al., 2014; Sezgin et al., 2022) as well as generally in older patients for different leukemia types (Safdar et al., 2011; Hassan et al., 2014; Peterson et al., 2019).

HCT serves as a cornerstone in leukemia treatment, demonstrating remarkable efficacy despite the evolving landscape of anti-leukemic therapies (Slavin et al., 1998; Schlenk et al., 2008; Casper et al., 2012; Grossman et al., 2014). Nevertheless, the persistent challenge in this context is the susceptibility of HCT recipients to microbial infections (Cornelissen et al., 2016), which continues to be a leading cause of morbidity and mortality ranges from 19% to 44% despite early aggressive antimicrobial therapy (Yeh et al., 1999; Wang et al., 2018).

Interestingly, cells of several leukemia types, including B (Sun et al., 2018) and T (Mehta et al., 2023) cells, acute myeloid leukemia (AML), as well as chronic myeloid leukemia (CML) (Sánchez-Martín et al., 2007; Nirmalanandhan et al., 2015; Bandyopadhyay et al., 2017; Morel et al., 2017; Caliceti et al. 2017; Zhao et al. 2019), have disruptions in the lipid composition of their outer cell membrane. Noteworthy, these lipid abnormalities were proposed as a potential cellular target for hematological malignancies (Brendolan et al., 2022).

CPPs, exemplified by TP10, offer a promising avenue for improving therapeutic strategies (Regberg et al., 2012; Hilchie et al., 2013; Feni & Neundorf, 2017; Zhang et al., 2018; Habault & Poyet, 2019; Zhou et al., 2022; Zorko et al., 2022). TP10, renowned for its efficient cellular penetration (Pooga et al., 1998; Soomets et al., 2000), exhibits cytotoxicity towards cancer cells (Saar et al., 2005; Rusiecka et al., 2016; Ptaszyńska et al., 2020) while preserving the survival of healthy cells (Ptaszyńska et al., 2020). Importantly, TP10 does not show any significant toxic or immunogenic effects in in vitro and in vivo experiments (Suhorutsenko et al., 2011; Urandur et al., 2023).

In contrast, well-established fluoroquinolone antibiotics such as LVX and CIP (Patel et al., 2020; Gardner et al., 2022) function by disrupting essential bacterial enzymes, like topoisomerase IV (Hooper, 2001; Redgrave et al., 2014). It is worth noting that, at high concentrations, LVX inhibits cell proliferation and triggers senescence of lung cancer cells by inducing mitochondrial dysfunction and oxidative damage (Song et al., 2016).

Recent studies have delved into the development of TP10 conjugates, aiming to harness their combined antibacterial and/or anticancer properties (Regberg et al., 2012; Ruczyński et al., 2019; Ptaszyńska et al., 2020). Notably, TP10 conjugates exhibit reduced generation of ROS and demonstrate antifungal activity against pathogenic yeasts (Ptaszyńska et al., 2020).

This sets the stage for exploring TP10 conjugates as a promising solution to the persistent challenge of microbial infections in leukemia patients, also those undergoing HCT. The innovative approach presented herein holds significant potential to enhance the overall efficacy of leukemia treatment.
MATERIALS AND METHODS

Data and statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al. Unless stated otherwise, n= 5 independent biological replicates were performed for each data point, expressed as mean ± SEM. Statistical analysis was performed either using Prism 8 (GraphPad, USA). Uniform significance level was applied through the entire text: * p < 0.05. As all data was assumed as normally distributed, statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test or two-way ANOVA followed by Dunnett’s multiple comparisons test (for analysis of multiple treatment means). Outliers were identified by ROUT test (Q = 1%).

LC-MS analysis

Before MS analyses, the samples were desalted using ZipTip® C18 Pipette Tips (Merck KGaA, Germany) according to the manufacturer’s protocol. LC-MS experiments were performed on a Qtof lcms9030 spectrometer (Shimadzu, Japan) equipped with an electropress ion source, LC Nexera X2 module with autosampler. Samples were dissolved in water and analysed on an XB C18 Aeris Peptide column (100 mm × 2.1 mm, Phenomenex®); 3.6 µm bead diameter. The LC system was operated with mobile phase: solvent A: 0.1% formic acid in H₂O and solvent B: 0.1% formic acid in MeCN. Samples were separated with a linear gradient (optimised for the best separation of the analysed samples), maintained at a flow rate of 0.2 mL min⁻¹. The injection volume was between 0.1 and 0.5 µL.

Drugs

Following compounds were purchased from Sigma-Aldrich (USA): 7-aminoactinomycin (Cat. A9400), CCCP (Cat. 215911), ciprofloxacin (Cat. 17850), levofloxacin (Cat. 1362103), DMSO (Cat. D8418). All drugs were dissolved in DMSO to concentrations of 2 to 10 mM and stored at −20 °C. 7-AAD was dissolved in MeOH:H₂O (4:6) and stored at 4 °C.

Cell Cultures

HL60, CEM and A549 were acquired from ATCC. The HL60, Jurkat, K562, and A549 cell lines were cultured in RPMI-1640 medium (Cat. R5158, Merck) and HEK293 Eagle’s Minimum Essential Medium (Cat. M5775, Merck). All cell lines were supplemented with 10% FBS (Cat. F7524, Sigma-Aldrich), 2 mM L-glutamine (Cat. G8540, Sigma-Aldrich), and the antibiotics penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. All cell lines were routinely screened for mycoplasma contamination. Cell density was measured using Coulter Z2 (Beckman, USA) equipped with 100 µm aperture or using the flow cytometer Guava EasyCyte 8HT (Merck-Millipore, USA).
**TP10 and Conjugate Synthesis**

