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BRIEF COMMUNICATION

Activity of DNA-targeted C8-linked pyrrolobenzodiazepine–heterocyclic polyamide conjugates against aerobically and hypoxically grown *Mycobacterium tuberculosis* under acidic and neutral conditions

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Abstract

Mycobacterium tuberculosis (*Mtb*) is the aetiological agent of tuberculosis, the leading cause of death worldwide from a single infectious agent. *Mtb* is a highly adaptable human pathogen that might enter a dormant non-replicating (NR), drug-tolerant stage. Reactivation of dormant *Mtb* can lead to active disease. Antibiotic treatments of active and latent tuberculosis are long, complex and may fail to fully eradicate the infection. Therefore, it is imperative to identify novel compounds with new mechanisms of action active against NR bacilli. Dormant *Mtb* habitat is mostly thought to be the pH-neutral and hypoxic caseous granuloma. We have used the Wayne culture model to reproduce this environment and tested the activities of two DNA-targeted agents, C8-linked-pyrrolobenzodiazepine(PBD)–polyamide conjugates **1** and **2**, against *Mtb* grown in aerobic and hypoxic conditions in both acidic and pH-neutral media. PBD **2** showed growth inhibitory activity at 5.1 µg/ml against 19-day-old hypoxic NR *Mtb* cultures with 1.8 $\log_{10}$ CFU reduction on day 21 at pH 7.3. PBD **2** was particularly effective against 5-day-old aerobic cells at pH 7.3, with CFU reduction (>6.8 $\log_{10}$) on day 21 at 5.1 µg/ml being identical to that of rifampin at 8 µg/ml. PBD **2** qualifies as a promising lead against aerobic and NR *Mtb*.

Note

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis (TB). In 2016, the WHO estimates of the global burden of TB were 10.4 million new cases and 1.7 million deaths [1]. Furthermore, about 2 billion people are latently infected with *Mtb*, with 10% of this population experiencing TB reactivation under favourable conditions for microorganisms' growth [2]. The complex pathology of TB is mostly attributable to the ability of *Mtb* to evade host immune system response by entering a dormant non-replicating (NR) state that allows the bacilli to develop phenotypic drug resistance (drug tolerance) [3–5].

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Poor adherence to drug-sensitive TB treatment, which consists of a combination of the first-line drugs isoniazid (INH), rifampin (RIF), pyrazinamide (PZA) and ethambutol (EMB) given daily for 2 months, followed by RIF and INH given daily for 4 months, severely compromises patient outcomes leading to the emergence of multidrug-resistant (MDR) *Mtb* strains (i.e. resistant at least to INH and RIF) and extensively drug-resistant (XDR) strains (i.e. MDR strains resistant to any fluoroquinolone and to at least one injectable second-line drug, kanamycin, amikacin or capreomycin) [6]. The four-drug cocktail (INH, RIF, PZA and EMB) regimen is effective against actively replicating (AR) *Mtb* in cellular granulomas at acidic pH, whereas NR bacilli localised in hypoxic, pH-neutral caseous granulomas, are refractory to drug action [7–9]. Hence, identifying novel drugs inhibiting both AR and NR *Mtb* is of great importance for the fight against TB.

In previous works, we employed the in vitro Wayne model of hypoxia-induced dormancy at pH 5.8 and 7.3 to reproduce environments of cellular and caseous granulomas, respectively. Indeed, the pH of activated macrophages present in cellular granulomas is acidic, while in the caseous

