In Vivo Efficacy Assessment and Pharmacokinetics of Systemic PBT2 in Combination with Doxycycline in a Neutropenic Mouse Model of Lung Infection.

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Introduction

Acinetobacter baumannii

- An opportunistic Gram-negative organism.[1]
- A major cause of nosocomial infections due to its ability to colonize medical devices and exhibits excellent genomic plasticity, enabling development of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains , which significantly limit available treatment options.[1]
- Classified by the WHO as a **Priority 1 (Critical) pathogen** for the development of new antibiotics.[2]

PBT2:

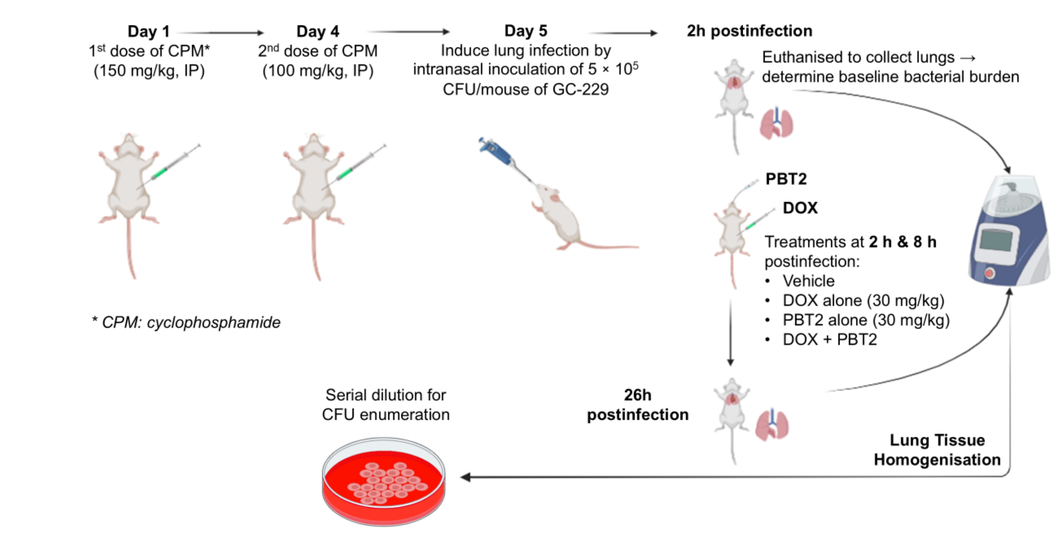
- a hydroxyquinoline ionophore initially developed for treating neurodegenerative diseases. In human, phase 2 clinical trials reported its safety and good tolerability with once-daily oral dose of 250 mg for up to 6 months.[3]
- Has recently been investigated as a potentially repurposed antibiotic adjuvant.
- Previous *in vitro* studies show that this ionophore can break antibiotic resistance of a wide range of bacteria.[4,5,6]
- *Mechanism of action:* PBT2 cause zinc intoxication and disruption to metal ion homeostasis.[4,5,6]
- In a recent study, PBT2 can rescue tetracycline antibiotic efficacy for treating *A. baumannii* lung infection in mouse model.[4]

Hypothesis: *"Co-administration of PBT2 in combination with doxycycline, is a potential antimicrobial therapy for the treatment of multidrug-resistant bacterial lung infection with A. baumannii."*