TP10 and conjugates 2-6 were synthesized according to the method detailed prescribed in our previous work (Ptaszyńska et al., 2020). Briefly, TP10 and CTP10 were synthesized applying a Prelude peptide synthesiser (Gyros Protein Technology, Inc., USA) and purified with RP-HPLC (PLC 2050 Gilson HPLC, France) to a purity of at least 95% and the authenticity of the peptides were confirmed by mass spectrometry analysis carried out by a MALDI-TOF/TOF, Autoflex MAX spectrometer, (Bruker, Billerica, MA, USA) with an α-cyano-4-hydroxycinnamic acid (CCA) and/or 2,5-dihydroxybenzoic acid (DHB) matrix as described previously (Ptaszyńska et al., 2020). The conjugates 2-6 (Figure 1a) were synthesized as detailed elsewhere (Ptaszyńska et al., 2020). Briefly, in the case of conjugate 2 and 4, CIP and LVX were manually attached to the N- end of peptidyl-resin using standard coupling reagents as was described in (Ptaszyńska et al., 2020). In order to obtain conjugate 3, a solution of Lomant’s reagent (3,3’-dithiodipropionic acid di(N-hydroxysuccinimide ester, Cat. D3669, Sigma-Aldrich))-DSP, was added to the peptidyl-resin and in the next step ciprofloxacin was added. To obtain conjugate 5, the disulphide bridge between LVX-Cys(Npys) and CTP10-NH₂ was formed in solution. In the case of conjugate 6, the disulphide bridge was formed during the reaction of CTP10-NH₂ and Cys(Npys)-CIP as described above for 5. After completing the syntheses, the solvent was removed in vacuo, and the conjugates 5 and 6 were purified by semi-preparative HPLC: PLC 2050 Gilson HPLC with Gilson Glider Prep. Software (Gilson, France), equipped with GraceVydac C18 (218TP) HPLC column (22 × 250 mm, 10 μm, 300 Å, Resolution Systems). The solvent systems were 0.1% trifluoroacetic acid (TFA) in water (A) and 0.1% TFA in 80% acetonitrile in water (B). Different linear gradients were applied (flow rate 5.6- or 20-mL min⁻¹, monitored at 226 nm). The purities of the synthesised peptides were checked with an HPLC Pro Star system (Varian, Australia) and use of a Kinetex 5 μm XB-C18 100 Å column (4.6 × 150 mm, Phenomenex®, Torrance, CA, USA). The solvent system was as described above. A linear gradient from 10% to 90% B for 40 min, flow rate 1 mL min⁻¹, monitored at 226 nm was used. All described peptides had purities of at least 95%. In order to confirm the correctness of the molecular masses of the synthesised peptides, mass spectrometry analysis was carried out by MALDI-TOF/TOF, Autoflex MAX spectrometer, (Bruker, Billerica, MA, USA) with an α-cyano-4-hydroxycinnamic acid (CCA) and/or 2,5-dihydroxybenzoic acid (DHB) matrix. (Ptaszyńska et al., 2020).

**Flow cytometry**

**Cell death**

HL60 were stained with Annexin-V-FLUOS (Cat. 11828681001, Roche) according to the manufacturer’s instruction. Dead and necrotic cells were counterstained with 1 mg/mL 7-AAD (Cat. A9400, Sigma) and analysed by Guava easyCyte flow cytometer (Merck-Millipore, USA). Results of flow cytometry were analysed with Flowing Software 2.5.1 (Turku Bioscience, Finland).
**Mitochondrial potential**

HL60 was incubated with 100 nM 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3)) (Cat. D273, Thermo Fischer Scientific). Cells were stained under culture conditions for 15 min, washed with prewarmed PBS, and performed Guava easyCyte flow cytometer (Merck-Millipore, USA). Results of flow cytometry were analysed with Flowing Software 2.5.1 (Turku Bioscience, Finland).

**Intracellular ROS generation**

Cell lines were cultured as mentioned above and seeded in Petri dishes (Falcon, USA) in the amount of 25x10^3 per Φ35 dish. A549 were allowed to attach overnight. ROS generation potential was tested for selected compounds after 0.25, 0.5, 1, 3, 6 and 24 h of incubation. CM-H2DCFDA molecular probe (Thermo Fischer Scientific, Waltham, USA) at final concentration 1 µM was added 15 min before the analysis. After staining A549 cells were trypsinised, harvested and suspended in fresh media. 7AAD (Cat. A9400, Sigma Aldrich) was added just before analysis. Analyses were carried out with Guava easyCyte flow cytometer (Merck-Millipore, USA). Flow cytometry data was processed with Flowing Software 2.5.1 (Turku Bioscience, Finland).

**Confocal Microscopy**

**Conjugates Uptake**

The tested compound was added at IC_{90} concentration to HL60 culture on Petri dish culture. Samples were observed using the Zeiss LSM 800 Axio Observer 7 inverted microscope with AiryScan detector and CCD camera, applying appropriate filters (Zeiss, Germany). Observation was done in chamber with temperature and CO₂ regulation (Zeiss, Germany). An objective with 63x optical magnification was used for observations. Images were processed in ZEN Blue 2.6 software (Zeiss, Germany).

**Nucleus and Mitochondria**

A day before the experiment, A549 cells were seed in the amount of 25 x10^3 per Φ35 mm Petri dish (Falcon, USA) containing 2-3 coverslips Φ12 mm (Marienfeld, Germany). The tested compound was added at IC_{90} concentration. Cells were stained with SYTO 62 (Cat. S11344, Termofisher Scientific) (final concentration 250 nM) (final concentration) as well JC-1 (Cat. T3168, Termofisher Scientific) (final concentration 500 nM) (final concentration). Samples were observed using the Zeiss LSM 800 Axio Observer 7 microscope with AiryScan detector and CCD camera applying appropriate filters (Zeiss, Germany). Incubation chamber mounted on the microscope allowed for temperature and CO₂ regulation (Zeiss, Germany). An objective with 63x optical magnification was used for observations. Images were acquired and processed in ZEN Blue 2.6 software (Zeiss, Germany).

**Cytoskeleton Immunofluorescence**
25 ×10³ of A549 were seeded on Petri dish (Falcon, USA) containing 2-3 coverslips Φ12 mm (Marienfeld, Germany) and incubated 24 h for adhesion. Test compounds dissolved in DMSO (Merck, Germany) were added at IC₉₀ concentration and incubated for another 24 h. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) for 10 min in RT. Then cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at RT. After that the samples were blocked with 3% BSA (Sigma-Aldrich, USA) in PBST (PBS with 0.2% Tween 20 (Sigma-Aldrich, USA). Then they were incubated with mouse monoclonal anti-β-tubulin primary antibody (1:300 in 3% BSA/PBST; Cat.T8328; Sigma-Aldrich, USA) for 1 h at 37 °C following incubation with goat anti-mouse monoclonal secondary antibody conjugated to a fluorochrome Dylight 488 (1:250 in 3% BSA/PBST; Invitrogen, USA) for 1 h at 37 °C. Successively, cells were incubated with phalloidin conjugated to Alexa Fluor 647 (1:200 in 3% BSA/PBST; Invitrogen, USA). Finally, the samples were stained with DAPI solution (0.5 µg/mL in PBS; Sigma, USA) for 10 min at RT. Coverslips were applied to slides with a layer of mounting medium on the base glycerol (90% glycerol/10% 1x PBS, 25 mg/mL DABCO). Protected against drying out nail polish. Slides were observed under an LSM 800 Axio Observer 7 confocal inverted microscope (Zeiss, Germany). Micrographs were processed in the ZEN 2.6 application (Zeiss, Germany).

**Metabolism and Cellular Stability Study LC-MS analysis**

Extracellular samples

Compounds were added at concentrations IC₉₀. The cultures were cultured in NuAir Incubator (37 °C, 5% CO₂). After 15 min, 3 h, 24 h incubation the samples were prepared as followed. For HL60, the suspension cultures were centrifuged (250 g, 5 min, 4 °C). The supernatant was transferred to new tubes. For HEK293, the medium was transferred to new tubes and centrifuged (500 g, 5 min, 4 °C) to remove cell debris. The protease inhibitor cocktail (Roche) was added and from this moment the samples were kept on ice. The samples were stored at -20 °C until the MS analysis.

