

Enzymic degradation of a β -lactam antibiotic, ampicillin, in the gut: a novel treatment modality

Jaana Harmoinen^{1*}, Kirsi Vaali², Pertti Koski³, Kaisa Syrjänen³, Outi Laitinen¹, Kai Lindevall³ and Elias Westermarck¹

¹Faculty of Veterinary Medicine, Department of Clinical Veterinary Sciences, PO Box 57, Helsinki;

²Institute of Biomedicine/Pharmacology, PO Box 63, University of Helsinki, FIN-00014 Helsinki;

³Ipsat Therapies Ltd, Sinimäentie 10B, FIN-02630 Espoo, Finland

Received 8 August 2002; returned 29 October 2002; revised 12 November 2002; accepted 20 November 2002

Antibiotics can cause severe alterations in the gut microflora and promote diarrhoea and overgrowth of pathogenic bacteria. The present study investigated the potency of targeted recombinant β -lactamase (TRBL) to degrade a β -lactam antibiotic in the jejunum of fistula-operated beagles. We used different peroral doses of purified β -lactamase (PenP) of *Bacillus licheniformis* in enteric-coated pellets together with intravenous ampicillin. Serum and jejunal samples were collected for ampicillin and β -lactamase analysis. A dose–response effect of TRBL on ampicillin concentrations in the jejunal samples could be observed. The highest doses applied decreased the jejunal ampicillin concentrations to undetectable levels. In the serum samples, the ampicillin concentrations were not affected by the β -lactamase dose used. Our results indicate that it may be possible to evolve a targeted treatment to degrade β -lactam antibiotics intestinally and, thus, decrease antibiotic-induced adverse effects on the gut microflora.

Keywords: β -lactamase, β -lactams, jejunum, microflora

Introduction

Normal gastrointestinal microflora form a relatively stable ecosystem.¹ However, certain factors, like the administration of antibiotics, can profoundly disrupt this microbial balance.² The most common antibiotic-associated adverse effects include overgrowth of pre-existing microorganisms as a consequence of a systemic infection or severe diarrhoea.^{3–5}

Although β -lactam antibiotics are by far the most effective, safe and widely used antibiotics, they may alter the normal intestinal microflora.⁵ The disturbance in bacterial composition reflects the antibacterial spectrum and the activity of the drug used.⁶ After parenteral injection, ampicillin is distributed rapidly and widely, resulting in a high concentration of the drug in bile.⁷ From bile it is excreted into the gut and is known to cause disruption of the normal intestinal microflora,⁸ by diminishing the main flora and increasing the number of yeasts⁹ as well as inducing a high risk of *Clostridium difficile* colitis.¹⁰

The purpose of our research was to evaluate a novel enzymic therapy, namely targeted recombinant β -lactamase (TRBL), and establish whether this could reduce the concentrations of parenterally administered β -lactam antimicrobials in the gut lumen. The hypothesis is if the intestinal concentrations of these agents can be reduced, then the adverse effects on the normal flora might also be reduced. The study was conducted in a jejunum–fistulated beagle model,^{11,12} which permits ready access to the intestinal contents, with the animals receiving the β -lactamase therapy by the oral route and ampicillin by the parenteral route.

Materials and methods

Form of TRBL dosage

Recombinant β -lactamase containing amino acid residues 41–268 of the PenP protein of *Bacillus licheniformis* 749/C¹³ was manufactured by Ipsat Therapies Oy (Espoo, Finland).

*Corresponding author. Tel: +358-9-191-49551; Fax: +358-9-191-49670; E-mail: jaana.harmoinen@helsinki.fi

The purified β -lactamase was freeze-dried and used as the biologically active substance in manufacturing enteric-coated Eudragit L 30D-55 pellets (methacrylic acid/ethyl acrylate copolymer, total diameter 0.9 mm), which dissolve above pH 5.5.