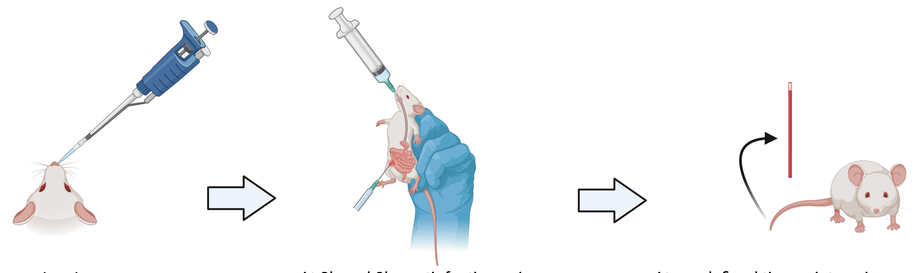
Method

- Doxycycline hyclate (D9891-10G) was purchased from Sigma-Aldrich RTC , and and PBT2 (HY-105321) was obtained from MedChemExpress.
- The doxycycline-resistant *A. baumannii* isolate GC-229 obtained from the GAME-CHANGER clinical trial was used in the study. The MICs of doxycycline alone and in the presence of 18 µg/mL of PBT2 against GC-229 were 32 µg/mL and 4 µg/mL, respectively.
- Male and female BALB/c mice (4-5 weeks old) purchased from the Australian Bio Resources (Sydney, Australia) were acclimatised at the CIPDD animal facility for at least 1 week prior to experimentation. Mice were housed in groups of five per cage in a temperature-controlled room (21 ± 2° C) with a 12 h/12 h light/dark cycle and had *ad libitum* access to standard laboratory rodent enrichment, food, and water.

In vivo Efficacy Evaluation:



Plasma Pharmacokinetics investigation



Neutropenic mice were intranasally infected with 5 × 10^5 CFU/mouse of GC-229

At 2h and 8h postinfection, mice were treated with PBT2 (30 mg/kg, PO), DOX (30 mg/kg, IP), or the combination

At pre-defined timepoints, microsamples of whole blood (~ 40 µL) were collected into K_2EDTA-coated capillaries.

- PK studies of PBT2 and doxycycline were performed to evaluate potential PK-PK interactions between these two compounds.
- A semi-serial sampling design was employed to minimise the number of animals used compared with traditional mouse PK studies
- Timepoints include: **pre-dose (t = 0 h) and 0.25, 0.5, 1, 2, 4, 6, 6.5, 7, 8, 10, 12, 16, 20, and 24 h** after the first dose. At each timepoint, a group of 3 mice per treatment was used for blood collection.
- Blood samples were centrifuge to yield plasma; and the resulting plasma samples were stored at −80 °C until analysis.

Epithelial Lining Fluifd (ELF) Pharmacokinetics Investigation

Neutropenic mice were intranasally infected with 5 × 10^5 CFU/mouse of GC-229

At 2h postinfection, mice were treated with the combination of PBT2 (30 mg/kg, PO) and DOX (30 mg/kg, IP)

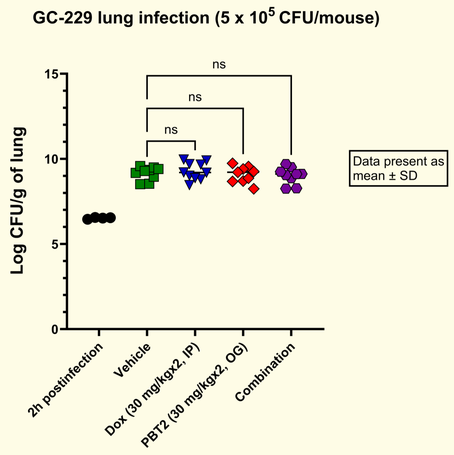
At pre-defined timepoints, whole blood samples were collected into K_2EDTA tubes followed by centrifugation for plasma separation. BALF samples were also collected. Plasma and BALF samples were stored at -80°C prior to analysis.

- ELF pharmacokinetics were assessed to determine pulmonary drug exposure, providing data to assist with interpretation of the *in vivo* efficacy observed in the neutropenic mouse lung infection model.
- 5 timepoints were involved, including **0 h (pre-dose), 0.5 h, 1 h, 2 h, and 6 h** post-dosing. At each time point, a group of mice (n = 3) was euthanised for blood and bronchoalveolar lavage fluid (BALF) collection.
- Urea concentrations in plasma and BALF samples were quantified for calculating ELF drug concentrations by correcting the PBT2 and doxycycline concentrations in BALF samples with the plasma and BALF urea concentrations using the following equation[7]: $C_{ELF} = C_{BALF} \times Urea_{plasma}/Urea_{BALF}$ where C_{ELF}, C_{BALF}, $Urea_{plasma}$ and $Urea_{BALF}$ are the concentration of PBT2 or doxycline in ELF and in BALF, and the concentration of urea in plasma and in BALF, respectively.