Cell fractionation

For HEK293, the cells were incubated in 1 mM EDTA in PBS for 5 min at RT. Then the cells were suspended by vigorous pipetting and centrifuged (250 g, 5 min, 4 °C). The cell pellets were suspended in Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% (v/v) glycerol, 0.1% Triton X-100, protease inhibitor cocktail (Cat. 11697498001, Roche)). Samples were incubated for 10 min on ice and centrifuged (1300 g, 4 °C, 4 min) to pellet down the nuclei. The supernatant was transferred to new tubes and centrifuged (16100 g, 4 °C, 15 min). Next, the supernatant (cytosolic fraction) was transferred to new tubes and stored. The pellet was washed in Buffer A, resuspended in Buffer B (3 mM EDTA, 0.2 mM EGTA, protease inhibitor cocktail (Roche)) and sonicated. The samples were centrifuged (16100 g, 4 °C, 15 min) to clear from the debris and the supernatant (nucleic fraction) was transferred to new tubes. The samples were stored at -20 °C until the LC-MS analysis.
Inhibition of S. Aureus Gyrase and Topoisomerase IV Activities

Inhibitory concentrations for three gyrase and topoisomerase IV activities were assessed: gyrase supercoiling, TOPO IV relaxation and TOPO IV decatenation. For this, commercially available kits were used (S. aureus gyrase supercoiling assay kit, S. aureus topoisomerase IV relaxation assay kit and topoisomerase IV decatenation kit all purchased from Inspiralis (UK)). The procedure was carried out according to the manufacturer’s instructions. The substrates for the reactions were: 500 ng of the relaxed pBR322 plasmid, 500 ng of supercoiled pBR322 or 200 ng of kinetoplast DNA (kDNA) from Crithidia fasciculate, respectively. The studied compounds were dissolved and diluted in dd H$_2$O at the experimentally selected concentrations (gyrase: 2000-250 μM for CIP, 1000-50 μM – LVX, 500-50 μM conjugates, topoisomerase: 500-5 μM CIP/LVX, 250-10 μM – conjugates). Appropriate controls were included (with or without the enzyme or without the addition of the tested compound). After the addition of the indicated amount of the enzyme, the reaction proceeded at 37 °C for 30 min. Loading buffer was used for reaction termination. The studied compounds were extracted from the reaction mixtures with 30 µL of chloroform/isoamyl alcohol solution (24:1; v/v). After centrifugation (3 min, 20,000 g), the upper aqueous phase was loaded on 1% agarose gels and run at 90 V for 4 h in TBE buffer (90 mM Tris-base, 70 mM boric acid, 1 mM EDTA, pH 8). Gels were stained with 1 μg/mL ethidium bromide (Sigma, USA) for 15 min to visualize DNA and unbound ethidium bromide was removed by washing gel in dd H$_2$O for 15 min.

Inhibition of DNA Relaxation Mediated by DNA Topoisomerase IIα and β

Human DNA topoisomerase II α and β Relaxation Assay Kits were purchased from Inspiralis (UK). Assays were performed according to manufacturer protocol. Briefly, the reaction mixture was prepared by mixing 500 ng of pBR322 DNA in reaction buffer (50 mM Tris-HCl, pH 7.5, 125 mM NaCl, 10 mM MgCl$_2$, 5 mM DTT, 100 μg/mL albumin, 30 mM ATP). Studied compounds were diluted in DMSO in the indicated concentrations. The reaction was initiated by the addition of topoisomerase IIα or β and allowed to proceed at 37 °C for 30 min. Reactions were terminated by addition of 5 μL of a loading buffer (Cat.B7024, NEB, UK). Studied compounds were extracted from reaction mixtures on vortex (for 30 sec) with 30 μL of chloroform/isoamyl alcohol solution (24:1; v/v). After centrifugation (2 min, 20000 g) half of upper aqueous phases were separated in 1% agarose gels at 15 V overnight in TBE buffer (90 mM Tris-base, 70 mM boric acid, 1 mM EDTA, pH 8). Gels were stained with 1 μg/mL ethidium bromide for 15 min to visualize DNA and unbound EtBr was removed by washing gel in 1 mM MgSO$_4$ solution in dd H$_2$O for 15 min. Gels were photographed under UV illumination with an ESSENTIAL V6 (UVITEC, UK).
RESULTS

1. TP10 conjugates synthesis

TP10 conjugates and constituent compounds (named here 2, 3, 4, 5, and 6) were synthesized as detailed previously (Ptaszyńska et al., 2020). Their structures are presented in Figure 1a.

2. Biological activities of TP10 and its conjugates

2.1. Activity against cancer cells

The antiproliferative activity of TP10 and its conjugates was evaluated using the MTT assay on a panel of five malignant cell lines: Jurkat, HL60, K562, CEM, and A549. Among them, Jurkat and CEM represented ALL, HL60 represented AML, K562 represented CML, and A549, the non-small lung cancer cell line, served as a representative of epithelial cancers (Supp. Table 1). The cellular response to all tested compounds was as indicated, with no more than a 10-fold difference for calculated Inhibitory Concentration (IC_{50} and IC_{90}) values, and in some cases even less than a 2-fold difference (Supp. Table 1). The tested compounds displayed IC_{50} and Effective Concentration (EC_{50}) values in the low-micromolar or nanomolar range in all tested cell lines (Supp. Table 1). The lowest concentrations of conjugates inducing the EC_{50} and EC_{90} effects were observed for 6 in Jurkat and were approximately 1 and 4 nM, respectively. The highest EC_{50} (7 nM) and EC_{90} (25 nM) were calculated for 5, in A549 and K562, respectively. In the average activity analysis of conjugates against cell lines, Jurkat proved to be the most sensitive with an average EC_{50} of 1 nM, followed by K562 with 2 nM, HL60 with 3 nM, and CEM with 5 nM (Supp. Table 1). The cell line A549 was least sensitive for 5 and 6 with an EC_{50} of ~6 and ~7 nM, respectively (Supp. Table 1). In Jurkat cells, the cytostatic effect of TP10 indicated by its EC_{50}, was on average ~10 times higher compared to the tested conjugates, whereas in K562, it was reduced ~2-fold for compounds 2, 4, and 6. In the case of HL60 cell line, the only such difference was observed for compound 4 (Supp. Table 1). In HL60, the EC_{50} and EC_{90} differences between compounds were around 2-fold with the exception for conjugate 4 with the highest activity with EC_{50}/EC_{90} at ~1/2 nM (Supp. Table 1). Although for CEM, the differences in EC_{50}/EC_{90} were less than 2-fold, none of the tested compounds was more active than TP10 itself. To assess the conjugates activity relative to TP10 and the antibiotic mixture, a comparative probe was included. For this purpose, in the experiment performed on Jurkat cells we added dedicated wells containing TP10 at EC_{50} concentration along with matching molar amounts of the CIP or LVX (Supp. Table 2). It is worth noting, that in the TP10 mixture combination of with an antibiotic, resulted in approximately 8-fold increased cell sensitivity measured by EC_{50} when compared to TP10 alone (Supp. Table 2a). Examining the TP10 mixture within the experimental cell culture well environment, we obtained EC_{50} values for 2, 4, and 6 that were respectively 30, 40 and 60 percent more efficient for the conjugates compared to the corresponding TP10 and antibiotic mixture (Supp. Table 2b). Out of
these three, a uniquely sulfur linked compound 6 was the most cytostatic (Supp. Table 2b).

