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A drug candidate for Alzheimer's and Huntington's disease, PBT2, can be repurposed to render *Neisseria gonorrhoeae* susceptible to natural cationic antimicrobial peptides

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Background: *Neisseria gonorrhoeae* is a Gram-negative bacterial pathogen that causes gonorrhoea. No vaccine is available to prevent gonorrhoea and the emergence of MDR *N. gonorrhoeae* strains represents an immediate public health threat.

Objectives: To evaluate whether PBT2/zinc may sensitize MDR *N. gonorrhoeae* to natural cationic antimicrobial peptides.

Methods: MDR strains that contain differing resistance mechanisms against numerous antibiotics were tested in MIC assays. MIC assays were performed using the broth microdilution method according to CLSI guidelines in a microtitre plate. Serially diluted LL-37 or PG-1 was tested in combination with a sub-inhibitory concentration of PBT2/zinc. Serially diluted tetracycline was also tested with sub-inhibitory concentrations of PBT2/zinc and LL-37. SWATH-MS proteomic analysis of *N. gonorrhoeae* treated with PBT2/zinc, LL-37 and/or tetracycline was performed to determine the mechanism(s) of *N. gonorrhoeae* susceptibility to antibiotics and peptides.

Results: Sub-inhibitory concentrations of LL-37 and PBT2/zinc synergized to render strain WHO-Z susceptible to tetracycline, whereas the killing effect of PG-1 and PBT2/zinc was additive. SWATH-MS proteomic analysis suggested that PBT2/zinc most likely leads to a loss of membrane integrity and increased protein misfolding and, in turn, results in bacterial death.

Conclusions: Here we show that PBT2, a candidate Alzheimer's and Huntington's disease drug, can be repurposed to render MDR *N. gonorrhoeae* more susceptible to the endogenous antimicrobial peptides LL-37 and PG-1. In the presence of LL-37, PBT2/zinc can synergize with tetracycline to restore tetracycline susceptibility to gonococci resistant to this antibiotic.

Introduction

Neisseria gonorrhoeae is a Gram-negative bacterium that causes the sexually transmitted disease gonorrhoea. Gonococcal infections can cause urethritis in men and cervicitis in women and also have the capacity to cause upper genital tract or disseminated infections.[1] *N. gonorrhoeae* can cause asymptomatically infections.[2] If untreated, infections in men may lead to epididymitis.[3] Asymptomatic infections in women can cause serious sequelae, such as infections of the uterus and fallopian tubes and acute pelvic inflammatory disease, which can lead to infertility or an increased risk for ectopic pregnancies. Mothers with gonorrhoea can also pass the bacteria to infants during birth, resulting in neonatal blindness. More importantly, *N. gonorrhoeae* infection has been implicated in increased susceptibility to HIV infection, reviewed in Edwards *et al.*[4]

The ability of *N. gonorrhoeae* to develop resistance to antibiotics significantly complicates the treatment of gonorrhoea. *N. gonorrhoeae* has developed resistance to the historically used therapeutics[5] and treatment options are currently limited to dual therapy with ceftriaxone and azithromycin. However, gonococcal strains exhibiting resistance to all recommended antimicrobial agents

have recently been identified.[5] The US CDC estimates that there are 550 000 *N. gonorrhoeae* infections each year in the USA (CDC, Biggest Threats and Data). The emergence of MDR *N. gonorrhoeae* has led global public health agencies to identify new gonococcal treatments as a critical unmet need.

PBT2 is a hydroxyquinoline-based ionophore that was developed as a potential treatment for Alzheimer's and Huntington's diseases.[6] The ionophoric properties of PBT2 aid the transport of metal ions, such as zinc, across biological membranes and alter intracellular metal homeostasis.[6] PBT2/zinc can reverse antibiotic resistance in Gram-positive bacteria.[7] Previously, we showed that a combination of PBT2/zinc can disrupt metal homeostasis, resulting in the inactivation of the Zn-containing protein lipooligosaccharide phosphoethanolamine transferase A [LptA; also called phosphoethanolamine transferase (EptA)[8]]. Loss of LptA function reduces the level of phosphoethanolamine on lipid A, thereby altering the charge on the bacterial cell surface and increasing the susceptibility of *N. gonorrhoeae* to the polycationic peptide antibiotics polymyxin B and colistin.[9] The use of polymyxin B and colistin as antimicrobials is restricted because of renal and neurological side effects.[10] It is reported that mutations in the *N. meningitidis lptA* gene can increase meningococcal susceptibility to two cationic antimicrobial peptides, LL-37 and protegrin-1 (PG-1),[11] which occur naturally in mammals and thus are unlikely to cause adverse effects. Here we assess the potential of PBT2/zinc to sensitize *N. gonorrhoeae* to LL-37 and PG-1, and the impact of PBT2/zinc to synergize with other antibiotics in the presence of these endogenous antimicrobial peptides.