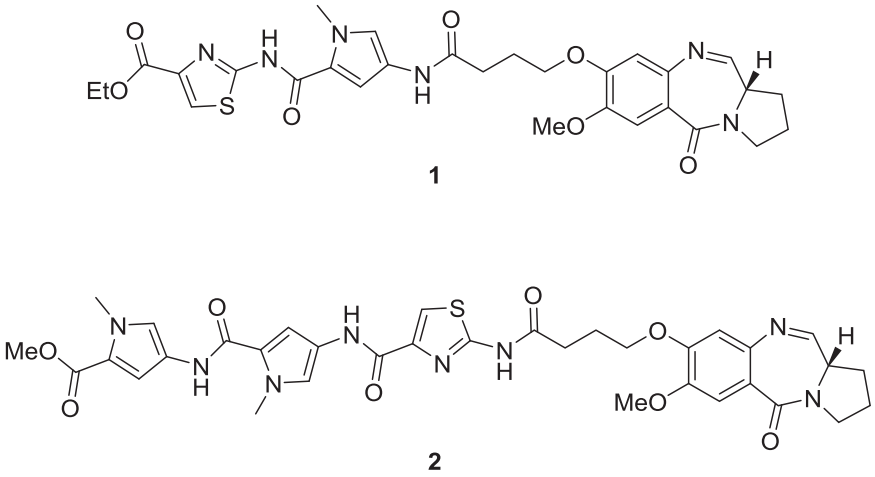

Fig. 1 Structures of pyrrole/thiazole-containing PBD-conjugates **1** and **2**

granulomas, where the NR drug-tolerant *Mtb* lives, the pH is neutral ranging from 7.2 to 7.5 [8, 9]. This approach allowed us to evaluate the anti-tubercular activity of drugs under conditions in which *Mtb* is phenotypically resistant to drug action [10–13]. Out of the 12 drugs tested, we found that only the rifamycin antibiotics RIF and rifapentine killed NR *Mtb* at pH 7.3, while the other tested drugs, i.e. inhibitors of mycolic acid synthesis (INH, PA-824), DNA synthesis (moxifloxacin), protein synthesis (amikacin), ATP synthesis (bedaquiline) and membrane functions (PZA, nitazoxanide, clofazimine), showed negligible activity (≤0.9 $\log_{10}$ CFU reduction on day 21) in these caseous granuloma-like conditions. On the other hand, most of TB drugs tested were active against NR *Mtb* at pH 5.8 [13]. Overall, our in vitro observations were in keeping with a recent in vivo report showing that only rifamycins fully sterilised the caseum of *Mtb*-infected rabbits, which exhibited extreme drug tolerance to first-line and second-line TB drugs [14]. This indicates that our stringent Wayne model of hypoxia at pH 7.3 is a reliable method for testing activity of drugs/drug combinations against NR *Mtb*.

These findings prompted us to select and screen diverse chemical scaffolds against *Mtb* in AR and NR stages using the Wayne models at pH 5.8 and 7.3. As part of our ongoing efforts to investigate new anti-tubercular probes with novel mode of action, we have recently identified a class of DNA sequence-selective agents, C8-linked pyrrolobenzodiazepine (PBD)–polyamide conjugates [15], with notable growth inhibitory activity against AR *Mtb* reference strain H37Rv, with MICs ranging from 0.08 to 5.20 μg/ml. PBDs are a family of antitumor antibiotics first isolated from *Streptomyces* species [16], and have a unique mode of action involving the covalent binding to guanine residues within the DNA-minor groove. The latter feature can be exploited to target discrete DNA sequences within the guanine-cytosine (GC)-rich mycobacterial genome and ultimately disrupt key enzymes and transcription factors. PBDs snugly fit within the DNA-minor groove and escape DNA repairing enzymes activities, a salient feature that can be exploited to overcome drug resistance issues related to existing anti-tubercular drugs. Moreover, PBDs permeate the *Mtb* cell envelope and this is a crucial requirement for effective anti-tuberculosis molecular probes.

In this study, we tested and compared with RIF and INH the ability of representative pyrrole/thiazole-containing PBD-conjugates **1** and **2** (Fig. 1) [shown in reference [15] as compounds 2 ($MIC_{H37Rv} = 0.63$ μg/ml) and 9 ($MIC_{H37Rv} = 0.16$ μg/ml), respectively] [15], to kill AR *Mtb* and NR *Mtb* under hypoxic granuloma-like conditions, using our previously described methods [10–13].

Briefly, AR and NR *Mtb* H37Rv was grown at 37 °C in 20×125 mm screw-cap tubes containing Dubos-Tween-albumin broth (DTAB) and the pH adjusted to 5.8 and 7.3. For the preparation of AR aerobic (A) cells, logarithmically growing cultures were diluted in DTAB and transferred to tubes with loosened screw caps. For the preparation of NR hypoxic (H) cells, log-phase cultures were diluted in DTAB and incubated in tubes with the caps tightly screwed and tight rubber seals put under the caps. The growth in the tubes was monitored by measuring CFU/ml on Middlebrook 7H10 (7H10) agar plates incubated at 37°C for 3 weeks. To determine drug activity, 5-day-old A cultures (A5) and 12-day-old and 19-day-old H cultures (H12 and H19, respectively) were grown with and without drugs for 7, 14 and 21 days at maximum concentrations in serum (C_{max}) of RIF and INH (8 and 2 μg/ml, respectively) [11] and at concentrations corresponding to ×2, ×8 and ×32 MICs of PBD **1** (1.3, 3.1 and 20.2 μg/ml, respectively) and PBD **2** (0.3, 1.3 and 5.1 μg/ml, respectively) [15]. After drug exposure, 1 ml of A5, H12 and H19 cultures at pH 7.3 and 5.8 was washed and resuspended in 1 ml of DTAB and 0.2 ml of the dilutions was inoculated in 7H10 plates for CFU/ml determination.