2.2. Selectivity against bacteria

We recently showed that the fluoroquinolones conjugated to TP10 retain selectivity against microbes normalized to noncancer cells (Ptaszyńska et al., 2020). To assess if that is still the case with other cell types, we calculated Bacteriostatic Selectivity Index ($B_{SI}$) values in relation to leukemia and epithelial cancer cells as $B_{SI} = \frac{MIC_{90}}{IC_{50}}$ (Supp. Table 3). Intriguingly, TP10 maintained a favorable $B_{SI}$ for the majority of the tested bacterial strains across all examined cell lines, as shown in Figure 1b. Conjugates had a favorable $B_{SI}$ level in almost all cell lines with the exception for Jurkat (Figure 1b). In the case of Jurkat, all conjugates besides 3 displayed cytostatic effect greater than bacteriostatic (Figure 1b). For the remaining cell lines tested with conjugates 4, 5, and 6, the least desirable (lower than 0.8) $B_{SI}$ values were obtained for $P. aeruginosa$ species. Interestingly, in case of conjugate 3, selectivity against $E. coli$ normalized to IC$_{50}$ against leukemic cells was 300-fold higher (Supp. Table 3).

2.3. Selectivity against leukemia

As shown previously, the cytotoxic activity of TP10 and its conjugates is higher in malignant Hep2G (hepatocellular cancer) than in healthy cells HEK293 (human embryonic kidney) and LLC-PK1 (pig kidney) (Ptaszyńska et al., 2020). To evaluate their effect on leukemia, the Malignancy Selectivity Index $M_{SI} = \frac{EC_{50}(\text{non-cancerous})}{EC_{50}(\text{leukemic or cancerous})}$ or $EC_{50}(\text{leukemic or cancer}) / EC_{50}(\text{leukemic or cancerous})$ (Supp. Table 4) and visualized in Figure 1c. Most compounds had a higher $M_{SI}$ not only for healthy cells than leukemic, but also for leukemic than cancer (Figure 1c, Supp. Table 4). The activity of the tested compounds besides compound 3 against Jurkat was higher compared to other cell lines, including healthy cells. Conjugate 3 displayed this non-desirable $M_{SI}$ also in other cell types (Figure 1c). Compared to other cell lines, compounds 5 and 6 were most active in Jurkat and K562. On the contrary, compound 4 showed the broadest spectrum of leukemic selectivity, when compared to HEK293 and LLC-PK1. Furthermore, conjugation by a disulfide bridge connecting cysteine TP10 derivative (CTP10) in conjugates 5 and 6 typically yielded compounds with higher $M_{SI}$ values than their non-cysteine 4 and 3 respective counterparts (Figure 1c). Interestingly, taken into account CEM and HL60 cell lines, conjugates 2 and 3 exhibit similarly worse selectivity towards solid tumor cells (Figure 1c).

Additionally, for conjugates 2, 5 and 6, a Hemolytic Selectivity Index $H_{SI}$ ($H_{SI} = \frac{HC_{50}}{IC_{50}}$ for leukemia or epithelial-cancerous) was calculated (Supp. Table 5) and presented in Figure 1d. Unmodified TP10 displayed the highest $H_{SI}$ for leukemia than cancer (Figure 1d). The highest $H_{SI}= 47$ was determined in K562, while for CEM and HL60 leukemia cells, this value was no less than 12 (Figure 1d, Supp. Table 5). TP10 exhibited a relatively low $H_{SI}= 2$ only in Jurkat. Conjugate 5 retained nearly 2-times higher selectivity for AML (HL60 $H_{SI}= 6$) than for ALL (CEM...
H$_{SI}$ = 3), whereas CML (K562 H$_{SI}$ = 52) and ALL (Jurkat H$_{SI}$ = 48) were similar to each other (Supp. Table 5). Interestingly, 5 and 6 retained favorable H$_{SI}$ values with leukemic cell types. The H$_{SI}$ of these conjugates for Hep2G and A549 were negative, indicating values of less than 0.8.

3. Study of potential toxicity

3.1. Suppression of intracellular ROS generation by fluoroquinolones

The ROS-generating activity of TP10 and its conjugates with LVX was evaluated in HL60 (Figure 2a) and HEK293 (Figure 2b). ROS were detected using fluorescent CM-H$_2$DCFDA probe, while unconjugated LVX, a known ROS inducer, was used as a positive control (Ptaszyńska et al., 2020). Interestingly, incubation with 4 and 5 (both at 2-fold IC$_{90}$) resulted in negligible levels of cellular ROS (Figure 2). Additionally, ROS generation activity was evaluated in the Mature HL60, enriched by ROS positive mature stages (Lica et al., 2018). Therefore, the effect was similar for sublines (Figure 2a).

3.2. TP10 and its conjugates have negligible impact on mitochondria

Since our data suggested that the observed cell death was ROS-independent (Figure 2a), we tested whether it could be attributed to mitochondrial dysfunction. To verify this, we evaluated the mitochondrial potential using flow cytometry with HL60 stained with DIOC6(3) (Figure 3a). Surprisingly, none of the tested compounds significantly affected the mitochondrial potential. To further validate this observation, treated A549 cells were stained with JC-1 and examined using confocal microscopy (Supp. Figure 1). The effect on mitochondrial potential and morphology compared to untreated control was not obvious (Supp. Figure 1).

4. Mechanistic Study

4.1. Treatment with TP10 or conjugates results in swift cell death with membrane phospholipid asymmetry loss

It was shown that TP10 can induce membrane disruption (He et al., 2016; Ptaszyńska et al., 2020, Anselmo et al., 2021; Anselmo et al., 2023). Following cell exposure to TP10, we performed a standard Annexin-V flow cytometry assay. As shown in Figure 3b-c, TP10 and conjugates induced quick membrane phospholipid asymmetry loss. For TP10 and conjugates, the effect was particularly pronounced, with a significant Annexin-V increase within 15 min of incubation. It was followed by a massive loss of membrane integrity (Figure 3b-c). Based on Figure 3b-c and on previous findings in Figure 2a and Figure 3a we concluded that the observed membrane disruption is ROS-independent and with minor impact on mitochondria function.

