

Dysregulation of bacterial proteolytic machinery by a new class of antibiotics

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Here we show that a new class of antibiotics—acyldepsipeptides—has antibacterial activity against Gram-positive bacteria *in vitro* and in several rodent models of bacterial infection. The acyldepsipeptides are active against isolates that are resistant to antibiotics in clinical application, implying a new target, which we identify as ClpP, the core unit of a major bacterial protease complex. ClpP is usually tightly regulated and strictly requires a member of the family of Clp-ATPases and often further accessory proteins for proteolytic activation. Binding of acyldepsipeptides to ClpP eliminates these safeguards. The acyldepsipeptide-activated ClpP core is capable of proteolytic degradation in the absence of the regulatory Clp-ATPases. Such uncontrolled proteolysis leads to inhibition of bacterial cell division and eventually cell death.

Worldwide spread of antibiotic resistance greatly impairs the treatment of life-threatening infections and antibacterial agents with new mechanisms of action are urgently needed^{1,2}. Among Gram-positive bacteria, multidrug-resistant isolates of *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus* cause special concern because of their rising prevalence in hospitals and community settings^{2–5}.

A group of eight closely related acyldepsipeptides (ADEPs) was previously isolated from the fermentation broth of *Streptococcus hawaiiensis* NRRL 15010 and briefly described as the 'A54556 complex' in a patent⁶. The report suggested notable *in vitro* activity against staphylococci and streptococci but provided no information about *in vivo* potency or mechanism of action. To assess the antibacterial activity of this class of compounds and to exploit their full potential, we determined the correct structure of 'factor A', the main component of the A54556 complex, which we designate here ADEP 1, established a route for its *de novo* synthesis and synthesized largely improved congeners in a derivatization program. Here, we describe the antibacterial activity of these optimized ADEPs as well as the mechanism of action of this new class of antibiotics.

RESULTS

ADEPs are potent against multidrug-resistant bacteria

Our improved congeners, exemplified here by ADEP 2 and ADEP 4 (Fig. 1), showed potent antibacterial activity against a broad range of Gram-positive bacteria, including multidrug-resistant clinical isolates (minimal inhibitory concentrations (MIC), 0.01–0.05 µg/ml) and largely surpassed the activity of the natural product ADEP 1

(Table 1). Gram-negative bacteria were only affected when efflux pumps were deleted or permeabilizing agents were added to the culture broth, indicating that penetration across the outer membrane is hampered, as expected for molecules of that size.

In vivo pharmacology, pharmacokinetics and toxicology

ADEP 2 and ADEP 4 proved active in the treatment of bacterial infections in rodents and surpassed in all models the activity of the marketed competitor linezolid. When we challenged mice with a lethal systemic infection of *E. faecalis*, 1 mg/kg ADEP 2 or 0.5 mg/kg ADEP 4 were sufficient for 100% survival (Fig. 2a). In lethal sepsis caused by *S. aureus*, 12.5 mg/kg ADEP 4 rescued 80% of the mice (Fig. 2b) and reduced the bacterial loads in liver, spleen and lung by 2–3 log units compared to an untreated control (Fig. 2c). Moreover, in a *S. pneumoniae* bacteremia in rats, ADEP 4 was again superior to linezolid (Fig. 2d), thus showing promising efficacy in two rodent species and against three major problem pathogens among Gram-positive bacteria.

Exploratory pharmacokinetic studies in mice and dogs showed moderate-to-high clearances, a moderate-to-high volume of distribution and half-lives of 1–2 h (Supplementary Note online). In addition, initial toxicological studies yielded promising results (Supplementary Note and Supplementary Table 1 online).