Determination of active β -lactamase content in enteric-coated pellets

β -Lactamase was extracted from the pellets with 20 mM sodium citrate buffer (pH 6.5), shaking the mixture occasionally at room temperature for 60 min. Insoluble material was removed by centrifugation (8000g, 10 min at room temperature). For quality control the β -lactamase activity of the supernatant was determined spectrophotometrically by using nitrocefin (Oxoid) as the substrate.¹⁴

Experimental protocol

The laboratory beagles used in the study were obtained from the National Laboratory Animal Centre (University of Kuopio, Finland) and the Faculty of Veterinary Medicine (University of Helsinki, Finland). The dogs ranged from 1 to 6 years of age and their weight from 9 to 16.5 kg. The dogs were given 1.5 cans of commercial, canned dog food (Pedigree, Fortivil 400 g; Waltham, Masterfoods Oy, Helsinki, Finland) twice a day.

The experimental protocol had been approved by the local ethics committee for animal experiment action in Helsinki, Finland, and was conducted in accordance with valid guidelines.¹⁵

An intussuscepted nipple valve fistula was operated into the jejunum of each dog.^{11,12} To study the dose–response effect of TRBL on the jejunum, we used intravenous (iv) Na-ampicillin 40 mg/kg (A-PEN inject 1 g; Orion, Espoo, Finland) and peroral TRBL. The TRBL was administered at the following doses: 0 mg/kg (placebo, $n = 6$), 0.003 mg/kg ($n = 5$), 0.03 mg/kg ($n = 6$) and 0.3 mg/kg ($n = 5$). Each dog was given a standard dose of ampicillin (40 mg/kg) and the dogs received a single TRBL/ampicillin combination.

The β -lactamase pellets were packed into gelatin capsules (Capsugel, size 0; Warner-Lambert, Zaventem, Belgium) according to the treatment dose for each dog. Empty capsules were used as the peroral placebo treatment. The TRBL/placebo capsules were administered perorally 3 min before the iv ampicillin, which was injected 30 min after feeding.

Serum samples were collected at 15, 30, 45, 60 and 120 min after iv ampicillin administration, and jejunal samples every 15 min during the first 2 h, then every 30 min during the next 3 h. After collection, the blood samples were centrifuged (room temperature, 1000g, 15 min). The samples were then kept at -80°C to await analysis.

Jejunal sample pH was measured immediately after collection (Sentron pH-meter; Sentron Europe B.V., Roden, The

Netherlands). Thereafter, the samples were stored at -80°C until analysed. For analysis, the jejunal samples were centrifuged (4°C , 1800g, 15 min) and filtered through 0.22 μm filters (Millex-GP; Millipore, Bedford, MA, USA). Faecal samples were prepared for analysis by mincing and mixing 0.5–1.5 g of stool with a five-fold amount of NaCl (Braun 9 mg/mL, 0.9%, 100 mL; Braun Medical Oy, Espoo, Finland), and then centrifuged (4°C , 1800g, 20 min). After centrifugation, the faecal samples were handled in the same manner as the intestinal samples.

Traces of ampicillin were measured from all of the samples by HPLC. The detection limit for serum samples was 1 mg/L and the detection limit for both jejunal and faecal samples was 0.5 mg/L (United Laboratories, Helsinki, Finland). The method used for ampicillin determination is a modification of a method described previously.¹⁶ β -Lactamase activity was determined in duplicate by a microtitre plate assay (Microtitre plate, 96-well/flat bottom; Bibby Sterilin, Staffordshire, UK)^{14,17} using nitrocefin (Oxoid) as the substrate (detection limit 10 ng/mL; quantification limit 30 ng/mL). The spectrophotometer measured the linear activity every 10 s at 10 time points, 20 s after the nitrocefin substrate had been added to the microtitre plate by a pipetting machine (Labsystems Multiskan RC reader, Finland).

Statistical analysis

The mean concentrations \pm S.E.M. of ampicillin and β -lactamase were computed for the samples in each treatment group at each time point, and the areas under the concentration–time curves (AUCs) were calculated. ANOVA for the results was carried out using the program S-plus 2000 Mixed Effects Linear Models.

Results

Serum ampicillin was detectable in all the dogs during the experiment period, and maximal concentrations were reached 15 min after iv dosing. There was no significant difference in serum ampicillin concentrations between the different treatment groups ($P = 0.32$; Figure 1). The mean AUCs of serum ampicillin from the different treatment groups are presented in Table 1.