4.2. Localization and Cytoskeleton Function

The fluorescent properties of fluoroquinolones allowed for microscopic observation of these conjugates in UV light. In striking contrast to parent fluoroquinolones, both 5 and 6 accumulated in
HL60 (Figure 4a). After 15 min of incubation with the compound, strong fluorescence localized in the nuclear membrane is observed, as shown on 3D confocal microscopy reconstructions (Figure 4b). Notably, the substantial morphological cell alterations were not mediated by a cytoskeleton disruption (Supp. Figure 2). CIP and LVX alone did not accumulate in eukaryotic cells, which appears to be a critical factor determining their low cytotoxic activity (Figure 4a).

4.3. Cellular Stability and Metabolism

To assess the metabolic fate of the conjugates in vitro, HEK293 and HL60 cells were incubated with 5 and cell lysates were collected after 0.25, 3 and 24 h of incubation. MALDI-TOF MS analyses of lysates showed that the conjugate reached the nucleus of both cell types within the first 15 min of incubation (Supp. Figure 3). MS spectra of the nuclear protein fractions recorded for all time points contained two signals, identified as unmodified conjugate 5 (with m/z 2746.425; [M+H]+) and the constituent peptide CTP10 (with m/z 2284.305) (Supp. Figure 3). In the low range of m/z, the signal corresponding to the cleaved LVX-Cys (m/z 465.274; [M+H]+) was also found on top of the background (coming from the lysate, lysate medium kit, matrix etc.) (Supp. Figure 3). As the only exception, the nuclear fraction of HEK293 lysate obtained after 15 min of incubation yielded only one signal corresponding to the intact conjugate (with m/z 2746.398; [M+H]+) (Supp. Figure 4), suggesting a slower cleavage kinetics. The signals corresponding to the conjugate, the constituent CTP10 peptide, or their degradation products, were not found in MS spectra of the cytoplasmic protein fractions (Supp. Figure 4a). It is worth noting that the cell culture medium did not degrade the conjugate before entering the cells. MS spectra recorded for the medium that was separated from the cells at the end of incubation time with conjugate 4 showed that only the intact conjugate was presented (Supp. Figure 4c).

4.4. Different systematic domain topoisomerases inhibition

We have previously reported the inhibitory activity of conjugates against yeast topoisomerase II (Ptaszyńska et al., 2020). Here, the studies were expanded by in vitro evaluation of activity towards topoisomerases originating from bacterial and human (IIα and β) species. As shown in Supp. Table 6, unconjugated peptide TP10 itself was active against bacterial topoisomerase IV, comparable to bare fluoroquinolones, while the impact of TP10 on the inhibition of gyrase activity was more pronounced. Direct conjugation of CIP or LVX to TP10 in 2 and 4 also improved activity against topoisomerase IV, retaining TP10 level of activity against gyrase. We then tested 5 against human topoisomerases IIα and β and observed that it was also able to inhibit these enzymes in a dose-dependent manner (Figure 5a-b). In light of our experimental data, the impact of LVX on topoisomerase-mediated DNA relaxation is much lower in the case human enzymes (Figure 5 and Supp. Table 6), (Ptaszyńska et al., 2020), consistently with its predominantly antibacterial mode of action.

4.5. Conjugate 5 membrane interactions - computational studies

To investigate possible origins of the observed selectivity of TP10 and its conjugates towards
leukemia, we used computational models of cell membranes to study the drug- and conjugate-membrane interactions on the atomistic level (Fanghänel et al., 2014). We first used free energy methods to evaluate the impact of TP10 conjugation on the membrane permeability of LVX by passive diffusion. Since the two pKa values reported for titratable groups in LVX 6.02 and 8.12 according to National Center for Biotechnology Information (PubChem Compound Summary for CID 149096, Levofloxacain, 2022) are both close to 7, we used the neutral protonation state for both variants, assuming the free energy cost of attaining the neutral state is marginal, within 1-2 kcal/mol. By comparing the 1D free energy profile for LVX with the analogous profile for 5 (Figure 6a-b), it is evident that TP10 does not help in membrane permeation of an isolated drug molecule, substituting a free energy barrier at the membrane midplane of ca. 5 kcal/mol and a free energy well of ca. 9 kcal/mol (that has to be overcome to reach the other end of the membrane) with values of ca. 10 and 7 kcal/mol, respectively.

Instead, equilibrium simulations of multiple membrane-embedded molecules of 5 indicate that at sufficiently high concentrations, it is local membrane disruption that facilitates the passage of the drug through the membrane midplane (Figure 6c). Even then, the high energetic cost of detachment of TP10 from the membrane (ca. 20 kcal/mol) indicates that efficient release of functional LVX towards the cytoplasm and the nucleus would require either hydrolysis or reduction of the conjugate at the membrane surface.

We then studied the behavior of membrane-bound oligomers of the 5 in two different types of model membranes – cholesterol-rich (30% cholesterol and 70% POPC) and cholesterol-free (100% POPC) – using equilibrium simulations (Supp. movie 1). Our simulation results, while circumstantial, showed that (1) the conjugates have a propensity to aggregate into oligomers, as exemplified by the upward trends in green lines in Figure 6d; (2) dimers and trimers already facilitate the penetration of water into the bilayer; (3) water pores initially established by tetramers remain stable over 500 ns, converging to a similar water occupancy in both cases (red/pink lines in Figure 6d); (4) the permeating water assumes a glass-shaped profile.

4.6. Interactions of conjugate 5 and its metabolites with nucleic acids - computational studies

To identify the possible modes of action of LVX derivatives related to DNA binding, we sampled the various interaction modes of LVX, LVX-Cys and conjugate 5 with a DNA 16-mer. Here, we used the protonated (cationic) version of the drug, likely to prevail in the vicinity of negatively charged DNA that typically shifts pKa values by ca. 2 units. In the free energy maps in Figure 7, one can identify regions corresponding to minor- and major-groove-bound states, as well as the intercalated state. Interestingly, intercalation was only observed in the case of “bare” LVX, although it is highly thermodynamically unfavorable (region marked by red dashed line in Figure 7a); protonated LVX mostly remains bound to DNA grooves, major with high affinity and minor with a significant kinetic barrier of 6-8 kcal/mol (Figure 7a, Supp. movie 2). LVX-Cys showed an even smaller
propensity to intercalate (no events observed within 20×500-ns multiple-walker metadynamics), instead binding to the minor and major groove with comparably moderate affinity and notably lower kinetic barriers (Figure 7a). Conjugate 5 had the lowest affinity for DNA overall (judged from the free energy differences between the bound and unbound basins), and hardly distinguished between minor and major groove binding due to non-specific interactions with the peptide component.

We also used a crystal structure of human topoisomerase II beta in complex with etoposide, a known anticancer drug (PDB code 3QX3), substituting the ligand with LVX-Cys to probe the stability of the resulting complex. Intercalated binding pose remains stable over at least 300 ns in both sampled positions (Figure 7b-c). This confirms that LVX-Cys, the primary metabolite of conjugate 5, can just as easily intercalate into the activated topoisomerase complex, acting as a topoisomerase IIβ poison. At the same time, its affinity for DNA intercalation is at least as low as that of LVX, thereby ensuring low mutagenicity. While we could not observe a rearrangement of the magnesium binding site compared to the crystal structure, we note that the presence of a C-terminal carboxyl from the cysteine suggests possible formation of a coordinated complex stabilizing the protein-ligand interaction, a common feature of topoisomerase poisons (Staker et al., 2002; Redgrave et al., 2014).