Figure 2 shows the activities of RIF, INH and PBD **2** against A5, H12 and H19 cells at pH 5.8 and 7.3. In untreated A5 cultures at pH 5.8 and 7.3 the CFUs increased from day 0 to 21

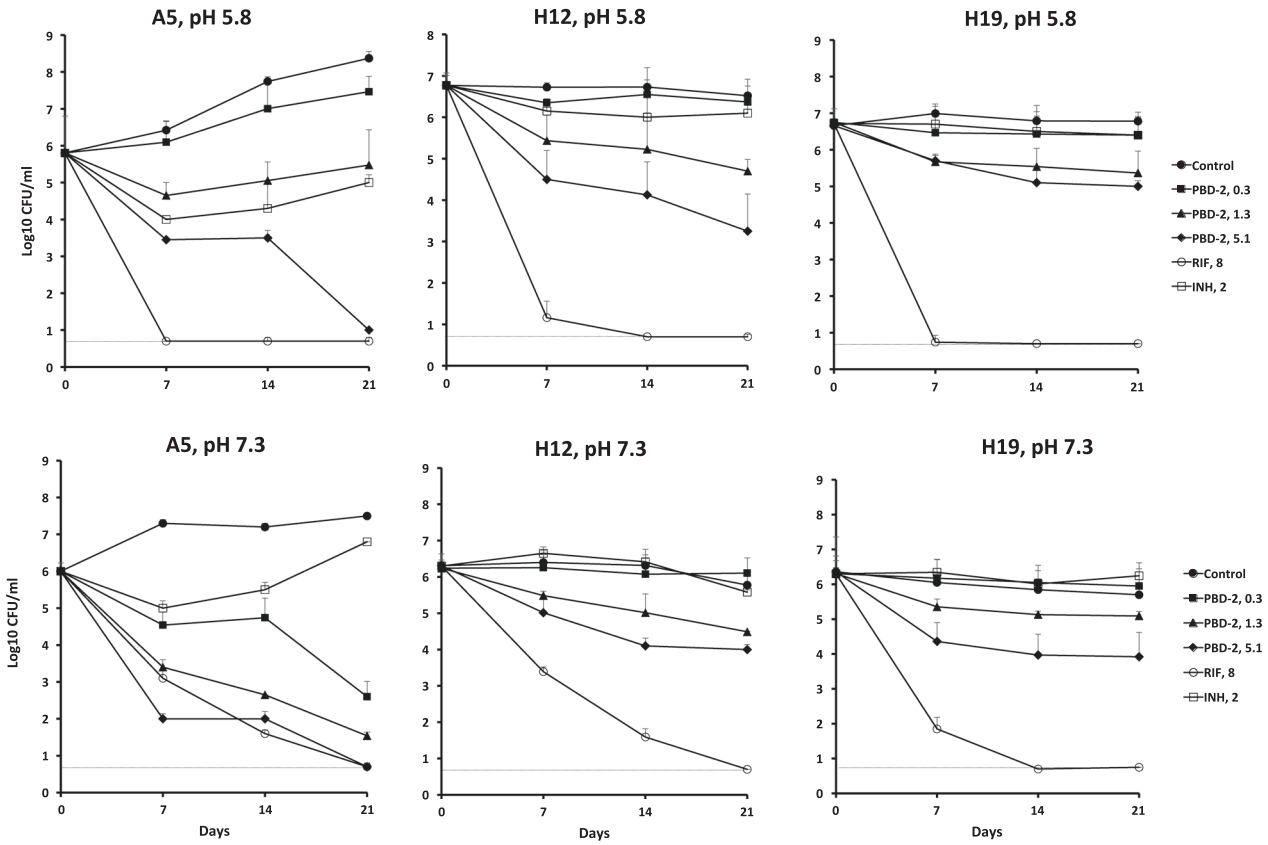

Fig. 2 Activity of drugs against aerobic and hypoxic *M. tuberculosis*. CFU of *M. tuberculosis* grown in aerobic and hypoxic acidic conditions after 0, 7, 14 and 21 days of drug exposure are shown. Five-day-old aerobic (A5) cultures, and 12-day-old, and 19-day-old hypoxic (H12, and H19, respectively) cultures were incubated with drugs at pH 5.8 and 7.3. The thiazole-containing PBD-conjugate **2** (PBD 2) was tested at ×2, ×8 and ×32 MICs (0.3, 1.3 and 5.1 μg/ml, respectively). Rifampin (RIF) and isoniazid (INH) were tested at 8 and 2 μg/ml, respectively. Dashed lines indicate the limit of detection (5 CFU/ml). Mean and standard deviations from two experiments are shown