A dose–response effect of TRBL on ampicillin concentrations in the jejunal samples was observed (Figure 2). The highest concentration of secreted ampicillin was detected in the placebo group 15 min after antibiotic administration. The lowest dose of TRBL (0.003 mg/kg) used reduced the jejunal ampicillin concentration below that of placebo. However, concentrations remained detectable for the first 2 h of sampling. With a 0.03 mg/kg dose of TRBL, the ampicillin concentrations in the jejunal samples were close to the detection limit of the assay (0.5 mg/L) and several concentrations

Degradation of ampicillin in the gut

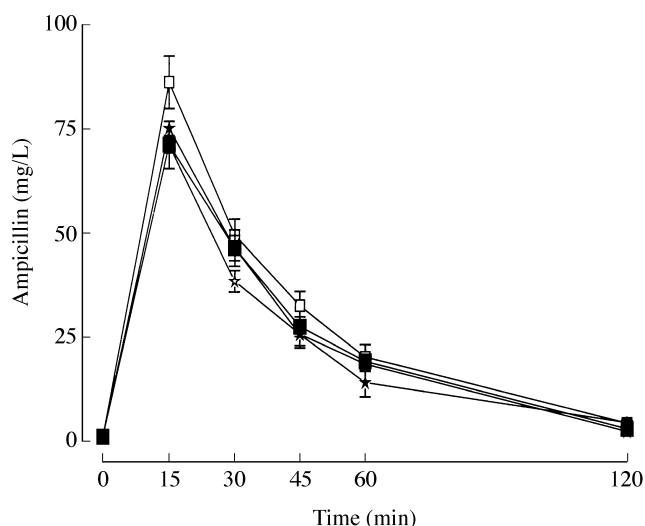


Figure 1. Effect of orally administered β -lactamase pellets on the serum ampicillin level in beagle dogs. Different doses of encapsulated enteric-coated β -lactamase pellets were given orally 3 min before iv-administered ampicillin (40 mg/kg). The values for each test group [white stars, ampicillin 40 mg/kg iv + placebo per os ($n = 6$); white squares, ampicillin 40 mg/kg iv + TRBL 0.003 mg/kg per os ($n = 5$); black squares, ampicillin 40 mg/kg iv + TRBL 0.03 mg/kg per os ($n = 6$); black stars, ampicillin 40 mg/kg iv + TRBL 0.3 mg/kg per os ($n = 5$)] are presented as mean serum ampicillin concentrations \pm S.E.M. at different time points.

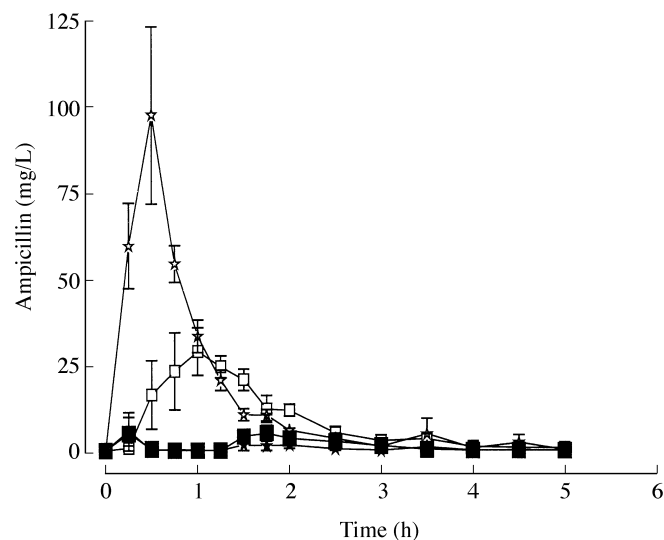


Figure 2. Effect of orally administered β -lactamase pellets on the concentrations of ampicillin in the jejunum of beagle dogs after iv administration of ampicillin (40 mg/kg). The values for each test group [white stars, ampicillin 40 mg/kg iv + placebo per os ($n = 6$); white squares, ampicillin 40 mg/kg iv + TRBL 0.003 mg/kg per os ($n = 5$); black squares, ampicillin 40 mg/kg iv + TRBL 0.03 mg/kg per os ($n = 6$); black stars, ampicillin 40 mg/kg iv + TRBL 0.3 mg/kg per os ($n = 5$)] are presented as mean jejunal ampicillin concentrations \pm S.E.M. at different time points.

slightly above this were observed during the study period (Figure 2). With a TRBL dose of 0.3 mg/kg, the jejunal ampicillin concentration was above the detection limit at the first sampling point (15 min), after which the concentrations dropped and remained below the detection level throughout the 5 h study period (Figure 2 and Table 1). There was a significant relationship between the concentration of ampicillin in the jejunal fluid and the β -lactamase dose ($P = 0.001$).

