Published in final edited form as:

Biopolymers. 2011 ; 96(2): 126–129. doi:10.1002/bip.21443.

Intramuscularly Administered Peptide A3-APO Is Effective Against Carbapenem-Resistant *Acinetobacter baumannii* in Mouse Models of Systemic Infections

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Abstract

Most antibacterial peptides exhibit low therapeutic indices in vivo. Peptide A3-APO was shown to exhibit high potency against *Escherichia coli* bacteremia when added intraperitoneally. To extend the studies to systemic infections against multidrug-resistant organisms, we studied the efficacy of A3-APO in mouse models of carbapenem-resistant *Acinetobacter baumannii* infection. When administered either intravenously at 2.5 mg/kg or intramuscularly (im) at 5 mg/kg twice or three times to mice infected with a carbapenem-resistant *A. baumannii* strain, peptide A3-APO reduced the bacterial counts by at least two log₁₀ units and increased the survival rate compared with untreated animals or mice treated with 40 mg/kg imipenem. Unlike after intraperitoneal or intravenous administration, A3-APO did not show toxic effects at 60 mg/kg dose im.

Keywords

carbapenem resistant; intramuscular; peptide antibiotics; sepsis model; survival rate

INTRODUCTION

Despite the public concern about methicillin-resistant *Staphylococcus aureus*, in reality gram-negative bacteria acquired in hospitals are more common pathogens in resistant infections.¹ The most dreadful bacteria include *Acinetobacter baumannii* and *Klebsiella pneumoniae* strains unaffected even by carbapenems, the last clinical resort against gram-negative infections in hospitals. In a two-year period starting from October 2005, resistance to imipenem (a carbapenem-type antibiotic) in military hospitals increased from 13% to 46%.² Equally disturbing, resistance to the second best agent, the quite toxic yet powerful cyclic peptide antibiotic colistin, similarly increased.² Antimicrobial peptides rarely exhibit activity in animal models of systemic infections,³ or the therapeutic index is too low for future clinical applications.⁴ A3-APO [(Chex-Arg-Pro-Asp-Lys-Pro-Arg-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-Pro-Pro-Arg-Pro-Val-Arg)₂-Dab] is a representative of the designer proline-rich antibacterial peptide family that shows in vitro efficacy against multidrug-resistant (MDR) *Enterobacteriaceae* alone or in combination with conventional antibiotics.⁵ A3-APO kills bacteria by a dual mode of action: membrane disintegration and inhibition of DnaK-mediated protein folding.⁶ A3-APO is inactive when added subcutaneously,⁷ but upon intraperitoneal (ip) administration at 10 mg/kg exhibits survival and bacterial count reduction figures comparable to imipenem added at 40 mg/kg.⁸ However, the low 50 mg/kg ip lethal dose limits its utility as a clinically viable antimicrobial agent. The A3-APO dimer also functions as a prodrug where the active metabolite is a Chex1-Arg20 single-chain fragment.⁹ Unfortunately Chex1-Arg20, unlike the dimer, rapidly induces resistance in vitro due to reduced level of activity on bacterial membranes.¹⁰ In the current study we were looking for alternative administration modes and extended the efficacy studies to a MDR gram-negative clinical isolate.

MATERIALS AND METHODS

Peptides

The details of the synthesis of the A3-APO dimer and its Chex1-Arg single chain metabolite have been published previously.⁸ In brief, the peptides were assembled on a CEM Liberty automated peptide synthesizer with standard Fmoc-chemistry and after cleavage with trifluoroacetic acid (TFA) were purified by reversed-phase high performance liquid chromatography in a water/acetonitrile/TFA system. Matrix-assisted laser desorption/ionization mass spectrometry verified the high purity of the peptide preparations.

