In vivo and in vitro activity of the siderophore monosulfactam BAL30072 against Acinetobacter baumannii

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Objectives: New antibiotics that are active against multidrug-resistant (MDR) Acinetobacter baumannii are urgently needed. BAL30072, a siderophore monosulfactam antibiotic that rapidly penetrates the outer membrane of A. baumannii and has potent activity against most isolates, including those harbouring AmpC β-lactamases and metallo- (class B) or OXA- (class D) carbapenemases, is being developed to meet that need.

Methods: We assessed the in vitro activity of BAL30072, meropenem and the combination of BAL30072 and meropenem (2:1 and 1:1 ratios) by MIC and time–kill studies. Proof-of-principle in vivo efficacy was determined using a rat soft-tissue infection model. Five diverse strains with defined phenotypic and genetic profiles were tested (AB307-0294, AB8407, AB1697, AB3340 and AB0057).

Results: In microdilution assays, combining BAL30072 with meropenem lowered meropenem MICs 2–8-fold. In time–kill studies, the BAL30072 and meropenem combinations resulted in bactericidal concentrations 2–8-fold lower than those of meropenem or BAL30072 alone. In the rat model, BAL30072 was active against four of five strains (AB307-0294, AB8407, AB1697 and AB3340), including meropenem-susceptible and -non-susceptible strains. AB0057 was the only strain resistant to BAL30072 in vivo and in vitro (MIC >64 mg/L). Meropenem was active in vivo against two of the five strains tested (AB307-0294 and AB3340). Both BAL30072 and BAL30072 with meropenem were equally effective in vivo.

Conclusions: These data support the continued evaluation of BAL30072 for use in the treatment of infections caused by MDR A. baumannii.

Keywords: MICs, time–kill assays, drug susceptibility testing, multidrug resistant

Introduction

In the last decade, the changing epidemiology and prevalence of infections due to Acinetobacter baumannii and its propensity to be multidrug resistant (MDR) has established it as a pathogen of increasing medical importance.1,2 Crude mortality rates associated with A. baumannii infection range from 19% to 54%.3 Notably, death associated with Gram-negative bacteraemia is also significantly higher when caused by A. baumannii compared with other Gram-negative pathogens.4,5 Lastly, colonization or infection with MDR Acinetobacter spp. is associated with an increased incidence of adverse outcomes when compared with MDR Pseudomonas aeruginosa.6 Particularly worrisome is the high degree of antimicrobial resistance demonstrated by many strains of A. baumannii.7 Resistance to aminoglycosides (gentamicin, tobramycin and
amikacin), penicillins (piperacillin), cephalosporins (ceftazidime and cefepime), monobactams (aztreonam) and fluoroquinolones (ciprofloxacin, levofloxacin and moxifloxacin) is disturbingly frequent. Moreover, resistance to carbapenems (imipenem, meropenem, ertapenem and doripenem) and β-lactam/β-lactamase inhibitor combinations (e.g. piperacillin/tazobactam and, even, ampicillin/subactam) presents a significant therapeutic challenge. Of foremost concern are MDR (resistant to three or more classes of antibiotics) or ‘pan-resistant’ strains, i.e. resistant to all antibiotics tested.10–12

In many tertiary care centres, the incidence of infections due to MDR strains is making treatment very challenging.13–17 The polymyxins [polymyxin B and polymyxin E (colistin)], despite being abandoned for decades because of their nephrotoxicity (incidence ranging from 8% to 36%), are becoming the critical ‘last-line’ agents. Unfortunately, our understanding of the pharmacodynamics and pharmacokinetics of polymyxins is still preliminary. Furthermore, resistance to colistin is being reported.19,20 Tigecycline, a tetracycline with broad spectrum activity, demonstrates in vitro activity, but its use for clinical treatment has been disappointing, with reports of emergence of resistance being common.21