Interestingly, querying LVX in the cheminformatic tool Chemical Checker (Duran-Frigola, et al., 2020) indicated etoposide and mitoxantrone – two chemotherapeutic agents used in leukemia treatment themselves – as the closest non-fluoroquinolone drugs in terms of predicted molecular targets. This suggests a potential synergy between the antibacterial action of LVX leukemic activity might prevent relapse in HCT patients, as also evidenced by the above mechanistic studies.

**DISCUSSION**

*Current knowledge*

The most effective leukemia treatments still rely on conventional, low-specificity chemotherapy followed by HCT (Cornelissen et al., 2016; Zhou et al., 2020). Importantly, HCT treatment requires immunosuppressive drugs increased risk of life-threatening microbial infections (Poutsiaka et al., 2007). High mortality from HCT infections makes the use of standard antimicrobials, like CIP and LVX, a necessity (Wang et al., 2018; Patel et al., 2020; Gardner et al., 2022; Gardner et al., 2022; Regberg et al., 2012). As a downside, though, antibacterial treatment with CIP and LVX generates high levels of intracellular ROS (Ptaszyńska et al., 2020), contributing to their general toxicity. Meanwhile, it is well-established that the antibacterial activity of CIP and LVX strongly depends on topoisomerase inhibition (Redgrave et al., 2014).

In parallel, it was recently highlighted that CPPs, including both TP10 and its fluoroquinolone conjugates, can combine antibacterial and anticancer function (Poutsiaka et al., 2007; Felício et al., 2017; Duan et al., 2017; Tornesello et al., 2020; Burdkiewicz et al., 2020; Reissmann & Filatova, 2021; Zeiders & Chmielewski, 2021; Rusiecka et al., 2022). These conjugates offer a dual pharmacological effect, targeting both bacterial infections and leukemia cells (Patel et al., 2020;
Conjugation of fluoroquinolones with TP10 decreases intracellular ROS generation, making these compounds more attractive for potential HCT application as combined antimicrobial-immunosuppressive agents (Figure 2 and Gardner et al., 2022).

Previously, we showed that conjugating CIP or LVX with CPP led to selective antibacterial activity against both Gram-negative and -positive bacteria (Ptaszyńska et al., 2020). Importantly, these conjugates exhibit cytotoxicity against S. cerevisiae, C. albicans and cancer cells with low drug sensitivity (Ptaszyńska et al., 2020). The antifungal effect was much more robust in conjugates where TP10 was linked to the fluoroquinolone by a disulfide bond, suggesting that disulfide bond reduction inside the cell might have allowed the interaction of parental drugs with DNA and nuclear enzymes (Yandek et al., 2007; He et al. 2016; Jia & Zhao, 2021).

What is the difference between combination TP10 and antimicrobial from TP10 conjugates?

Chemically, the difference between conjugates and a mixture of TP10 and an antibacterial (CIP or LVX) is that in conjugates, a covalent bond links TP10 to the antibacterial agent. In such an approach, the coupling linker has the ability to confer additional functionalities.

As we showed in our previous works (Ptaszyńska et al 2019a, 2019b), simple mixtures of components of a conjugate do not always act synergistically, sometimes even showing antagonistic effects. A similar pattern was observed for three chimera peptides composed of bovine lactoferramin and the analogue of truncated human neutrophil peptide 1 (Ptaszyńska et al., 2018): the antimicrobial activity of the equimolar mixtures of studied peptides was lower than that of the individual peptides and chimeras.

In all these cases, the addition of a covalent linker – permanent or subject to disintegration inside the cells, like the redox-sensitive disulfide bond – had a significant positive impact on the sought-after properties, even if the precise molecular reason was hard to pinpoint. This indicates that the antagonistic effects of constituent peptides can be overcome by connecting them with a covalent linker, with different linker types modulating the synergistic effect. We thus expected that combining two compounds with distinct chemistry and mechanism of antibacterial action into a single functional molecule may yield compounds with a beneficial biological activity.

We acknowledge that the exact mechanism by which conjugates enhance the individual properties of their constituents is not fully understood, but on a molecular level, several notable differences can be mentioned. Firstly, intracellular delivery is synchronized, and potentially enhanced as conjugate oligomers have the capacity of permeabilizing the cell membrane. Secondly, depending on the kinetics of cleavage, intracellular release of individual components can be prolonged, leading to a longer-lasting effect. Finally, the delayed release of components might selectively delay binding to certain targets, yielding a different cellular response.

Several mechanisms might also make conjugates more favorable in vivo, while being impossible to determine in an in vitro setting. These include co-targeting to affected tissues and higher metabolic stability, lower toxicity prior to linker cleavage, and a lower chance of developing resistance.
upon long-term treatment. These, however, remain speculative until promising candidate drugs are
selected for animal studies.

**Previous study and recent data**

In this report, we present that the antimicrobial effect of TP10 and its conjugates is selective
against bacteria and correlates with anticancer and, in particular, antileukemic activity (Figure 1b-c). Comparative cytostatic activity of TP10 and conjugates on Jurkat, CEM, HL60, K562, A549, Hep2G, HEK293, LLC-PK1, and human erythrocytes show that the effect in most cases is selective against leukemias (Figure 1b-d and Supp. Table 4). Conjugates 4 and 5 perform very well in terms of M_{50} and H_{50} values (Supp. Tables 4 and 5). Additionally, in Jurkat cell line (the cells most sensitive for tested conjugates) we have demonstrated that even in environment without specific substance trapping and elimination of substances (including substances returning to the environment after cell death) compounds 2, 4 and 6 was more effective than TP10 and antibiotic mixture (Supp. Table 2).

In a previous article, we also documented that conjugation of TP10 with ROS-generating CIP and LXV significantly reduced intracellular ROS levels (Ptaszyńska et al., 2020). We now observe the same effect for 4 and 5, which strongly suggests that the induction of cell death is ROS-independent (Figure 2a-b).

The fluorescent properties of LVX and CIP allowed microscopic observations of the cellular accumulation of TP10 conjugates (Figure 4). After 15-min incubation with 5 and 6, 2D as well 3D-reconstruction microphotographs showed cells with critical cell membrane defects (Figure 4) but no effect on cytoskeleton (Supp. Figure 2). In both living and dead cells, a strong signal is emitted from the nuclear membrane and the nucleus (Figure 4). Importantly, flow cytometry and fluorescent confocal microscopy demonstrated no observable effect of the conjugates on mitochondrial structure and function (Figure 3a and Supp. Figure 1).