ADEPs act through a novel antibacterial mechanism

Here we focus on our discovery of the molecular mechanism by which ADEPs cause bacterial death. The best-characterized Gram-positive

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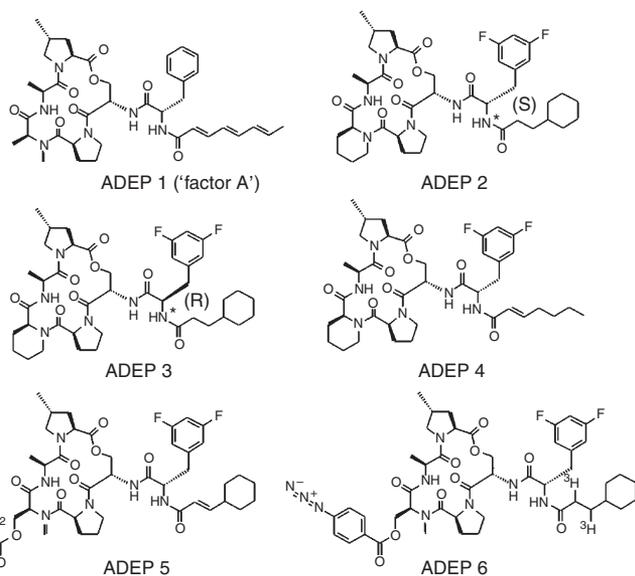


Figure 1 Structures of ADEPs. Natural product ADEP 1 ('factor A') compared to its optimized congeners ADEP 2 and ADEP 4. The *R*-epimer ADEP 3, which differs from ADEP 2 (*S* configuration) only by the conformation of the difluorophenylalanine side chain (stereocenter indicated by an asterisk), is antibacterially inactive and was included as a negative control in the mode-of-action studies described here. ADEP 5 and ADEP 6 are further tools for mode-of-action studies. The NH_2 functionality introduced in the southwestern region of ADEP 5 allows coupling to NHS-activated Sepharose and ADEP 6 carries a tritium label and an arylazide moiety for crosslinking studies.

species, *Bacillus subtilis*, served as a model bacterium for our studies. Treatment of *B. subtilis* with 1.6 $\mu\text{g/ml}$ of ADEP 1 (eightfold the MIC) reduced the number of viable cells by 2 log units over a period of 6 h. In incorporation assays with radiolabeled precursors, the biosynthesis of DNA, RNA, protein, cell wall and fatty acid proceeded unhindered for 1 h at 2 $\mu\text{g/ml}$ ADEP 1, whereas classical antibiotics were clearly distinguished by preferential inhibition of their target pathway in this type of assay. Microscopic examination showed that immediately after addition of ADEP 1 at concentrations as low as 0.4 $\mu\text{g/ml}$, *B. subtilis* started to form filaments that reached a length of 200 μm after 5 h (Fig. 3a). These results indicate a mechanism of action that does not fall into one of the classical target areas, but involves direct or indirect inhibition of cell division.

The target of ADEPs is ClpP

To search for the molecular target we constructed a genomic library from an ADEP-resistant mutant. For ease of genetic manipulation, we performed this experiment in an *Escherichia coli* efflux pump mutant (*E. coli* HN818, ΔacrA)⁷, which was susceptible to ADEP 1 in the presence of the outer membrane permeabilizer polymyxin B nonapeptide⁸ and showed the same filamentation phenotype as *B. subtilis*. Selection on ADEP-containing agar plates yielded a colony

Figure 2 *In vivo* efficacy of optimized ADEPs. Treatment of lethal systemic infections in mice caused by *E. faecalis* (a) or *S. aureus* (b) with a single dose of antibiotic (amounts indicated). Untreated control animals died within 24 h after infection. Survival is depicted 5 d after infection. (c) Effect of ADEP 4 therapy on the viable bacterial organ load during an *S. aureus* sepsis in mice. (d) Lethal *S. pneumoniae* sepsis in the rat. * $P < 0.05$, ** $P < 0.01$. i.p., intraperitoneal application; i.v., intravenous application.