Safe and reliable agents with predictable antimicrobial activity against ‘pan-resistant’ or MDR A. baumannii and other non-fermenting pathogens remain an important goal for drug discovery. Designing or discovering a potent β-lactam antibiotic that rapidly penetrates the outer membrane of Gram-negative bacteria, retains stability against β-lactamases and is relatively resistant to efflux pumps is essential to the quest for novel β-lactam antibiotics.22 To test BAL30072 (3S)-3-[(2Z)-2-amino(1,3-thiazol-4-yl))-3-[{1,5-dihydroxy-4-oxo(2-hydropyridyl)}methoxy]-3-azaprop-2-enoylamino]-4,4-dimethyl-2-oxazetidinyl hydroxysulfonate; Figure 1) is a new monocyclic siderophore in its side chain, which is believed to contribute to accelerated flux across the Gram-negative outer membrane.23 Further, the sulphated monocyclic β-lactam nucleus is a very poor substrate for class B metallo-β-lactamases and acts as a mechanism-based inhibitor of class C β-lactamases.24 To test the efficacy of BAL30072 against MDR A. baumannii, we assessed five strains, in which genetic determinants of β-lactam resistance were previously defined, utilizing in vitro microdilution and time–kill studies. Further, as proof of principle we evaluated whether BAL30072 was active in an in vivo rat soft-tissue infection model.25

Materials and methods

Bacterial strains and media

MICS were determined according to CLSI methods. For all commercially available antibiotics, MIC interpretation is based on CLSI breakpoints. BAL30072 and meropenem were tested alone, and in 2:1 and 1:1 ratios (w/w). BAL30072 was synthesized in the laboratories of Basilea Pharmaceutica International Ltd.

Time–kill synergy studies

Assays were performed using a standard method in cation-supplemented MH broth in 10 mL volumes.26 BAL30072 and meropenem were tested alone and in 2:1 and 1:1 ratios against 1 × 10⁸ cfu/mL of the strain being assessed. In each case, concentrations up to four doubling dilution steps higher than and four dilution steps lower than the MIC were tested. Viability counts were performed at 0, 12 and 24 h. Bacteriostatic activity was defined as a decrease of ≥3 log₁₀ cfu/mL and bactericidal activity as a ≥3 log₁₀ cfu/mL reduction over 24 h. Synergy was defined as an increase of ≥1 log₁₀ cfu/mL over the microbiocidal activity of the two agents. The phenotypic and genetic characteristics of A. baumannii strains AB307-0294, AB0057, AB1697, AB8407 and AB3340 are described in Table 1. All strains were grown on trypticase soy agar plates or in Luria–Bertani (LB) or cation-supplemented Mueller–Hinton (MH) broth, and were maintained at −80°C in 50% LB broth and 50% glycerol. The sequence type (ST) of each of the strains used in this study was previously determined by a multilocus sequence typing method using PCR followed by electrospray ionization mass spectrometry (PCR/ESI-MS), as previously described.27

Table 1. A. baumannii strains studied

<table>
<thead>
<tr>
<th>A. baumannii strain</th>
<th>PCR/ESI-MS strain type/EICT</th>
<th>Phenotype</th>
<th>bla gene content</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB307-0294 ST16/I</td>
<td>MEM⁴, BAL30072⁵, blaADC, blaOxa-69, blaOxa-58</td>
<td>blaTEM</td>
<td>blaADC, blaOxa-69, blaOxa-58</td>
</tr>
<tr>
<td>AB1697 ST24/assigned</td>
<td>MEM⁴, BAL30072⁵</td>
<td>blaADC, blaOxa-69, blaOxa-58</td>
<td>blaADC, blaOxa-69, blaOxa-58</td>
</tr>
<tr>
<td>AB0057 ST15/I</td>
<td>MEM⁴, BAL30072⁵</td>
<td>blaADC, blaOxa-69, blaOxa-23</td>
<td>blaADC, blaOxa-69, blaOxa-23</td>
</tr>
<tr>
<td>AB8407 ST86/probably II</td>
<td>MEM⁴, BAL30072⁵</td>
<td>blaADC, blaOxa-69, blaOxa-23</td>
<td>blaADC, blaOxa-69, blaOxa-23</td>
</tr>
<tr>
<td>AB3340 ST15/I</td>
<td>MEM⁴, BAL30072⁵</td>
<td>blaADC, blaOxa-69, blaOxa-23</td>
<td>blaADC, blaOxa-69, blaOxa-23</td>
</tr>
</tbody>
</table>

EICT, European-International clone type; MEM, meropenem; S, susceptible; R, resistant.
aPCR products were not fully sequenced, but amplicons were detected with specific primers.
bST86 is an uncommon ST that is similar to ST10 and is probably part of European clone II.
defined as a 2 log₁₀ decrease in the viable count compared with the initial inoculum at 24 h with the combination, provided each component alone has little effect at the concentration used in the combination.