In Vitro Antibacterial Assay

Conventional antibacterial growth inhibition assays were performed using sterile 96-well polypropylene plates in a final volume of 100 μ L.⁶ Briefly, 50 μ L of a suspension of midlogarithmic phase bacterial cultures diluted to 5×10^5 cfu/mL in Mueller-Hinton broth was added to 50 μ L of serially diluted peptides. The highest peptide concentration was 64 mg/L. Cultures were then incubated at 37°C for 16–20 h without shaking, and growth inhibition was measured by recording the absorbance at 600 nm using a microplate reader. Minimal inhibitory concentrations (MICs) were identified as the lowest antimicrobial doses

when the 600 nm absorbance did not exceed that of the negative control medium only values.

Animals

Female CD-1 mice of 20–25 g (Charles River Hungary, Budapest) were used for the in vivo efficacy studies. Mice were housed in plastic type 2 cages, three to five mice per cage on softwood granules as bedding. The room was kept between 21°C and 25°C with 12 h : 12 h light : dark cycles. The animals had free access to tap water and pelleted rodent food. The assays were initiated after a three-week acclimatization period. Upon completion of the experiments, the mice were euthanized with 150 mg/kg sodium barbiturate (Sigma-Aldrich Hungary, Budapest) ip.

Toxicity and In Vivo Efficacy Studies

For acute toxicity studies, peptide A3-APO was dissolved in water and given as bolus injection at 20, 40, or 60 mg/kg intramuscularly (im).

Efficacy Assays: Intravenous (iv) Assay

Totally, 4×10^7 cfu/g *A. baumannii* BAA-1805 was injected ip into CD-1 mice. A3-APO or imipenem were administered iv at a 2.5 mg/kg dose at 4, 8, and 12 h postinfection.

Intramuscular (im) assay A: Mice were challenged ip with 2×10^6 cfu/g *A. baumannii* BAA-1805. Four, eight, and twelve hours after challenge 5 mg/kg A3-APO or the Chex1-Arg20 peptide or 40 mg/kg imipenem were administered im to their right and left hind and right front legs (in this order). In im assay B mice were infected ip with 3×10^7 cfu/g *A. baumannii* BAA-1805, and received 5 mg/kg A3-APO or 40 mg/kg imipenem 4 and 8 h after the infection. Survival rate was recorded 24 h post-treatment. Prior to drug administration at all timepoints and 4 h later, 10 µL blood was taken from the tail vein of 1–4 mice for determining blood bacterial counts.

Animals were maintained and handled in accordance with the recommendations of the Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of Semmelweis University (permission No.: 399/003/2005).

RESULTS AND DISCUSSION

When looking for alternative administration routes, we noticed that im administered A3-APO apparently lacked systemic toxicity. Healthy CD-1 mice (five per dose group) experienced only local discomfort when given 20, 40, or 60 mg/kg doses but quickly recovered without any visible negative effects (Table I). The lack of dose dependence indicated that the animals suffered from the inoculation procedure itself (perhaps the residual trifluoroacetate counterions in the preparation) and not the systemic toxicity of the peptide. Encouraged by this finding we relyophilized the peptide from 1% acetic acid and tested the ability of A3-APO to eradicate MDR gram-negative nonfermenting strains by im route of administration. We selected *A. baumannii*, for which resistance to carbapenems, the “silver bullets” against gram-negative pathogens¹¹ is rapidly increasing.¹² Current treatment of carbapenem-resistant *A. baumannii* infections employs im administered

colistin.^{13,14} While im drug delivery is desirable in many settings ranging from combat field to soft tissue infections,¹⁵ colistin, a heavily modified cyclic peptide antibiotic suffers from high toxicity with an im LD₅₀ value of <40 mg/kg in CD-1 mice while the efficacious dose is 2.5–5 mg/kg twice daily.¹⁶