Table 1 Antibacterial activity of selected ADEPs

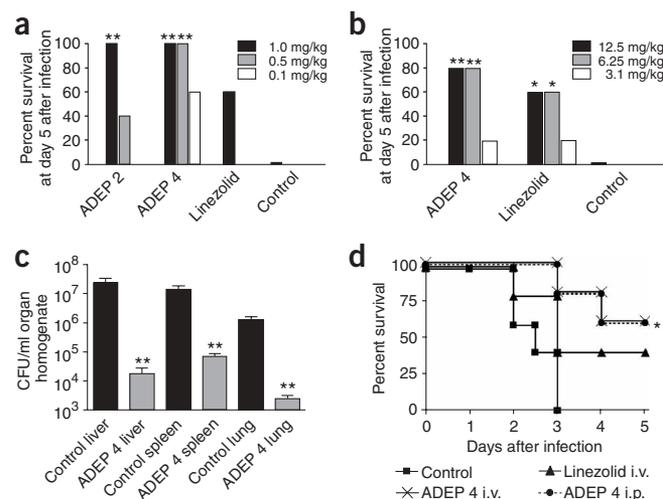
Strain	Resistance phenotype	IC50 ($\mu\text{g/ml}$)			
		ADEP 1	ADEP 2	ADEP 3	ADEP 4
<i>Bacillus subtilis</i> 168		0.2	0.05	>100	0.01
<i>Streptococcus pneumoniae</i> 665	PRSP	1.6	0.05	>100	0.02
<i>Streptococcus pyogenes</i> Wacker		0.4	0.01	>100	0.02
<i>Enterococcus faecalis</i> ICB 27159		0.4	≤ 0.01	>100	≤ 0.01
<i>Enterococcus faecium</i> L 4001	VRE	0.4	0.02	>100	≤ 0.01
<i>Staphylococcus aureus</i> NRS 119	MRSA	6.3	0.4	>100	0.05

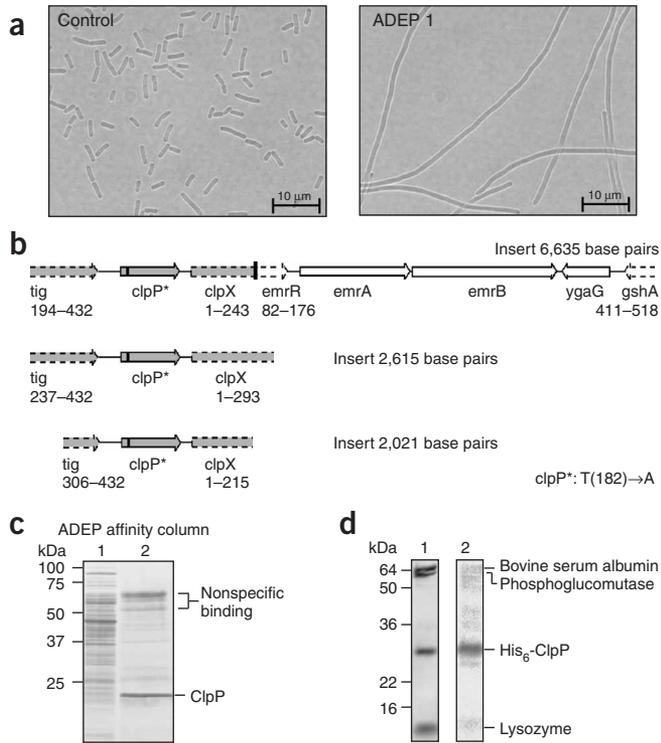
MIC values for representative multidrug-resistant clinical isolates as a measure of antibacterial *in vitro* potency. PRSP, penicillin-resistant *S. pneumoniae*; VRE, vancomycin-resistant *E. faecium*; MRSA, methicillin-resistant *S. aureus*.

with stable resistance to ADEPs (MIC $\geq 100 \mu\text{g/ml}$ versus 3 $\mu\text{g/ml}$ for the susceptible wild-type). Transformation of the susceptible HN818 wild-type strain with a genomic library of the resistant isolate (on a plasmid vector) generated daughter clones with plasmid-encoded high-level resistance to ADEPs. Sequencing of the respective plasmids identified ClpP, the catalytic core unit of a major bacterial protease (caseinolytic protease)^{9,10}, as the resistance determinant (Fig. 3b). All daughter clones carried a point mutation leading to an amino-acid exchange (Thr182Ala) close to the active center. Sequencing of the gene encoding ClpP of the resistant parent clone confirmed the mutation, suggesting that the activity of ClpP is impaired and that intact ClpP is indispensable for ADEP-mediated bacterial death.