**Rat soft-tissue infection model**

An established rat (Long–Evans) soft-tissue infection model was employed as previously reported. The rat soft-tissue infection model animal studies were reviewed and approved by the University at Buffalo and Veterans Administration Institutional Animal Care Committee. In brief, a subcutaneous pouch was created on the dorsal surface of Long–Evans rats that contains 8–12 mL of exudative fluid. The desired bacterial inoculum was introduced into the pouch and pouch fluid removed over time, enabling the determination of bacterial cfu/mL. A. baumannii strains were injected into the pouch to achieve a starting titre of $\approx 1 \times 10^5$ or $1 \times 10^7$ cfu/mL, and pouch fluid was removed to enumerate bacterial counts at 1 min, 3 h, 6 h and 24 h. Animals were then treated with BAL30072, meropenem or both, with agents administered intraperitoneally (ip) at 2, 4 and 6 h post-bacterial challenge, as follows: (i) BAL30072 alone (50 mg/kg/dose); (ii) meropenem alone (50 mg/kg/dose); (iii) BAL30072 plus meropenem (25 mg/kg/dose); and (iv) BAL30072 plus meropenem (50 mg/kg/dose each). The activity of BAL30072 in combination with meropenem was studied as a result of previous observations of additive or synergistic effects of such combinations against other species of Gram-negative bacteria in vitro. Two ratios for BAL30072 to meropenem (1:1 and 2:1) were investigated, because the earlier studies had suggested that synergy might be obtained over a range of concentrations, with different proportions being more or less effective for the various species studied. The antibiotics were dissolved in sodium acetate buffer pH 5.5 and filter sterilized. Sterile acetate buffer alone was administered ip at 2, 4 and 6 h post-bacterial challenge to control animals. Each treatment group contained three to four animals for each A. baumannii strain tested. Since this was a proof-of-principle study to assess activity in vivo, and not a formal pharmacokinetic study, drug concentrations were not measured. For each strain assessed, three to four independent experimental (per antimicrobial regimen) and three to four independent control animals were utilized.

**Statistical analyses**

Data are presented as means ± SEM. P values of 0.05/n (n = the number of comparisons) are considered statistically significant based on the Bonferroni correction for multiple comparisons. A statistical trend was defined as a P value of <0.1. In vivo activity of BAL30072 was defined as a difference in the survival and growth of the test strain in animals treated with antimicrobial compared with those treated with buffer alone. To normalize in vitro and in vivo data, log₁₀ transformed values were utilized, the area under each curve was calculated and these areas were compared using two-tailed unpaired t-tests (Prism 4 for Macintosh, GraphPad Software Inc.).

**Results**

**In vitro susceptibility**

Table 2 summarizes the results of the in vitro microdilution and time–kill susceptibility studies. Only AB0057 was not inhibited by BAL30072 at concentrations <16 mg/L, whereas AB0057, AB1697 and AB3340 were resistant to meropenem as assessed by microdilution MIC assays. In microdilution MIC assays, combining BAL30072 and meropenem (2:1 and 1:1) lowered meropenem MICs 2–8-fold.

In time–kill studies, the BAL30072 and meropenem combinations (2:1 and 1:1) were bactericidal at concentrations 8- and 4-fold lower, respectively, than meropenem alone against the carbapenem-susceptible strain AB307-0294. BAL30072 and meropenem (2:1 and 1:1) were bactericidal at concentrations 4-fold lower than meropenem alone against AB8407. The combination of BAL30072 and meropenem (2:1 and 1:1) lowered bactericidal concentrations 2-fold for the meropenem- and BAL30072-resistant strain AB0057 and the meropenem-resistant strain AB3340, and 4-fold for the meropenem-resistant strain AB1697 (Table 3).