while in untreated H12 and H19 cultures the CFUs stabilised or slightly decreased. Rifampin was very active against A5, H12 and H19 cells with no CFUs remaining on day 21 irrespective of the pH used. Isoniazid was active against A5 cells over the first 7 days, then *Mtb* regrew after ≥14 days. On day 21, INH showed a small activity against H12 cells and H19 cells at pH 5.8, although it was inactive at pH 7.3.

As to the new compounds, **1** showed no consistent activity against A5, H12 and H19 cells (data not shown). Instead, dose-dependent and time-dependent activities of **2** against A5, H12 and H19 cells were observed. PBD **2** was particularly effective against A5 cells at pH 7.3, with CFU reduction on day 21 by 5.1 μg/ml of **2** (>6.8 $\log_{10}$ CFU) being identical to that attained by 8 μg/ml of RIF, and reduction by 1.3 μg/ml of **2** being about 0.8 $\log_{10}$ CFU lower than that obtained by 8 μg/ml of RIF. When tested at its lowest concentration (0.3 μg/ml), PBD **2** was found to be much more active against A5 cells at pH 7.3 than at pH 5.8 (4.9 and 0.9 $\log_{10}$ CFU reduction, respectively).

Consistent dose-dependent and time-dependent activities of **2** against H12 and H19 cells were observed on day 21. PBD **2** attained $\log_{10}$ CFU reductions at concentrations of 1.3 and 5.1 μg/ml at both pH 5.8 (H12: 1.8 and 3.3 $\log_{10}$ reduction; H19: 1.4 and 1.8 $\log_{10}$ reduction, respectively) and pH 7.3 (H12: 1.3 and 1.8 $\log_{10}$ reduction; H19: 0.6 and 1.8 $\log_{10}$ reduction, respectively).

The activity of **2** in hypoxia at pH 7.3 is particularly interesting since very few drugs are active in these hypoxic–pH-neutral conditions [13]. The low efficacy of several anti-tubercular drugs in this caseum-mimicking model stimulated us to explore the possibility that other molecules including DNA-damaging agents may inhibit NR *Mtb*. The C8-linked (PBD)–polyamide conjugates used in this study form DNA adducts responsible for cancer cell cytotoxicity and antibacterial efficacy and have a more favourable cytotoxicity profile than PBD dimers reported to have antistaphylococcal activity [17], but their therapeutic index needs to be certainly improved [15]. To this end, the PBDs' promiscuous bacterial/host DNA interactions can be obliterated by incorporating these agents in drug-delivery systems that direct the cidal activity solely towards the tubercle bacilli, resulting in no toxicity to *Mtb*-infected host cells. This was recently exemplified by DNA-minor groove binding agents, which after encapsulation in non-ionic

surfactant vesicles, showed only anti-tubercular activity with no macrophage toxicity [18].

Highly hydrophobic drugs (measured via their clogP values, i.e., the calculated octanol/water partitioning coefficient) like clofazimine and bedaquiline (clogP > 6) [12] bind to caseum macromolecules at the outer edge of the caseous core, thus preventing further diffusion toward the centre of necrotic areas [19]. Instead, less lipophilic drugs may diffuse more favourably through the caseum and accumulate inside it. This is the case of RIF (clogP = 3.85) [12] that is able to diffuse and accumulate in the necrotic core of the caseum [19, 20], and kill NR *Mtb* in a pH-neutral environment (the caseum pH), as shown in in vitro [13] and ex vivo studies [14].

Interestingly, the hydrophilic characters of PBDs **1** (clogP = 1.24) and **2** (clogP = 1.98) (the clogP values were calculated using the CambridgeSoft Chemdraw software) should theroretically favour their diffusion through the caseum and possibly concentrate inside it. To this end, it is anticipated that appropriate combinations of DNA-targeted compounds with rifamycins, the only drugs potentially able to sterilise caseum [14], might exert a synergistic antibacterial effect on AR and NR *Mtb* strains.