To confirm the requirement of functional ClpP for ADEP activity in a Gram-positive background, we next investigated a ClpP-deletion mutant of *B. subtilis*. Strain *B. subtilis* trpC2 QB4916 ($\Delta\text{clpP}::\text{spectinomycin}^R$)¹¹ was highly resistant to ADEP 1 (MIC $\geq 100 \mu\text{g/ml}$ versus 0.2 $\mu\text{g/ml}$ for the isogenic wild-type). Similarly, a ClpP-deletion strain of *S. aureus* was ADEP resistant (MIC > 50 $\mu\text{g/ml}$ versus 0.6 $\mu\text{g/ml}$ for the wild-type) and analysis of resistant colonies of *E. faecalis* and *S. pneumoniae*, which occurred on ADEP-containing agar plates with frequencies in the range of 10^{-6} , showed point mutations in ClpP. These results indicate that the presence of functional ClpP is a requirement for ADEP activity in all species tested and that its absence or inactivity is sufficient for ADEP resistance.

ClpP forms the proteolytic core of major protein degradation machinery in eubacteria and is highly conserved and broadly





distributed¹². In previous studies with *E. coli*, *B. subtilis* and other species, it was shown that the ClpP core by itself is not capable of protein degradation¹³⁻¹⁵. Proteolysis can only take place when ATPases belonging to the Clp protease complex carry substrates to ClpP, unfold them in an ATP-dependent process and feed them into the small entry pores of the proteolytic chamber^{10,13}. But our experiments show that knockout mutants in all three Clp ATPases of *B. subtilis* are as susceptible to ADEP 1 as the wild-type (Supplementary Note and Supplementary Table 2 online). Thus, ClpP is the only member of the Clp family that is required for ADEP-mediated bacterial death and no Clp ATPase is necessary for this process.

ADEPs bind to ClpP

Next we performed two experiments to determine whether ADEPs and ClpP interact directly with each other. When we passed a cell lysate of *B. subtilis* through an ADEP 5 affinity column (Fig. 1 and Supplementary Methods online) only one protein remained specifically bound, which we identified as ClpP by N-terminal sequencing (Fig. 3c). In crosslinking studies, we incubated ³H-ADEP 6 (Fig. 1) with a mixture of purified ClpP and several randomly selected proteins and crosslinked by irradiation (Supplementary Methods online). Separation of the proteins by SDS-PAGE showed that ClpP was preferentially labeled (Fig. 3d). Both experiments indicate a direct