**In vivo susceptibility**

We assessed the efficacy of BAL30072 in vivo in a rat soft-tissue infection model as a proof of principle for the activity of BAL30072. The advantages of this infection model are that immunocompetent animals are utilized and multiple samplings can be obtained from each animal at set timepoints, making it temporally and economically efficient for initial in vivo proof-of-principle studies to assess activity.
assessment of antibacterial activity. Furthermore, assessing antimicrobial activity in an in vivo setting that includes multiple concomitant confounding host factors that cannot be reproduced in an in vitro evaluation is important. Lastly, this model is clinically relevant given that A. baumannii has been increasingly recognized as a cause of a variety of soft-tissue infections.29,30

As a first step, we evaluated a susceptible strain, AB307-0294, and employed a pragmatic dosing regimen that was administered ip at 2, 4 and 6 h post-bacterial challenge. Previous experiments showed that in the absence of treatment, a starting inoculum of \(1 \times 10^7\) cfu/mL of rat pouch fluid resulted in growth of AB307-0294.26 Under these experimental conditions, the treatment effect of BAL30072 alone (50 mg/kg/dose), meropenem alone (50 mg/kg/dose), BAL30072 (50 mg/kg/dose) plus meropenem (25 mg/kg/dose) (2:1 ratio) and BAL30072 plus meropenem (50 mg/kg/dose each) (1:1 ratio) administered at 2, 4 and 6 h post-challenge with AB307-0294 was assessed. AB307-0294 was significantly killed by each of these treatment regimens (Table 4).

Next, four MDR A. baumannii strains (AB8407, AB0057, AB1697 and AB3340; resistant to cephalosporins, aminoglycosides and fluoroquinolones) were studied. Three of these strains were also carbapenemase producing (AB0057, AB1697 and AB3340). Before assessing the effect of BAL30072 and meropenem on these strains in our soft-tissue infection model, we needed to determine the challenge inoculum necessary for their growth and survival in vivo in the absence of antimicrobial therapy. Although there were some strain-to-strain differences, an initial inoculum to achieve a titre of \(1 \times 10^7\) cfu/mL in pouch fluid was needed for the survival and growth of the four MDR A. baumannii strains assessed in this model (data not shown); a necessary attribute to assess the effect of antimicrobials. Therefore, for subsequent experiments the administration and dosing regimens previously established and a starting inoculum to achieve a titre of \(1 \times 10^7\) cfu/mL in pouch fluid were used for the four MDR strains and AB307-0294, so that results would be comparable.

Our results are summarized in Figure 2. Compared with drug-free, buffer-treated controls, BAL30072 (50 mg/kg/dose) showed in vivo activity against four of the five strains studied (AB307-0294, AB8407, AB1697 and AB3340). This difference was statistically significant for AB1697, a meropenem-resistant strain (\(P=0.018\)) (Figure 2c). In contrast, this difference was not significant for AB307-0294 (\(P=0.13\)) (Figure 2b), although it was significant with the low-challenge inoculum (\(P=0.0005\)) (Table 4). There was a trend towards significance for AB3340 (\(P=0.08\)) (Figure 2a) and there was a non-statistically significant difference for AB8407. In vivo activity of BAL30072 was not demonstrated against AB0057, the strain most resistant to this agent (MIC >64 mg/L). In this stringent in vivo model, BAL30072 was active against all four strains that were inhibited by BAL30072 in vitro.

Compared with drug-free, buffer-treated controls, meropenem (50 mg/kg/dose) was active in vivo against two of the five strains assessed (AB307-0294 and AB3340); however, this difference was statistically significant only for AB307-0294 (\(P=0.0005\); Figure 2e). There was non-statistically significant kill when AB8407 was treated with meropenem. AB0057, AB1697 and AB8407 were resistant to meropenem in vivo, with poor correlation between in vivo and in vitro activity. The effect of BAL30072 (50 mg/kg/dose) plus meropenem at two dosing regimens (25 and 50 mg/kg/dose; 2:1 and 1:1 ratios, respectively) was also assessed in this infection model. The addition of meropenem at either dose was not additive, synergistic or antagonistic (Table 4).