Like for several *Enterobacteriaceae*, A3-APO was only moderately efficacious against MDR *A. baumannii* (some of the strains described previously, Ref. 17) or *Enterobacter cloacae* in vitro (Table I). From the nine *A. baumannii* strains we tested, the MIC (measured by the liquid microdilution method in undiluted Mueller-Hinton broth, 6), varied from 32 to >64 mg/L compared to a 2–32 mg/L range for imipenem or meropenem. Against *E. cloacae* these figures were 4–16 mg/L for A3-APO and 4–8 mg/L for the carbapenems. The peptide showed no activity against *Proteus vulgaris* or *Enterococcus faecalis* controls (>64 mg/L) (Table I). The activity against *A. baumannii* can be explained by the conserved *Escherichia coli* homologous DnaK D-E helix sequence with a 3-residue frameshift, similar to the susceptible strain *Agrobacterium tumefaciens*.¹⁸ While this low in vitro activity level would normally be suggestive of no efficacy for small molecule antibiotics in vivo,⁴ A3-APO exhibiting comparable in vitro activity data (median MIC 30 mg/L) is quite efficacious in systemic *E. coli* infection models.⁸

After ip infection of mice, iv administered A3-APO reduced the blood bacterial counts by 0.5 log₁₀ units after two doses and 1.5 log₁₀ units after the third dose (see Figure 1). In this assay, CD-1 mice (7 per group, 20 g each) were infected with a 4 × 10⁷ cfu/g suspension of *A. baumannii* followed by addition of 2.5 mg/kg peptide 4, 8, and 12 h after infection. The bacterial strain showing MDR properties (including carbapenem-resistance) was isolated from a Canadian soldier injured in Afghanistan¹⁹ and was purchased from the American Type Culture Collection (designation BAA-1805). As Figure 1 indicates, A3-APO showed efficacy in the systemic mouse MDR *A. baumannii* infection assay.

We document im efficacy in two models. First a less stringent measure was used in which the mice received a 2 × 10⁶ cfu/g *A. baumannii* BAA-1805 bacterial inoculum ip. The mice then received 5 mg/kg peptide A3-APO (in 0.01 mL/g body weight physiological salt solution) at 4, 8, and 12 h after infection. The first dose of peptide reduced the blood bacterial counts by almost 2 log₁₀ units, and further dosing did not provide additional treatment benefits (Figure 1, im Assay A). During the second round of testing, mice were infected with a significantly higher bacterial load (3 × 10⁷ cfu/g) and received 5 mg/kg peptide treatment to their hind legs only 4 and 8 h after infection. After these two doses, the blood bacterial counts dropped to 2.5 × 10⁵ cfu/mL, by more than 2 log₁₀ units (Figure 1, im Assay B).

Our results also indicated that A3-APO was more efficacious than imipenem in the bacteremia models as measured by the survival rate after drug treatment. Along with the iv peptide dosing, a group of seven CD-1 mice was also treated with 2.5 mg/kg imipenem iv. Table II shows that the peptide prolonged the early survival rate better than imipenem with a statistically significant improvement at 12 h after infection compared to both untreated and imipenem treated mice. When comparing the efficacies after im drug administration, for imipenem we used the 40 mg/kg dosing recommended for clinical applications. During the

less stringent conditions of im Assay A, imipenem showed a statistically significant improvement over untreated animals only at 12 h after infection, compared to A3-APO that exhibited such advantage significantly longer, between 12 and 18 h (Table II, im Assay A). In the second infection model using a higher inoculum, only two of the seven imipenem-treated mice survived after the first dose of drug and none after the second. In contrast, all seven A3-APO-treated mice survived until the second treatment representing a statistically significant improvement over imipenem in hours 8–10 after infection (Table II, im Assay B).

As shown above, peptide A3-APO exhibited improved in vivo efficacy compared with imipenem in MDR *A. baumannii* infection models when used either iv or im. In addition, the im therapeutic index of the peptide (>12) appeared to be higher than that of colistin (around 5–6) indicating a potential role of A3-APO as a clinical therapy option.