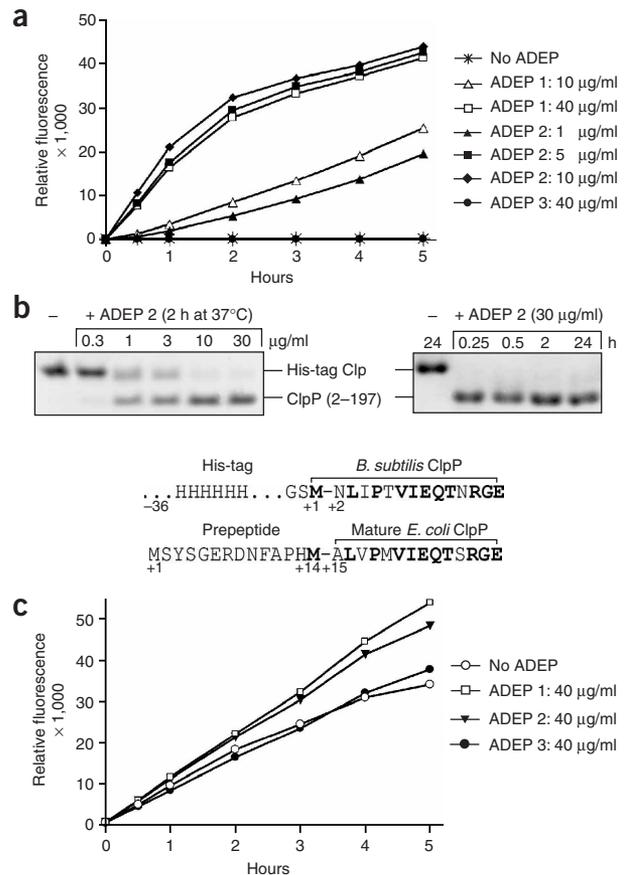
Figure 4 Effect of ADEPs on peptidase activity and autoproteolytic activity of ClpP. (a) Hydrolysis of the fluorogenic peptide *N*-succinyl-Leu-Tyr-amidomethylcoumarin by His-tagged ClpP of *B. subtilis*. (b) ADEP-induced autoproteolysis of *B. subtilis* His-tagged ClpP. Concentration series (left SDS gel) and time course (right SDS gel). Alignment of the N termini of *B. subtilis* His-tagged ClpP and the precursor of *E. coli* ClpP, the latter of which contains in its native form a 14 amino-acid leader peptide, which is autoproteolytically cleaved off during protein maturation. Corresponding processing sites are indicated. (c) Peptidase activity of native *B. subtilis* ClpP.

Figure 3 The target of ADEPs is ClpP. (a) Filamentation of *B. subtilis* 5 h after addition of ADEP 1. Scale bars, 10 μ m. (b) A genomic library on a plasmid vector was constructed from an ADEP-resistant *E. coli* mutant. Sequencing of the resistance-mediating plasmids showed the depicted inserts. Solid lines represent full genes, dashed lines interrupted genes, numbers below gene names represent amino-acid regions. The bar in ClpP marks the location of the mutation. In the uppermost clone, two fragments from independent chromosomal regions had fused prior to ligation into the vector (first region, tig to clpX; second region, emrR to gshA). (c) Adsorption of a *B. subtilis* cell lysate to an ADEP-affinity column (Coomassie-stained blot). Lane 1, crude cell lysate; lane 2, eluate after extensive washing. Nonspecific binding in the range of 55–70 kDa was identified in a control experiment with blank NHS-Sepharose (Supplementary Fig. 1 online). (d) Crosslinking of ³H-ADEP 6 to a ClpP-containing protein mixture. Lane 1, blot stained with Coomassie; lane 2, autoradiograph.

interaction between ADEPs and ClpP and identify, along with the mutation studies, ClpP as the target. We next asked how ADEPs cause bacterial death by binding to ClpP, the protease core, which by itself is devoid of proteolytic activity.

Effect of ADEPs on the peptidase activity of ClpP

In the absence of a Clp ATPase, purified *E. coli* ClpP was found to hydrolyze only short peptides of up to six amino acids, which are presumably small enough to squeeze through the entry pores^{13,14}. Thus, ClpP, as the catalytic core of the Clp-protease complex, can act alone as a peptidase. To investigate the effect of ADEPs on the peptidase activity of *B. subtilis* ClpP, we used the fluorogenic peptide *N*-succinyl-Leu-Tyr-amidomethylcoumarin, which was previously