Materials and methods

The MDR *N. gonorrhoeae* strains WHO L, WHO M, WHO X, WHO Y and WHO Z[5] were used in MIC assays, according to CLSI guidelines and as previously described[7,9,12] (the detailed Materials and methods for the MIC assay and the other methods described below are given in the Supplementary Materials and methods available as Supplementary data at *JAC* Online). LL-37 and PG-1-amide were synthesized and purified by Mimotopes (Clayton, Victoria, Australia). PBT2 was synthesized as described in Bohlmann *et al.*[7] Bacterial time–kill assays were performed as previously described.[9] Proteomic SWATH-MS (sequential window acquisition of all theoretical fragment ion spectra - mass spectrometry) studies were performed as previously described.[13] LL-37 production by primary human cervical epithelial (Pex) cells[14] was measured by ELISA, according to standard protocols, using the CAP18/LL-37-specific mouse monoclonal antibody 1-1C12 (Hycult Biotech, Wayne, PA, USA). LL-37 was quantified from a standard curve derived from 2-fold serial dilutions of LL-37 peptide (Hycult Biotech).

Results

LL-37 is the only human representative of the cathelicidin family of antimicrobial peptides.[15] LL-37 can be found throughout the body and has been shown to exhibit antimicrobial activity against numerous pathogens,[16] including *N. gonorrhoeae*.[17] To examine if PBT2/zinc can increase the susceptibility of *N. gonorrhoeae* to LL-37 (as it does for the antibiotic peptides polymyxin B and colistin[9]), the MIC of LL-37 in the presence or absence of PBT2/zinc was determined. The MDR *N. gonorrhoeae* strain WHO Z, which carries most of the currently known resistance genes,[5] was initially used in MIC assays. In the presence of PBT2/zinc, the susceptibility of WHO Z to LL-37 was lowered from an MIC of 8 mg/L to an MIC of 4 mg/L [FIC index (FICI) = 0.93]. This suggests an additive increase in the inhibitory activity of LL-37 against WHO Z in the presence of PBT2/zinc (Table 1).

In vivo, human cells can express a wide range of LL-37 concentrations. For example, <1.2 to >80 mg/L LL-37 is reported in nasal fluid.[18] Here we report that the concentration of LL-37 expressed by (uninfected) Pex cells into tissue culture medium is 16 mg/L (see the Supplementary Materials and methods), demonstrating the relevance of LL-37 in gonococcal infection in women. To determine whether PBT2/zinc may have a synergistic effect on the killing activity of LL-37 toward MDR *N. gonorrhoeae*, MIC assays were performed using LL-37 at reported *in vivo* concentrations and at an *in vitro* sub-inhibitory concentration (2 mg/L). Although strain WHO Z is resistant to tetracycline (MIC = 5 mg/L), PBT2/zinc acted synergistically with LL-37 to render WHO Z susceptible to tetracycline (MIC = 0.156 mg/L, FICI = 0.46) (Table 1). Notwithstanding the limitation of the 2-fold dilution resolution of the MIC assay, we observed reproducible sensitization of WHO Z to tetracycline in the presence of PBT2/zinc, while introducing the *in vivo* mimic condition with respect to the human antimicrobial peptide LL-37.

PG-1 is a cathelicidin antimicrobial peptide found in porcine leucocytes. Previous work suggests that PG-1 has potential utility as an antimicrobial agent for the treatment of local or systemic infections caused by clinically relevant pathogens, such as MRSA and *Pseudomonas aeruginosa*.[19] We tested whether a sub-inhibitory concentration of PBT2 (0.067 or 0.135 mg/L)/zinc (2.5 μM) could increase the susceptibility of *N. gonorrhoeae* to PG-1 (Table 1). The

Table 1. *N. gonorrhoeae* MICs (mg/L) of LL-37 and PG-1 in the presence and absence of PBT2/zinc and MICs (mg/L) of tetracycline in the presence of PBT2/zinc with and without LL-37