Measurement of PBT2 and doxycycline in mouse plasma and BALF

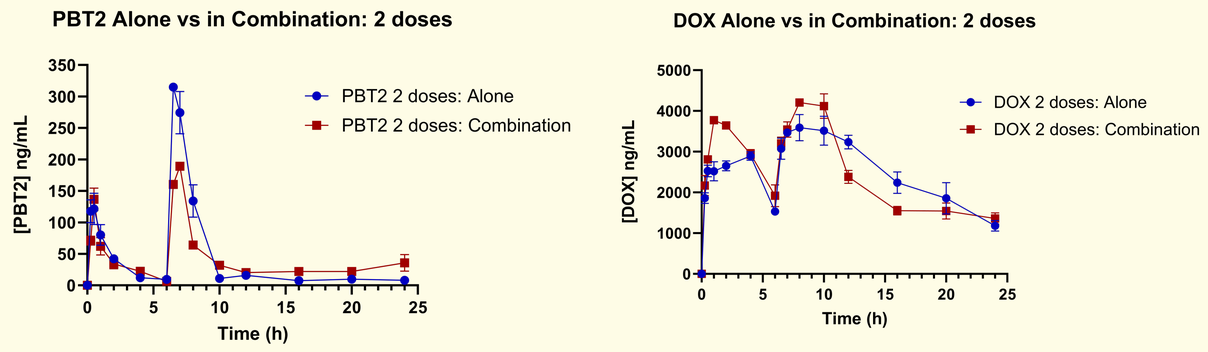
- The PBT2 and doxycycline concentrations in plasma and BALF samples were quantified by a validated UHPLC-MS/MS bioanalytical method.
- The quanfication range was 2 – 1000 ng/mL for PBT2 and 10 – 1000 ng/mL for doxycycline.
- ***Sample preparation:*** 5 µL plasma sample (or 20 µL BALF sample) aliquots were mixed with 40 µL (or 60 µL for BALF analysis) of acetonitrile containing 20 ng/mL of d3-doxycycline as the internal standard. The mixture was centrifuged at 13,400×*g* for 10 min to remove the protein precipitate before aliquots of the supernatants were transferred into autosampler vials. Afterwards, 4 µL aliquots (2 µL for BALF analysis) of the supernatant were injected into the UHPLC-MS/MS system for the analysis.

Results



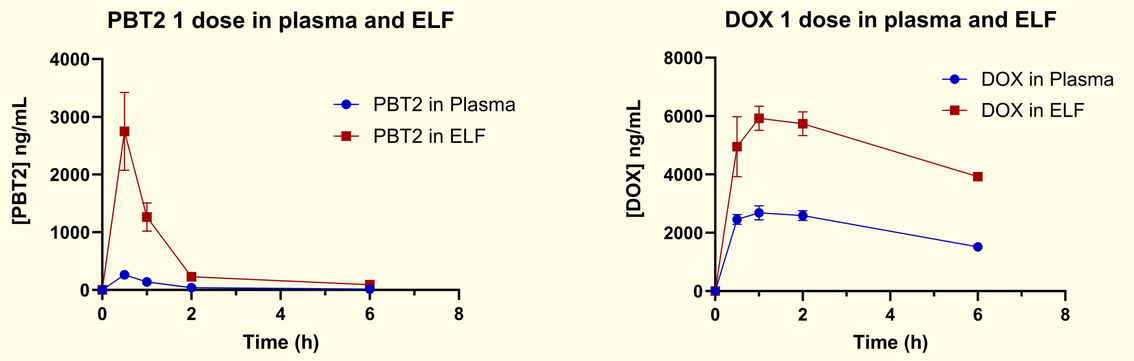
- Mice were rendered neutropenic by 2 IP injections of cyclophosphamide on day 1 (150 mg/kg) and on day 4 (100 mg/kg)[8] before being intranasally infected with 5×10^5 CFU/mouse to induce lung infection.
- The initial bacterial burden recovered from the lungs in mice at 2 h postinfection was 6.5 log_{10} CFU/g. The bacteria grew by 2.6 log_{10} to 9.1 log_{10} CFU/g at 26 h postinfection in the lungs in mice treated with vehicle.
- There was no significant difference in the lung bacterial burden between groups of mice treated with PBT2 alone, doxycycline alone, the PBT2/doxycycline combination or the vehicle control group.