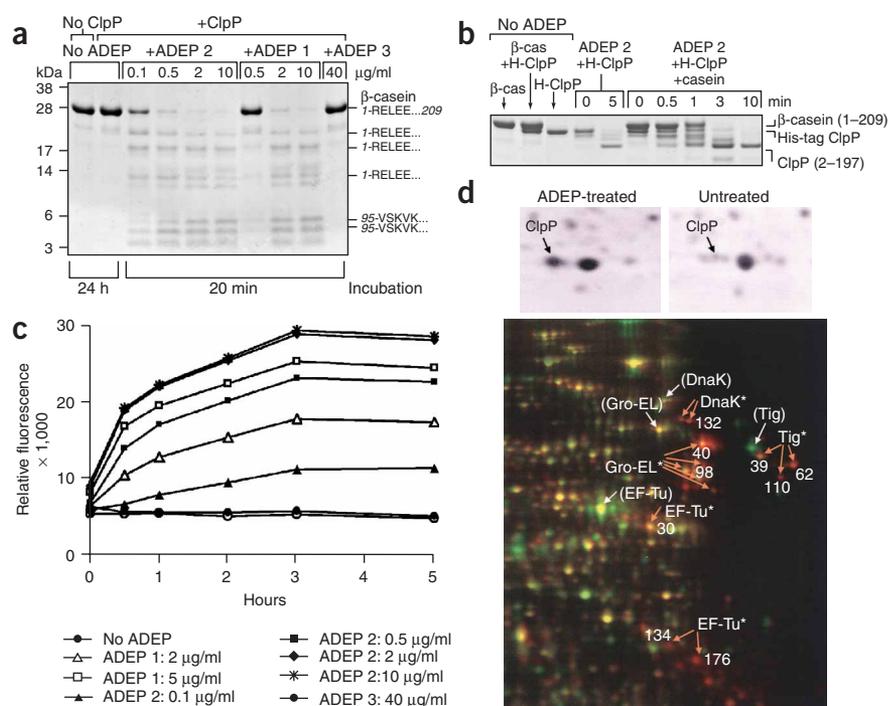


Figure 5 ADEPs activate the protease function of ClpP. **(a)** ADEP-induced degradation of β -casein by native ClpP from *B. subtilis*. **(b)** Time course of β -casein degradation by His-tagged ClpP from *B. subtilis*. β -cas, β -casein; H-ClpP, His-tagged ClpP. **(c)** Kinetics of ADEP-induced α -casein degradation by native ClpP from *B. subtilis*. **(d)** Proteome analysis of *B. subtilis* in response to treatment with ADEP 1. ClpP-containing region on a Coomassie-stained gel in ADEP-treated versus untreated cells (upper panels). Section of a two-dimensional gel bearing various fragments of the chaperones DnaK, GroEL and Tig as well as of the elongation factor EF-Tu (lower panels). Protein isoforms induced in response to antibiotic treatment appear red, repressed proteins appear green and proteins expressed at similar relative synthesis rates before and after antibiotic treatment appear yellow. White arrows mark intact proteins and orange arrows protein fragments. White labels in brackets designate the protein spots from which the fragments originate. Protein fragments accumulating to sufficient amounts were analyzed by N-terminal sequencing and the number of the N-terminal amino acids is indicated.

described as a model substrate for *E. coli* ClpP¹³. N-terminally His-tagged *B. subtilis* ClpP, which we used in a first approach for the ease of purification, was almost inactive in peptide hydrolysis; however, its peptidase activity was stimulated 300-fold by the addition of ADEP 1 and 2. The antibacterially inactive side-chain epimer ADEP 3 showed no effect (**Fig. 4a**). Closer investigation showed that *B. subtilis* ClpP cleaved off its 37 amino-acid N-terminal extension by autoproteolysis immediately after addition of ADEP 1 and 2 (**Fig. 4b**). No Clp-ATPase was present during this event. Processing was complete within a few minutes and the product (containing amino acids 2–197 of the native *B. subtilis* ClpP) was stable for more than 24 h at 37 °C. Two explanations for the observed peptidase-stimulating activity of ADEPs seemed plausible. Either ADEPs directly stimulate the ClpP peptidase activity or the histidine tag rendered ClpP inactive and its ADEP-mediated removal yielded active ClpP that was then able to hydrolyze the peptide substrate. To determine which explanation was correct, we purified *B. subtilis* ClpP in its native form and repeated the peptide hydrolysis assay (**Fig. 4c**). In contrast to the His-tagged protein, native *B. subtilis* ClpP easily hydrolyzed the fluorogenic peptide and this activity was only slightly stimulated by ADEPs, which proved the second hypothesis to be correct (**Supplementary Note** and **Supplementary Fig. 2** online).