	PBT2/zinc	No PBT2/zinc	FICI[a]
LL-37			
WHO Z	4	8	0.93
	PBT2/zinc/LL-37	No PBT2/zinc/LL-37	FICI[a]
Tetracycline			
WHO Z	0.156	5	0.46
	PBT2/zinc	No PBT2/zinc	FICI[a]
PG-1			
MS11	0.5	2	0.68
ATCC 49226	2	>16	<0.56
WHO L	2	>16	<0.56
WHO M	2	>16	<0.56
WHO X	2	>16	<0.56
WHO Y	2	>16	<0.56
WHO Z	2	>16	<0.56

Zinc is present in all assays at a concentration of 2.5 μM.
0.135 mg/L PBT2 was used in the assays for WHO Z, ATCC 49226 and WHO M.
0.067 mg/L PBT2 was used in the assays for MS11, WHO L, WHO X and WHO Y.
2 mg/L LL-37 was used in the assays for WHO Z.
[a]FICI was calculated as $A/MIC_A + B/MIC_B = FIC_A + FIC_B = FICI$. Synergy: the combination of compounds results in an FICI value of <0.5. Additive: the combination of compounds results in an FIC value of 0.5–4. See the Supplementary Materials and methods for details of FICI calculation.

MIC of PG-1 for all MDR strains tested decreased from >16 mg/L without PBT2/zinc to 2 mg/L with PBT2/zinc. The FICI was <0.56, which is just outside the 0.5 definition for synergistic activity, indicating that the observed increase in the combined inhibitory activity of PG-1 and PBT2/zinc on WHO Z was additive.

Time–kill studies showed that, although neither PBT2/zinc nor PG-1 alone displayed antibacterial activity against WHO Z at the concentrations used, PBT2/zinc+PG-1 induced a bactericidal effect on *N. gonorrhoeae* (Figure 1). This same phenotype was also observed for the CLSI *N. gonorrhoeae* reference strain ATCC 49226 and the laboratory strain MS11, in addition to other MDR strains (i.e. WHO L, WHO M, WHO X and WHO Y) (Table 1).

To determine the mechanism by which PBT2/zinc increased *N. gonorrhoeae* susceptibility to tetracycline plus LL-37, we performed SWATH-MS proteomic analysis using WHO Z cells grown under different treatment conditions (see the Materials and methods section and see the Supplementary Materials and methods). Cells treated with PBT2/zinc had 32 proteins (25 down- and 7 up-regulated) with more than a 1.5-fold change in expression relative to the untreated control ($P<0.01$). Of these, OmpR was up-regulated 1.63-fold (Table S1, available as Supplementary data at *JAC* Online). OmpR is the response regulator for the two-component system OmpR/EnvZ, which is involved in sensing changes in osmolarity.[20] Another protein detected at increased levels was IscS (1.68-fold), which is involved in maintaining iron-sulphur (4Fe-4S) cluster proteins,[21] such as NADH dehydrogenase. These findings suggest that PBT2/zinc can disrupt membrane integrity and iron-sulphur cluster proteins, which is consistent with its ionophoric function and its ability to aid first-row transition-metal ions across biological membranes.

In the presence of LL-37 alone, the expression of 24 proteins changed by more than 1.5-fold. These included the regulator OxyR (4.55-fold increase), which is involved in regulating genes involved in the oxidative stress response.[22] This observed increase in OxyR is consistent with the reported increase in oxygen-radical production in response to LL-37 treatment.[22]

PBT2/zinc and LL-37 combined resulted in a significant change in 91 proteins (79 down- and 12 up-regulated). The down-regulated proteins included: MinE (3.57-fold), a cell division protein; the outer-membrane protein NspA (3.46-fold); and the peptidyl-prolyl cis-trans isomerase PrsA (5.67-fold), which is involved in protein folding within the periplasm.[23] The down-regulation of periplasmic and outer-membrane proteins potentially suggests that protein secretion is dysregulated in the presence of combined PBT2/zinc and LL-37. Taken together, these SWATH-MS data suggest that the most probable mode of action for PBT2/zinc combined with LL-37 is the loss of membrane integrity, which results in an increase in misfolded proteins as well as in radical production plus a dysregulation of iron-sulphur cluster proteins, with the end result being cell death.