Abbreviations

Arg	L-arginine
Asp	L-aspartic acid
Chex	1-amino-cyclohexanecarboxylic acid
Dab	2,4-diamino-butyric acid
im	intramuscular
ip	intraperitoneal
iv	intravenous
Leu	L-leucine
Lys	L-lysine
MDR	multidrug-resistant
MIC	minimal inhibitory concentration
Pro	L-proline
TFA	trifluoroacetic acid
Tyr	L-tyrosine
Val	L-valine

REFERENCES

1. Murray CK, Wilkins K, Molter NC, Yun HC, Dubick MA, Spott MA, Jenkins D, Eastridge B, Holcomb JB, Blackburn LH, Hospenthal DR. J Trauma. 2009; 66:S138–S144. [PubMed: 19359957]
2. Hawley JS, Murray CK, Griffith ME, McElmeel ML, Fulcher LC, Hospenthal DR, Jorgensen JH. Antimicrob Agents Chemother. 2007; 51:376–378. [PubMed: 17043112]
3. Marr AK, Gooderham WJ, Hancock REW. Curr Opin Pharmacol. 2006; 6:468–742. [PubMed: 16890021]

4. Bush K, Macielag M, Weidner-Wells M. *Curr Opin Microbiol.* 2004; 7:466–476. [PubMed: 15451501]
5. Otvos L Jr, de Olivier Inacio V, Wade JD, Cudic P. *Antimicrob Agents Chemother.* 2006; 50:3146–3149. [PubMed: 16940114]
6. Otvos L Jr, Wade JD, Lin F, Condie BA, Hanrieder J, Hoffmann R. *J Med Chem.* 2005; 48:5349–5359. [PubMed: 16078852]
7. Rozgonyi F, Szabo D, Kocsis B, Ostorhazi E, Abbadessa G, Cassone M, Wade JD, Otvos L Jr. *Curr Med Chem.* 2009; 16:3996–4002. [PubMed: 19747127]
8. Szabo D, Ostorhazi E, Binas A, Rozgonyi F, Kocsis B, Cassone M, Wade JD, Nolte O, Otvos L Jr. *Int J Antimicrob Agents.* 2010; 35:357–361. [PubMed: 20031377]
9. Noto PB, Abbadessa G, Cassone M, Mateo GD, Agelan A, Wade JD, Szabo D, Kocsis B, Rozgonyi F, Otvos L Jr. *Protein Sci.* 2008; 17:1249–1255. [PubMed: 18413862]
10. Cassone M, Frith N, Vogiatzi P, Wade JD, Otvos L Jr. *Int J Pept Res Ther.* 2009; 15:121–128.
11. Edwards JR, Williams S, Nairn K. *J Antimicrob Chemother.* 1989; 24(Suppl A):279–285. [PubMed: 2681126]
12. Orsi GB, Franchi C, Giordano A, Rocco M, Ferraro F, Mancini C, Venditti M. *J Chemother.* 2008; 20:219–224. [PubMed: 18467249]
13. Pantopoulou A, Giamarellos-Bourboulis EJ, Raftogannis M, Tsaganos T, Dontas I, Koutoukas P, Baziaka F, Giamarellou H, Perrea D. *Int J Antimicrob Agents.* 2007; 29:51–55. [PubMed: 17189095]
14. Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, Bonomo RA. *Antimicrob Agents Chemother.* 2007; 51:3471–3484. [PubMed: 17646423]
15. Mellor SG, Cooper GJ, Bowyer GW. *J Trauma.* 1996; 40(3 Suppl):S128–S134. [PubMed: 8606393]
16. Lin B, Zhang C, Xiao X. *J Vet Pharmacol Ther.* 2005; 28:349–354. [PubMed: 16050814]
17. Hujer KM, Hujer AM, Hulten EA, Bajaksouzian S, Adams JM, Donskey CJ, Ecker DJ, Massire C, Eshoo MW, Sampath R, Thomson JM, Rather PN, Craft DW, Fishbain JT, Ewell AJ, Jacobs MR, Paterson DL, Bonomo RA. *Antimicrob Agents Chemother.* 2006; 50:4114–4123. [PubMed: 17000742]
18. Kragol G, Hoffmann R, Chattergoon MA, Lovas S, Cudic M, Bulet P, Condie BA, Rosengren KJ, Montaner LJ, Otvos L Jr. *Eur J Biochem.* 2002; 269:4226–4237. [PubMed: 12199701]
19. Tien HC, Battad A, Bryce EA, Fuller J, Mulvey M, Bernard K, Brisebois R, Doucet JJ, Rizoli SB, Fowler R, Simor A. *BMC Infect Dis.* 2007; 7:95. [PubMed: 17697345]

FIGURE 1.