In functional cells the nuclear membrane-bound aggregates formed by conjugates 5 or 6 appear to be good targets for enzymatic reduction of the disulfide bond between fluoroquinolone and TP10, leading to the penetration of nucleus by fluoroquinolones derivatives. Both oxidized (unmodified) and reduced (released) molecules can interact with nuclear compartments, making the damage more pronounced. 3D-reconstruction confocal microphotographs show that the nuclear membrane is less permeable for the conjugates than the cell membrane (Figure 4).

An LC-MS analysis of second-phase metabolites (nucleus and cytosolic fraction of HL60 and HEK293) shows a partial reduction of the disulfide in conjugate 5 already after 15 min (Supp. Figure 3 and 4). The unique lack thereof in HEK293 lysates indicates another route for selectivity against HL60. The dot plots presented in Figure 3b-c demonstrate that TP10, conjugates induce membrane damage through a switch of membrane protein composition. Based on the quantitative and structural changes in cellular components, some past reports highlighted an altered cholesterol metabolism in leukemic cells showing that these cells actively retrieve cholesterol from plasma and are often sensitive to cholesterol-lowering therapeutics such as statins (Klock & Pieprzyk, 1979; Vitols et al.,
This interesting link provides a possible mechanism for cell type-specific toxicity of membrane active agents towards leukemic cells: if cholesterol was actively consumed by the cell, its content in the plasma membrane would decrease, lowering membrane stiffness and facilitating the penetration by membrane active agents. Because of their involvement in cancer proliferation, survival, and invasion, cholesterol-enriched domains are arguably a significant factor in tumor progression and constitute a promising target in novel anticancer therapies (Maja & Tyteca, 2022; Brendolan et al., 2022).

To investigate possible origins of the observed selectivity of TP10 and its conjugates towards leukemic cells, we used free energy methods to evaluate the impact of TP10 conjugation on the membrane permeability of LVX by passive diffusion. By comparing the 1D free energy profile for LVX with an analogous profile for TP10 it is evident that TP10 does not help in membrane permeation of an isolated drug molecule, substituting a free energy barrier at the membrane midplane of ca. 5 kcal/mol and a free energy well of ca. 9 kcal/mol (that has to be overcome to reach the other end of the membrane) with values of ca. 10 and 7 kcal/mol, respectively.

Instead, equilibrium simulations of multiple membrane-embedded molecules of TP10 indicate that at sufficiently high concentrations, it is local membrane disruption that facilitates the passage of the drug through the membrane midplane. Even then, the high energetic cost of detachment of TP10 from the membrane (ca. 20 kcal/mol) indicates that efficient release of functional LVX towards the cytoplasm and the nucleus would require either hydrolysis or reduction of the conjugate at the membrane surface.

The simulated behavior of membrane-bound oligomers of TP10 in two different types of model membranes – cholesterol-rich (30% cholesterol/70% POPC) and cholesterol-free (100% POPC) – showed that the conjugates have a propensity to aggregate into oligomers, with dimers and trimers already facilitating the penetration of water into the bilayer. Hglass-shaped water pores established by tetramers remained stable over 500 ns, likely allowing for easier permeabilization of thin rather than thick membranes, especially at low concentrations where low oligomers prevail. It remains speculative but plausible that this feature of the pores contributes to specificity towards leukemic cells (Klock & Pieprzyk, 1979; Vitols et al., 1990; Li et al., 2003; Giacomini et al., 2021).

The search for therapeutics similar to LVX in Chemical Checker reveals interesting patterns. While its clinical fingerprint expectedly shows high similarity to other topoisomerase and gyrase inhibitors, its nearest non-fluoroquinolone neighbors in the space of cellular and molecular targets are mitoxantrone, a topoisomerase poison specifically used in leukemia therapy, and etoposide, an anticancer drug also used against leukemia. At high concentrations, LVX inhibits cell proliferation and triggers senescence of lung cancer cells by inducing mitochondrial dysfunction and oxidative damage (Song et al., 2016).

Indeed, as demonstrated in previous work (Ptaszyńska et al., 2020) on yeast topoisomerase, TP10 and conjugates as well as LVX and CIP inhibited the bacterial and human IIα and β isoforms in a
dose dependent manner (Figure 5 and Supp. Table 6). Our computational model showed that the intercalated binding pose of LVX-Cys in the binding site of topoisomerase IIβ remains stable, confirming that while the DNA intercalation affinity of LVX-Cys, the primary metabolite of 5, is at least as low as that of LVX – ensuring low mutagenicity – it can just as easily intercalate into the activated topoisomerase complex, acting as a topoisomerase IIβ poison. The DNA binding modes of LVX, LVX-Cys and 5 were mostly restricted to the major and minor grooves: LVX bound to major groove with high affinity and minor with a kinetic barrier of 6-8 kcal/mol, while conjugated LVX would bind to both grooves with moderate affinity and lower kinetic barriers. Intercalation, albeit highly unfavorable, was only observed in the LVX case.

Based on the study of DNA affinity of LVX and its derivatives, we highlight the important relationship between the propensity to intercalation and the ability to act as a topoisomerase poison. Specifically, making DNA intercalation unfavorable should decrease the formation of secondary lesions, as random intercalation events are usually mutagenic and unwanted. In turn, the ability to intercalate into the exposed active topoisomerase complex can be used to target proliferating cells with higher specificity. Such a design principle might prove useful in engineering of future drugs with a more desirable selectivity/toxicity profile.

**Conclusion**

Our selectivity, toxicity and mechanistic assays provide evidence that TP10 and its selected fluoroquinolones conjugates exhibit antimicrobial and selectively anti-leukemic properties with low level of ROS generation and mitochondrial disfunctions. These results strongly suggest that the leukemic specific activity is a result of fluidity-dependent disruption of membrane integrity by CPP aggregates, as well as specific inhibition of topoisomerase activity. This dual pharmacological property of TP10 conjugates is a promising starting point in the development of new therapeutic solutions for patients ongoing HCT, irrespective of myeloablative intensity (all age patients) or myeloid condition (patients 65+).

**Data Availability**

The authors declare that all the imaging, biological and mass spectrometry data as well as computational simulations supporting the findings of this study are available within the paper and its Supplemental Data.

**Author Contributions**

*Participated in research design:* Lica, Heldt, Wieczór, Chodnicki, Ptaszyńska, Maciejewska, Łęgowska, Stupak, Milewski, Bieniaszewska, Grabe, Hellmann, and Rolka.

*Conducted experiments:* Lica, Heldt, Wieczór, Chodnicki, Ptaszyńska, Maciejewska, Łęgowska, Brankiewicz, Gucwa, Domagalska and Dębowski.
Contributed new reagents or analytic tools: Lica., Heldt., Wieczór, Chodnicki, Ptaszyńska, Łęgowska., Hellmann and Rolka.

Performed data analysis: Lica, Heldt, Wieczór, Chodnicki, Ptaszyńska, Maciejewska, Łęgowska, Brankiewicz, Gucwa, Domagalska, Dębowski, Grabe, Hellmann and Rolka.