SWATH-MS analysis of tetracycline-treated cells revealed a different set of proteins with altered abundance (39 up- and 16 down- regulated; see Table S1). Tetracycline impairs translation; consequently, we observed changes in numerous proteins involved in various responses. These included: OxyR (4.68-fold increase); the stringent response protein SspB (1.88-fold increase); and a protein, HisB, involved in histidine biosynthesis (2.06-fold increase). When comparing PBT2/zinc/LL-37/tetracycline to tetracycline alone, 55 proteins changed in their abundance (12 up- and 43 down-regulated). Of those proteins that were up-regulated, of particular interest were: MurA (7.25-fold), a protein involved in the first step of peptidoglycan synthesis; SspA (3.2-fold), which is involved in the starvation response; and ProQ (2.07-fold), an osmoprotectant. This strongly implies that *N. gonorrhoeae* are stressed during PBT2/zinc/LL-37/tetracycline treatment and also that a loss of membrane integrity resulting from PBT2/zinc/LL-37 most likely enhances the ability of tetracycline to enter the bacterial cell.

Discussion

PBT2 has been shown to be safe and well tolerated in patients with Alzheimer's or Huntington's disease.[24] Previous studies showed

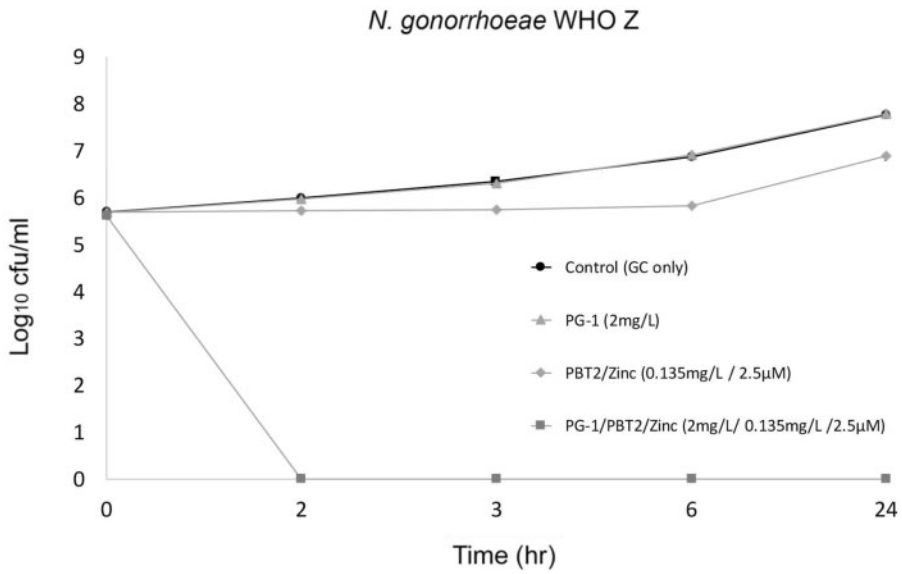

Figure 1. Time–kill curves of *N. gonorrhoeae* WHO Z in GC broth with or without PBT2/zinc [PBT2 (0.135 mg/L)/zinc (2.5 μM)] in the absence and presence of PG-1 (2 mg/L). Error bars indicate standard deviations from three biological replicates.

that PBT2/zinc can alter metal homeostasis in bacteria as well as disrupt the function of the metal-containing protein LptA, which, in turn, reduces *N. gonorrhoeae* resistance to cationic polymyxin antibiotic peptides.[9] Here we show that PBT/zinc can also increase the susceptibility of *N. gonorrhoeae* to two cationic antimicrobial peptides, LL-37 and PG-1, which are naturally present in mammals. Recently, it was reported that LL-37 can prevent gonococcal invasion of human ME-180 epithelial carcinoma cells and reduce the expression of gonococcal-induced TNF-α in THP-1 monocytes.[17] Our previous work showed that PBT2/zinc can break resistance to colistin, polymyxin B and tetracycline *in vitro*.[9] In that LL-37 is found throughout the human body,[15] *in vivo*, this effect may be further enhanced by LL-37. That is, we have now shown that LL-37 can synergize with PBT2/zinc to heighten the susceptibility of MDR *N. gonorrhoeae* to tetracycline, which is also a potential avenue of future study on other classes of antibiotics. The emergence of bacterial strains that are resistant to available antimicrobials is a current health emergency. Treatment with PBT2/zinc to sensitize a bacterium to a natural antimicrobial peptide, such as LL-37, may represent a new strategy to treat MDR *N. gonorrhoeae* infections and reinvigorate the use of currently available antibiotics.

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Transparency declarations

None to declare.

Supplementary data

Supplementary Materials and methods and Table S1 are available as Supplementary data at *JAC* Online.

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