ADEPs confer independent protease activity to ClpP

As ADEPs had only a minor effect on the peptidase activity of native ClpP, we hypothesized that the drug must bestow novel proteolytic properties to ClpP. We therefore investigated the ClpP-mediated degradation of a whole protein in the presence of ADEPs. Casein is widely used as model protein substrate for studies of the Clp protease complex. It is readily degraded by purified *E. coli* ClpP in the presence of a Clp-ATPase and ATP, but remains intact in the presence of ClpP alone¹³. In agreement with this, we found that purified native *Bacillus* ClpP was not able to degrade β -casein. But addition of ADEP 1 or ADEP 2 triggered immediate and complete casein degradation into various fragments (3–20 kDa) in the absence of

ATPase (**Fig. 5a**). The inactive congener ADEP 3 (**Fig. 1a**), showed no protease-stimulating effect, demonstrating the specificity of the interaction of ADEPs with ClpP. Analysis of the accumulated casein fragments by N-terminal sequencing showed that the degradation products carried the correct N terminus of mature β -casein (RELEE...), indicating C-terminal processing at various sites. When we incubated β -casein with His-tagged ClpP from *B. subtilis* in the presence of ADEP 2, proteolysis started with autoproteolytic activation of ClpP by removal of the interfering histidine tag followed by degradation of casein (**Fig. 5b**). Proteolysis of α -casein by native *B. subtilis* ClpP in the presence of an ADEP dilution series showed full activation of ClpP (2.5 μM) at an ADEP 2 concentration of 2.5 μM (2 $\mu\text{g/ml}$), indicating full stimulation at equimolar concentrations (**Fig. 5c**).

ADEPs trigger protein degradation in the bacterial cell

The preceding experiments indicated that ADEPs efficiently enable purified ClpP to degrade casein *in vitro* and confer proteolytic capabilities to the otherwise inactive core of the Clp-protease complex. Proteome analysis, which we performed in a two-pronged approach for target elucidation in parallel with the genetic strategy discussed above, led independently to the hypothesis that ADEPs might activate or deregulate a protease. Moreover, the holistic proteomic view of the bacterial response after ADEP exposure provides a first impression of the consequences that ClpP deregulation might have for the bacterial cell. We analyzed cytoplasmic proteins of an ADEP-treated *B. subtilis* culture by two-dimensional polyacrylamide gel electrophoresis and compared this expression profile with the proteome pattern of an untreated control. Thirty minutes after addition of ADEP 1, the protein spot representing ClpP was substantially induced (**Fig. 5d**). In addition to induction of ClpP, the proteome profile was dominated by the induction of chaperones (the Clp-ATPase ClpC, DnaK, GroEL and the trigger factor Tig) and of the peptidyl-prolyl isomerase PpiB (**Supplementary Fig. 3** and **Supplementary Note** online), which are known to be induced in *B. subtilis* under conditions

of protein damage^{16,17}. But the proteome profile of ADEP-exposed cells is unique and differs from those of 30 other antibacterial agents investigated previously¹⁶. Various novel spots appear on the ADEP gel in the vicinity of the spots representing intact GroEL, DnaK, Tig and the elongation factor EF-Tu, which we identified as their fragments by peptide mass fingerprinting and N-terminal sequencing (Fig. 5d). It seems plausible that truncated and defective protein forms appear in the cell as a result of ClpP deregulation, which then triggers the expression of the chaperones. But as ClpP might also digest these newly synthesized proteins, protein fragments rather than the native forms accumulate on the gel.