Plasma and ELF Pharmacokinetics of PBT2 and doxycycline



Visual inspection of the mean (± SEM) plasma concentration versus time curves and the corresponding C_{max}, $t_{1/2}$, and AUC data show that there was no PK-PK interaction between PBT2 and doxycycline in the neutropenic mouse model of lung infection induced with *A. baumannii.* The estimated PK parameters (mean ± SEM) are summarized in the table below.

PK parameters	PBT2			
	Alone		In combination with doxycycline	
	1st Dose	*2nd Dose*	*1st Dose*	*2nd Dose*
T_{max} (h)	0.5	0.5	0.5	1
C_{max} (ng/mL)	122 ± 25	315 ± 7	137 ± 18	189 ± 5
$t_{1/2}$ (h)	1.44	3.43	1.60	5.30
$AUC_{0\text{-}6h}$ (ng.h/mL)	231 ± 22	604 ± 60	217 ± 24	403 ± 14
Total $AUC_{0\text{-}24h}$ (ng.h/mL)	952 ± 66		909 ± 59	
PK parameters	**Doxycycline**			
	Alone		In combination with PBT2	
	1st Dose	*2nd Dose*	*1st Dose*	*2nd Dose*
T_{max} (h)	4	2	1	2
C_{max} (ng/mL)	2893 ± 105	3589 ± 321	3774 ± 45	4208 ± 63
$t_{1/2}$ (h)	N/A	9.06	N/A	9.16
$AUC_{0\text{-}6h}$ (ng.h/mL)	14595 ± 423	20172 ± 1120	17694 ± 548	21671 ± 834
Total $AUC_{0\text{-}24h}$ (ng.h/mL)	59985 ± 2676		59230 ± 1658	



- ELF pharmacokinetics for each of PBT2 and doxycycline shows that both drugs achieve excellent pulmonary penetration. The ELF-to-plasma ratio for PBT2 was approximately 8.5, and that for doxycycline was ~2.3.
- Despite the high ELF penetration observed, the pulmonary exposure of PBT2 generated following oral administration of PBT2 at the dose of 30 mg/kg was insufficient to break antibiotic resistance of the *A. baumannii* isolate GC-229.

	PBT2		Doxycycline	
	In plasma	In ELF	In plasma	In ELF
T_{max} (h)	0.5	0.5	1	1
C_{max} (ng/mL)	261.5 ± 32.1	2747 ± 674.4	2685 ± 243	5921 ± 418.6
$AUC_{0\text{-}6h}$ (ng.h/mL)	362.0 ± 29.6	3071 ± 547.1	12753 ± 672.8	29108 ± 1653
ELF-to-plasma Ratio	8.5		2.3	

Conclusion

In summary, two combined doses of oral PBT2 at 30 mg/kg with IP doxycycline at 30 mg/kg with the doses given 6 h apart in neutropenic mice with *A. baumannii* GC-229-induced lung infection, did not induce significant bacterial killing. This result may be explained potentially by insufficient PBT2 exposure at the site of infection in the lung to be able to disrupt doxycycline resistance in this pathogen.

A potential solution to address this issue is to change the PBT2 dosing route to intratracheal instillation. By giving PBT2 directly into the lung of neutropenic mice, exposure at the site of infection can be markedly elevated, potentially to a level sufficient to disrupt *A. baumannii* resistance to doxycycline.

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Acknowledgements

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