The β -lactamase activities determined in the jejunal samples of the different groups were directly related to dose given (Figure 3 and Table 1), with most of the activity seen within the first hour after administration.

No ampicillin or β -lactamase was detectable in the faecal samples, either in the time zero samples or in the samples taken the following morning.

Discussion

We introduce a novel peroral treatment modality, TRBL, for inactivating a parenterally administered and secreted but unabsorbed β -lactam antibiotic, ampicillin, in the canine jejunum.

Antibiotic treatment frequently alters the normal intestinal flora and may result in diarrhoea and overgrowth of poten-

Table 1. Mean area under the concentration–time curve (AUC) for serum and jejunal ampicillin and β -lactamase (TRBL) concentrations in each test group

<i>n</i>	Oral treatment, TRBL (mg/kg)	Intravenous treatment, ampicillin (mg/kg)	Mean serum ampicillin AUC (mg/L)·h	Mean jejunal ampicillin AUC (mg/L)·h	Mean jejunal TRBL AUC (mg/L)·min
6	0	40	2789	4779	0
5	0.003	40	3414	2548	2
6	0.03	40	2988	415	31
5	0.3	40	2842	213	105

n, number of animals in each test group. The AUCs were calculated by the trapezoidal method.

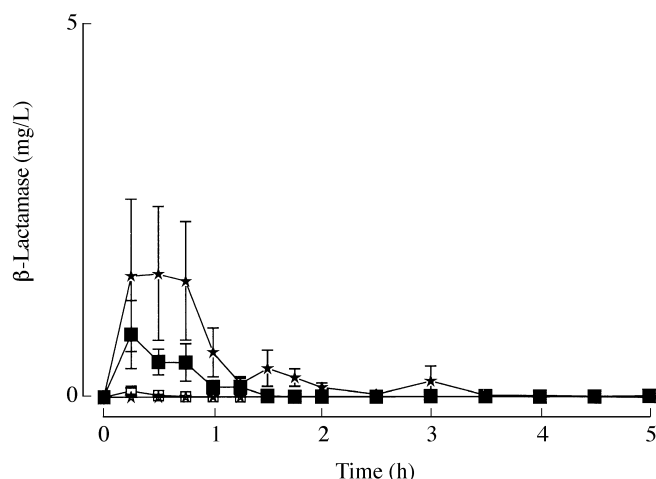


Figure 3. β-Lactamase activity in the jejunum of beagle dogs after different oral doses. The values for each test group [white stars, ampicillin 40 mg/kg iv + placebo per os ($n=6$); white squares, ampicillin 40 mg/kg iv + TRBL 0.003 mg/kg per os ($n=5$); black squares, ampicillin 40 mg/kg iv + TRBL 0.03 mg/kg per os ($n=6$); black stars, ampicillin 40 mg/kg iv + TRBL 0.3 mg/kg per os ($n=5$)] are presented as the mean jejunal β-lactamase activity ± S.E.M. at different time points.

tially pathogenic microorganisms.¹⁸ This effect largely occurs in the large intestine and colon, and if antibiotics can be prevented from reaching the luminal contents of these organs, many of the adverse effects may be prevented.

In this study, we used a gastroresistant formula of TRBL, which has been shown to release *in vitro* >80% of its total β-lactamase activity within 15 min at pH values >5.5 (data not shown). For an orally administered agent such as this, certain characteristics, such as a high capacity to degrade the desired β-lactam antibiotic and resistance to the action of proteolytic enzymes (so that it can remain active in the gut for a long time), are required. Our results show that recombinant β-lactamase of *B. licheniformis* 749/C resists the harsh intestinal conditions and is able to break down ampicillin *in vivo*, and therefore it would appear to be an excellent candidate for use in designing a drug substance to be applied in the inactivation of unabsorbed ampicillin in the gut.