### Discussion

BAL30072 is a siderophore monosulfactam antibiotic that holds promise for the treatment of MDR Gram-negative bacilli, including MDR isolates of A. baumannii. In this study, we assessed its activity in vitro and in vivo against five diverse strains of A. baumannii, which were from four unique STs (Table 1). Overall, BAL30072 appears to be active against...
selected strains of *A. baumannii*. The substrate profile of Acinetobacter-derived cephalosporinases could have an impact on the susceptibility of the host strain to the monosulfactam and/or to the carbapenem combination. However, such ‘extended-spectrum’ substitutions would not test susceptible to the single agent or a combination of agents. In microdilution assays, BAL30072 and meropenem (2:1 and 1:1) lowered meropenem MICs 2–8-fold (Table 2). In time–kill studies, BAL30072 alone and meropenem alone were less active than when combined [BAL30072 and meropenem (2:1 and 1:1), bactericidal concentrations 2–8-fold lower than BAL30072 or meropenem alone] (Tables 2 and 3). Interestingly, the enhancement of activity by the combination of BAL30072 and meropenem compared with either agent alone was similar for AB8407 and AB0057 in time–kill studies. However, in MIC studies, AB8407 and AB0057 were susceptible and resistant to BAL30072 and meropenem, respectively (Table 2). One explanation would be if meropenem and BAL30072 were targeting different penicillin-binding proteins. In the time–kill experiments (which are done under ‘dynamic’ conditions in short time frames and not ‘fixed’ as in MIC studies), the rates at which different penicillin-binding proteins are inactivated (acylated) relative to the growth of the pathogen may be different for susceptible and resistant strains. In addition, the mechanisms for resistance to carbapenems (class D carbapenemases, efflux) and the siderophore monosulfactam BAL30072 are probably different.

We next established that the rat soft-tissue infection model could be used to assess the *in vivo* efficacy of various antimicrobial agents, including BAL30072. However, a relatively high initial bacterial challenge inoculum is required, depending on the ability of the strain being assessed to survive and grow in this *in vivo* model system. For four of five strains initially tested in this study, which interestingly were all MDR, a relatively high-challenge inoculum was necessary (≏1 × 10⁷ cfu/mL). The antimicrobial-susceptible strain AB307-0294 required a 100-fold lower challenge inoculum for growth and survival. Although the number of strains evaluated is too small to make any definitive conclusions, these data are consistent with a hypothesis that MDR in *A. baumannii* may be associated with decreased biological fitness *in vivo*. Further, this soft-tissue infection model is a closed-space infection. Treatment of such infections without drainage is challenging and usually results in clinical failure. In addition, antimicrobials were administered ip 2, 4 and 6 h after bacterial challenge, and final titres were enumerated at 24 h post-
challenge. This initial pragmatic dosing regimen was used because the goal of this study was to establish a proof of principle for in vivo BAL30072 activity. However, an alternative dosing regimen based on pharmacokinetic data will almost certainly enhance bacterial killing, particularly for AB3340 and AB8407. Lastly, the relatively small animal number used for each condition (n = 3–4) and the inherent increased magnitude of variability that occurs in in vivo models compared with in vitro studies makes the demonstration of statistically significant activity more challenging. The enhancement of in vitro activity of meropenem by BAL30072 in time–kill studies was not observed in vivo. Potential explanations include bacterial factors, such as phenotypic or genotypic alterations in A. baumannii in vivo (e.g. heteroresistance), or an effect of the in vivo environment, such as protein binding. Nonetheless, despite the use of a rigorous in vivo model and a non-optimized treatment regimen, there was a clear demonstration of in vivo activity for BAL30072 against MDR A. baumannii. These encouraging results lend cautious optimism to the notion that these findings have the potential to translate into clinical efficacy in humans.

In conclusion, BAL30072 performed well against this group of MDR A. baumannii strains. In a stringent in vivo model, compared with drug-free, buffer controls, BAL30072 was active against four of the five A. baumannii strains tested and these four strains were inhibited by BAL30072 in vitro. In this in vivo model, meropenem was active against two of the five A. baumannii strains studied, with poor correlation between in vivo and in vitro activity. These data support the potential use of the siderophore sulfactam with poor correlation between in vivo bacterial factors, such as phenotypic or genotypic alterations in the pharmacokinetic/pharmacodynamic properties of BAL30072 in vivo, and the goal of this study was to establish a proof of principle for in vitro experiments of in vivo efficacy. T. A. R., M. G. P. P., M. J. and R. A. B. participated in the experimental execution of in vivo experiments. T. A. R., M. G. P. P., M. J. and R. A. B. participated in the experimental execution of in vivo experiments. T. A. R., M. G. P. P., M. J. and R. A. B. participated in the experimental execution of in vivo experiments.

References