In conclusion, PBD **2** efficiently killed AR bacilli at the same order of magnitude of RIF, and inhibited to some extent the growth of NR bacilli under stringent conditions of hypoxia at pH 7.3. Overall, these observations qualify this compound as a promising lead to be improved in further medicinal chemistry work to decrease its cytotoxicity and generate new, safer rationally designed analogues targeting NR *Mtb* DNA's structures and functions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. World Health Organization. Global Tuberculosis Report 2017. Geneva: WHO; 2017. WHO/HTM/TB/2017.23.
2. Getahun H, Matteelli A, Chaisson RE, Raviglione M. Latent *Mycobacterium tuberculosis* infection. N Engl J Med. 2015;372:2127–35.
3. Prosser G, Brandenburg J, Reiling N, Barry CE 3rd, Wilkinson RJ, et al. The bacillary and macrophage response to hypoxia in tuberculosis and the consequences for T cell antigen recognition. Microbes Infect. 2017;19:177–92.
4. Caño-Muñiz S, Anthony R, Niemann S, Alffenaar JC. New approaches and therapeutic options for *Mycobacterium tuberculosis* in a dormant state. Clin Microbiol Rev. 2017;31 https://doi.org/10.1128/CMR.00060-17.
5. Gold B, Nathan C. Targeting phenotypically tolerant *Mycobacterium tuberculosis*. Microbiol Spectr. 2017;5 https://doi.org/10.1128/microbiolspec.TBTB2-0031-2016.
6. Dheda K, Gumbo T, Maartens G, Dooley KE, McNerney R, et al. The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. Lancet Respir Med. 2017;5:291–360.
7. Dartois V. The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. Nat Rev Microbiol. 2014;12:159–67.
8. Lanoix JP, Lenaerts AJ, Nuermberger EL. Heterogeneous disease progression and treatment response in a C3HeB/FeJ mouse model of tuberculosis. Dis Model Mech. 2015;8:603–10.
9. Iacobino A, Piccaro G, Giannoni F, Mustazzolu A, Fattorini L. Fighting tuberculosis by drugs targeting nonreplicating *Mycobacterium tuberculosis* bacilli. Int J Mycobacteriol. 2017;6:213–21.
10. Wayne LG, Hayes LG. An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. Infect Immun. 1996;64:2062–9.
11. Piccaro G, Giannoni F, Filippini P, Mustazzolu A, Fattorini L. Activities of drug combinations against *Mycobacterium tuberculosis* grown in aerobic and hypoxic acidic conditions. Antimicrob Agents Chemother. 2013;57:1428–33.
12. Piccaro G, Poce G, Biava M, Giannoni F, Fattorini L. Activity of lipophilic and hydrophilic drugs against dormant and replicating *Mycobacterium tuberculosis*. J Antibiot. 2015;68:711–4.
13. Iacobino A, Piccaro G, Giannoni F, Mustazzolu A, Fattorini L. *Mycobacterium tuberculosis* is selectively killed by rifampin and rifapentine in hypoxia at neutral pH. Antimicrob Agents Chemother. 2017;61:pii: e02296–16.
14. Sarathy JP, Via LE, Weiner D, Blanc L, Boshoff H, et al. Extreme drug tolerance of *Mycobacterium tuberculosis* in caseum. Antimicrob Agents Chemother. 2018;62:e02266–17.
15. Brucoli F, Guzman JD, Basher MA, Evangelopoulos D, McMahon E, et al. DNA sequence-selective C8-linked pyrrolobenzodiazepine-heterocyclic polyamide conjugates show anti-tubercular-specific activities. J Antibiot. 2016;69:843–9.
16. Leimgruber W, Stefanović V, Schenker F, Karr A, Berger J. Isolation and characterization of anthramycin, a new antitumor antibiotic. J Am Chem Soc. 1965;187:5791–3.
17. Rahman KM, Rosado H, Moreira JB, Feuerbaum EA, Fox KR, et al. Antistaphylococcal activity of DNA-interactive pyrrolobenzodiazepine (PBD) dimers and PBD-biaryl conjugates. J Antimicrob Chemother. 2012;67:1683–96.
18. Hlaka L, Rosslee M, Ozturk M, Kumar S, Parihar SP, et al. Evaluation of minor groove binders (MGBs) as novel antimycobacterial agents and the effect of using non-ionic surfactant vesicles as a delivery system to improve their efficacy. J Antimicrob Chemother. 2017;72:3334–41.
19. Sarathy JP, Zuccotto F, Hsinpin H, Sandberg L, Via LE, et al. Prediction of drug penetration in tuberculosis lesions. ACS Infect Dis. 2016;2:552–63.
20. Prideaux B, Via LE, Zimmerman MD, Eum S, Sarathy J, et al. The association between sterilizing activity and drug distribution into tuberculosis lesions. Nat Med. 2015;21:1223–7.