There was some fluctuation in the jejunal pH values during the sampling period, even within an individual dog (data not shown). However, the pH value was within the required limits (pH 5.5–6.5) in all dogs at most of the time points for the dissolution of the TRBL-containing pellets used in the study. Furthermore, the diameter of the pellets was optimal for expeditious passage through the canine pylorus, as it is known that <1.2 mm granules can pass rapidly from the stomach into the gut.^{19–21} Because ampicillin is rapidly distributed after iv medication and its half-life is quite short ($t_{1/2}$ in human serum is 1.0–1.2 h), we timed the β-lactamase dosing 3 min before iv ampicillin injection to ensure that the enzyme would be available in the gut when ampicillin entered it. The highest concentrations of both ampicillin and β-lactamase were detected in

the jejunal samples 15 min to 1 h after dosing, suggesting that we had been successful in this respect, and that these two different medication forms, parenteral and oral, can be combined.

The results also very clearly indicate that ampicillin can be detected in jejunal samples by HPLC and that the ampicillin concentration in the jejunal samples depends on the β-lactamase dose used. With a β-lactamase dose of ≥ 0.03 mg/kg, we were able to lower the jejunal ampicillin concentration to an almost undetectable level, and thus prevent it from reaching the colon. To our knowledge, there are no published articles in which ampicillin has been quantified in faecal samples by HPLC, and in this study we were unable to measure any ampicillin in the faecal samples, even in the group treated with iv ampicillin and oral placebo.

It is likely that bacteria with β-lactamase activity, present in the gut, may have degraded the ampicillin, and had an agent with greater β-lactamase stability been used it is possible that we may have been able to detect it in the faecal samples. The absence of any β-lactamase activity in the faecal samples would suggest that the β-lactamase is broken down in the gut, in line with unpublished data we have obtained from studies with rats (data not shown).

Enzymic and non-enzymic inactivation of β-lactam antibiotics occurs naturally inside the intestinal tract,^{22–24} and it is not to be expected that any biological enzyme in its active form would penetrate the gastrointestinal tract and reach the parenteral system. Unfortunately, the nitrocefin assay as such does not enable the determination of β-lactamase activity from serum samples,¹⁴ so we were unable to look directly for β-lactamase activity. Nevertheless, our results give indirect evidence of the non-absorption of TRBL from the gastrointestinal tract into the systemic circulation, as even the highest β-lactamase dose used did not change the AUC of serum ampicillin. This is an important result considering the aim of parenteral antibiotic therapy, since it means that our treatment does not affect the antibiotic concentration in the systemic circulation and that the antibiotic is effective systemically.

In conclusion, the findings of this dose–response study suggest that this novel treatment modality could be developed into a method for degrading parenterally injected β-lactam antibiotics in the gut. This, for its part, offers the opportunity to prevent antibiotic-induced adverse effects on the gut. However, as we have only been able to demonstrate a reduction in the concentration of ampicillin in the gut lumen, further studies will be required to establish whether this actually prevents changes in the gut microflora.

Acknowledgements

We would like to thank Rafael Frias, DVM, for his assistance in preparing the samples for analysis, as well as laboratory technicians Seppo Lasanen, Pirkko Nokkala-Wahrman and

Degradation of ampicillin in the gut

Martti Siimekselä for their professional help in handling and taking care of the animals.