Reduction of blood bacterial counts after intravenous (iv) or intramuscular (im, Assays A and B) peptide A3-APO dosing to *Acinetobacter baumannii* BAA-1805-infected CD-1 mice. The drug was administered 4, 8, and 12 h after infection (iv treatment and im Assay A), or 4 and 8 h after infection (im Assay B) at 2.5 or 5 mg/kg doses during the iv and im treatment assays, respectively. For comparison, the 12-h blood bacterial counts of untreated mice infected simultaneously were 1.5×10^8 cfu/mL (iv treatment), 1.9×10^7 cfu/mL (im Assay A) and 3.9×10^7 cfu/mL (im Assay B). The blood of the single A3-APO treated surviving mouse in im Assay B was sterile. Error bars for iv assay and im Assay B represent standard deviation of data from 1 to 4 mice plated in duplicates. Because of the lower original bacterial load during infection in im Assay A, the blood bacterial counts greatly varied.

Table I

MICs and In Vivo Systemic Toxicity Parameters of Peptide A3-APO

Bacteria	In Vitro Activity Range (mg/L)	Toxicity in Mice Upon Intramuscular Administration (mg/kg, Single Dose)
<i>A. baumannii</i>	32, 32, 32, 64, 64, 64, >64, >64, >64	No Observed Adverse Effect Level
<i>E. cloacae</i>	4, 8, 8, 8, 16	>60
<i>P. vulgaris</i> (n = 5) <i>E. faecalis</i> (n = 3)	> 64(each)	

Carbapenems (imipenem or meropenem) killed the same *Acinetobacter baumannii* and *Enterobacter cloacae* strains in MIC ranges 2–32 and 4–8 mg/L, respectively. The MIC against *A. baumannii* strain BAA-1805 that was used for the in vivo efficacy studies was 32 mg/L for both imipenem and peptide A3-APO.

Survival of Mice After Intraperitoneal *A. baumannii* BAA-1805 Infection and Treatment With Peptide A3-APO, Its Single Chain Chex1-Arg20 Metabolite or Imipenem

Table II

Time After Infection (h)	Intravenous Drug Administration (Intraperitoneal Infection)			Intramuscular Drug Administration (Intraperitoneal Infection)		
	Untreated	2.5 mg/kg Imipenem	2.5 mg/kg A3-APO	Untreated (Assay A)	40 mg/kg Imipenem (Assays A/B)	5 mg/kg A3-APO (Assays A/B)
4	7	7	7	7	7/7	7/7
8	7	6	7	6	7/2	7/7**
12	4	4	7*	3	6*/0	6*/2
18	0	1	1	2	3/0	5*/1
24	0	1	1	2	2/0	3/1

Seven animals were used per group. Statistically significant improvements in survival data (Student's *t* test, 90% confidence limits) compared with untreated controls [intramuscular treatment (im) Assay A] or peptide efficacy compared to imipenem (im Assay B) are marked with single (Assay A) or double (Assay B) asterisks. The * in the intravenous (iv) column indicates statistically significant improvement over both untreated and imipenem-treated mice. The drugs were administered 4, 8, and 12 h after infection (iv treatment and im Assay A), or 4 and 8 h after infection (im Assay B). The survival figures after iv A3-APO administration and im A3-APO administration (Assay A) compared with untreated controls were statistically significant in the entire periods between 12 and 13 h and 12 and 18 h, respectively, after infection. The survival figures after im peptide treatment compared with imipenem treatment (Assay B) were statistically significant in hours 8–10 after infection. In the iv assay and in im Assay B, the bacterial inoculum contained an increased load of pathogens compared with im Assay A.