Wrote or contributed to the writing of the manuscript: Lica, Heldt, Wieczór, Chodnicki, Ptaszyńska, Maciejewska, Łęgowska, Brankiewicz, Gucwa, Stupak, Pradhan, Domagalska, Dębowski, Milewski, Bieniaszewska, Grabe, Hellmann and Rolka.

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And Antimicrobials - Design, Chemical Synthesis, And Evaluation Of Antimicrobial Activity And Mammalian Cytotoxicity. Peptides. 117, 170079. (2019a)


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**Conflicts of Interest**

The authors declare no conflict of interest.

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**Figure descriptions**

**Figure 1.** Compound structures and Selectivity Indexing Panels. (a) Chemical structures of conjugates and their constituent compounds. Conjugates numbering received from previous work (Ptaszyńska et al., 2020). (b) Bacteriostatic index normalized to IC50. (c) Malignancy index normalized to EC50. (d) Malignancy normalized to HC50. Selectivity indexing: Red – none, Yellow – moderate, Light green – good, Deep green – very good. IC50 - Inhibitory Concentration, EC50 - Effective Concentration (IC normalized to cell number) (Lica et al. 2021), HC50 – hemolytic concentration. For more details, please see also Supp. Tables 3, 4 and 5.

**Figure 2.** Activity of TP10 and selected conjugates on intracellular ROS generation. (a) Dot-plots of HL60 viability and ROS level as measured by 7-AAD and CM-H2DCFDA, respectively. (b) HL60 and HEK293,
the comparison of conjugates and their bare constituents. LB, left bottom; RB, right bottom; LT, left top; RT, right top. Flow cytometry data was analyzed by Flowing Software 2.5.1 (Turku Bioscience, Finland). Values represent averages ± SEM from five independent experiments.

**Figure 3.** The impact of TP10 and specific conjugates on cell death. (a) The conjugates do not affect HL60 mitochondrial potential as evaluated by DIOC6(3) staining. Dot-plots of viability measured by 7-AAD and lipid membrane asymmetry by an Annexin-V assay evaluated on HL60 (b), Jurkat and K562 (c). Flow cytometry data for (b) was analyzed by Flowing Software 2.5.1 (Turku Bioscience, Finland) and for (c) by FlowJo v9 (Becton Dickinson, New Jersey, USA). Values represent averages ± SEM from five independent experiments.

**Figure 4.** Conjugate 5 accumulation in HL60 visualised by fluorescence confocal microscopy after 15 min incubation time. (a) Contrary to LVX conjugate 5 accumulates in the nuclear membrane. Wide-field fluorescence imaging with DIC contrast is shown for reference. CTRL points to cells only control. (b) 3D confocal microscopy reconstruction. Scale bars correspond to 20 µm.

**Figure 5.** Human topoisomerase IIα (a) and IIβ (b) inhibition mediated by conjugate 5. LVX and conjugate 5 were tested at 500, 100, 50, 10, and 5 µM concentrations. Letters R and S stand for respective relaxed and supercoiled DNA forms. Minus and plus signs in control wells (CTRL) point to absence and presence of topoisomerase, respectively. Attached gels are representative of three independent experiments.

**Figure 6.** Simulations of the interaction of LVX and conjugate 5 with model biological membranes. (a) 2D free energy map for the translocation of a single molecule of conjugate 5 as a function of the Z-distances of the peptide part and the LVX ligand from membrane midplane (see miniatures for a visual explanation). (b) 1D free energy profiles comparing the energetic cost of translocation of conjugate 5 (dashed red line, corresponding to the minimal free energy path in panel (a)) and free neutral-charge LVX (solid black line) across a model membrane. (c) A representative snapshot showing a water pore stabilized by a tetramer of conjugate 5 in a POPC membrane. (d) Number of water molecules within the central 1 nm-thick slab around the membrane midplane in four 600-ns simulations, started either from four conjugate monomers or one pre-assembled tetramer, in either pure POPC or cholesterol-rich (raft-like) membranes.

**Figure 7.** Modes of interaction of LVX, LVX-Cys and conjugate 5 with DNA. (a) Free energy maps for the interaction of the respective molecules with a DNA dodecahexamer as a function of distances of the ligand’s centre of geometry to minor and major grooves. (b) Final geometry of LVX-Cys complexed with topoisomerase IIβ: a side and top view of the intercalated complex. (c) RMSD of the two LVX-Cys molecules in the intercalation sites after aligning the nucleic acid portion of the topoisomerase complex.
Figure 2

(a)

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15 min

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30 min

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1 h

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<td>0 ±0</td>
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24 h

(b)

< 15% ROS at all times | 15 min | 30 min | 1 h | 3 h | 6 h | 24 h
> 15% ROS at one point | 15 min | 30 min | 1 h | 3 h | 6 h | 24 h

ROS positive cells [%]
Figure 3

(a) Relative mitochondrial mass [%]

(b) HL-60

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<td>37 ± 10*</td>
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<td>2 ± 2</td>
<td>61 ± 8*</td>
<td>6 ± 3</td>
<td>22 ± 11*</td>
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<td>0 ± 0</td>
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</tr>
<tr>
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<tr>
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<td>5 ± 4</td>
<td>38 ± 2*</td>
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<td>8 ± 2*</td>
<td>8 ± 4*</td>
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<tr>
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<td>2 ± 3</td>
<td>2 ± 1*</td>
<td>2 ± 3*</td>
</tr>
<tr>
<td>RT 30 min</td>
<td>0 ± 0</td>
<td>2 ± 3</td>
<td>2 ± 1*</td>
<td>2 ± 3*</td>
</tr>
</tbody>
</table>

(c) Jurkat

(c) K562

<table>
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<th>Conjugate 5</th>
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<td>19 ± 2*</td>
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<td>38 ± 2*</td>
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<td>6 ± 1*</td>
<td>1 ± 1*</td>
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<tr>
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<td>7 ± 4*</td>
<td>8 ± 3*</td>
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<tr>
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<td>22 ± 1*</td>
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<tr>
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<td>22 ± 1*</td>
<td>28 ± 1*</td>
<td>3 ± 1*</td>
</tr>
<tr>
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<td>1 ± 1*</td>
</tr>
<tr>
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<td>61 ± 1*</td>
</tr>
<tr>
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<td>67 ± 1*</td>
<td>3 ± 1*</td>
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</tr>
<tr>
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</tbody>
</table>

7-AAD

Annexin V

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 4

(a) | CTRL | CIP | LVX | Conjugate 5 | Conjugate 6
---|------|-----|-----|-------------|-------------
DIC | ![DIC images](image1)
UV | ![UV images](image2)
Merge | ![Merge images](image3)

(b) | Conjugate 5  | Conjugate 6
---|-------------|-------------
2D | ![2D images](image4)
3D | ![3D images](image5)
Figure 5

(a) CTRL  CTRL  LVX  Conjugate 5

(b) CTRL  CTRL  LVX  Conjugate 5

R
S
Figure 6