References

1. Batt, R. M. (1996). Enteric bacteria: friend or foe? *European Journal of Comparative Gastroenterology* **1**, 19–24.
2. Nord, C. E. & Edlund, C. (1990). Impact of antimicrobial agents on human intestinal microflora. *Journal of Chemotherapy* **4**, 218–37.
3. Nord, C. E. (1993). The effect of antimicrobial agents on the ecology of the human intestinal microflora. *Veterinary Microbiology* **3**, 193–7.
4. van den Bogaard, A. E. & Stobberingh, E. E. (1999). Antibiotic usage in animals: impact on bacterial resistance and public health. *Drugs* **58**, 589–607.
5. Edlund, C., Stark, C. & Nord, C. E. (1994). The relationship between an increase in β -lactam activity after oral administration of three new cephalosporins and protection against intestinal ecological disturbances. *Journal of Antimicrobial Chemotherapy* **34**, 127–38.
6. Nakaya, R., Chida, T. & Sibaoka, H. (1981). Antimicrobial agents and intestinal microflora. *Bifidobacteria Microflora* **1**, 25–37.
7. Acred, P., Brown, D. M., Turner, D. H. & Wilson, M. J. (1962). Pharmacology and chemotherapy of ampicillin—a new broad-spectrum penicillin. *British Journal of Pharmacology* **18**, 356–69.
8. Sullivan, Å., Edlund, C. & Nord, C. E. (2001). Effect of antimicrobial agents on the ecological balance of human microflora. *Lancet Infectious Diseases* **1**, 101–14.
9. Amtsberg, G., Stock, V., Treschnak, E. & Ringel, U. (1989). Composition of intestinal microorganisms in the dog in relation to diet and decontamination of the intestinal tract with various antibacterial substances. *Advances in Animal Physiology and Animal Nutrition* **19**, 120–30.
10. Gorbach, S. L. (1993). Perturbation of intestinal microflora. *Veterinary and Human Toxicology* **35**, 15–23.
11. Harmoinen, J., Mättö, J., Rinkinen, M., Wilson-Rahmberg, M. & Westermarck, E. (2001). Permanent jejunum fistula: promising method for obtaining small intestinal chyme without disturbing intestinal function. *Comparative Medicine* **51**, 252–6.
12. Wilson-Rahmberg, M. & Jonsson, O. (1997). Method for long-term intestinal access in the dog. *Laboratory Animals* **31**, 231–40.
13. Neugebauer, K., Sprengel, R. & Schaller, H. (1981). Penicillinase from *Bacillus licheniformis*: nucleotide sequence of the gene and implications for the biosynthesis of a secretory protein in a Gram-positive bacterium. *Nucleic Acids Research* **9**, 2577–88.
14. O'Callaghan, C. H., Morris, A., Kirby, S. & Shingler, A. H. (1972). Novel method for detection of β -lactamase by using a chromogenic cephalosporin substrate. *Antimicrobial Agents and Chemotherapy* **1**, 282–8.
15. Legislation for the use of animals in scientific procedures: Animals (Scientific Procedures) Act 1986. http://www.homeoffice.gov.uk/new_indexes/index_anima.htm (25 June 2002, date last accessed).
16. Vree, T. B., Hekster, Y. A., Baars, A. M. & van der Kleijn, E. (1978). Rapid determination of amoxycillin and ampicillin in body fluids of many by means of high-performance liquid chromatography. *Journal of Chromatography* **145**, 496–501.
17. Simons, K., Sarvas, M., Garoff, H. & Helenius, A. (1975). Membrane-bound and secreted forms of penicillinase from *Bacillus licheniformis*. *Journal of Molecular Biology* **126**, 673–90.
18. Nord, C. E., Kager, L. & Heimdahl, A. (1984). Impact of antimicrobial agents on the gastrointestinal microflora and the risk of infections. *American Journal of Medicine* **76**, 99–106.
19. Davis, S. S., Hardy, J. G. & Hara, J. W. (1986). Transit of pharmaceutical dosage through the small intestine. *Gut* **27**, 886–92.
20. Guilford, W. G. & Strombeck, D. R. (1990). Gastric structure and function. In *Small Animal Gastroenterology*, 2nd edn (Strombeck, D. R. & Guilford, W. G., Eds), pp. 167–86. W. B. Saunders Company, Philadelphia, PA, USA.
21. Aoyagi, N., Ogata, H., Uchiyama, M., Yasuda, Y. & Tanioka, Y. (1992). Gastric emptying of tablets and granules in humans, dogs, pigs, and stomach-emptying-controlled rabbits. *Journal of Pharmacological Science* **81**, 1170–4.
22. van der Waaij, D. & Nord, C. E. (2000). Development and persistence of multi-resistance to antibiotics in bacteria; an analysis and a new approach to this urgent problem. *International Journal of Antimicrobial Agents* **16**, 191–7.
23. Hazenberg, M. P., Pennock-Schröder, A. M. & van der Merwe, J. P. (1984). Binding to and antibacterial effect of ampicillin, neomycin and polymyxin B on human faeces. *Journal of Hygiene Cambridge* **93**, 27–34.
24. Welling, G. W., Holtrop, A., Sloomaker-van der Meulen, C., Meijer-Severs, C. J., van Santen, E., Tonk, R. H. *et al.* (1992). Inactivation of ceftriaxone by fecal enzyme preparations during ceftriaxone treatment. *Journal of Antimicrobial Chemotherapy* **30**, 234–5.