DISCUSSION

New antibiotics with novel mechanisms of action are urgently needed to keep pace with the increasing incidence of bacterial resistance. The natural product ADEP 1 and its optimized congeners represent a new class of antibacterial acyldepsipeptides, which are currently in pre-clinical research. Our experiments show their promising antibacterial potential *in vitro* and *in vivo* and indicate a new mechanism of antibiotic action. Their target is ClpP, the catalytic core of a Clp protease complex. According to the crystal structure, the ClpP core of *E. coli* is a hollow, solid-walled cylinder ~90 Å in both height and diameter, and is formed by two stacked rings of seven symmetric subunits each¹⁰. The 14 catalytic sites of the ClpP homotetradecamer are located within a central, spacious chamber, access to which is controlled by two axial pores of ~10 Å. The second component of the Clp protease complex is an ATPase of the Clp/Hsp100 family, which assembles as a homohexameric ring on one or both sides of the ClpP barrel and which is strictly required for proteolytic processing. The function of the ATPase is to carry proteins to ClpP, to unfold them in an ATP-consuming process and to thread them into the tiny entrance pores of the ClpP core. Our knockout experiments show that the effect that ADEPs exert on ClpP is independent of the function of a Clp-ATPase. ADEPs bind to ClpP and confer novel proteolytic capabilities to the isolated protease core, which in the absence of the antibiotics hydrolyzes only small peptides.

As a demonstration of these new proteolytic features, ADEP-activated His-tagged ClpP of *B. subtilis* cleaved off its N-terminal extension, 37 amino acids in length, by autoproteolysis and ADEP-activated *B. subtilis* ClpP in its native form rapidly degraded casein, which we chose as an exemplary protein substrate. These observations are notable because they suggest that in the presence of ADEPs large polypeptides can gain access to the active sites, which are confined to the interior of the degradation chamber. The extent of this ADEP-mediated proteolytic deregulation becomes obvious in the light of previous studies. For casein degradation by *E. coli* ClpP, the Clp-ATPase ClpA is strictly required, and in the case of *B. subtilis* ClpP, not only the Clp-ATPase ClpC but also the additional adapter protein MecA is necessary^{13,15}.

What are the consequences of ClpP deregulation for the bacterial cell? In *B. subtilis*, the ClpP protease complex has two major functions: the rather general degradation of defective and misfolded proteins, and the targeted proteolysis of specific protein substrates, among these transcription factors and other key regulators of developmental programs^{11,18,19}. But growth and development of ClpP-deletion mutants in *B. subtilis* and other species are far less impaired than those of ADEP-treated cells, indicating that mere inhibition of the natural functions of ClpP is not a sufficient explanation for ADEP-mediated bacterial death (Supplementary Note online). Rather, ADEPs deregulate ClpP activity by efficiently relaxing the tight control mechanisms that normally safeguard cytoplasmic proteins from

random degradation by the ClpP machinery. Future studies will show whether some proteins are better targets than others and which cascade of events leads to inhibition of bacterial cell division (Supplementary Note online). With respect to the molecular mechanism of ClpP activation, it is tempting to speculate that binding of ADEPs to ClpP triggers allosteric activation and/or a conformational change that facilitates access of the substrates to the proteolytic chamber (Supplementary Note online). ADEPs are the first naturally occurring small-molecule activators of a proteasome-like proteolytic machinery and will serve as a tool for the molecular elucidation of ClpP function. In addition, ADEPs indicate a new mechanism by which an antibiotic can trigger bacterial death. In contrast to classical antibiotics that inhibit essential cellular functions, ADEPs trigger bactericidal activity by overactivating a bacterial protein. By turning a carefully regulated peptidase into a harmful protease, ADEPs show that there are more ways to combat bacteria than we may have anticipated.

A consequence of the fact that ClpP is not strictly essential for bacterial survival is the observation that ADEP-resistant mutants can be generated in the laboratory *in vitro* with moderate frequencies (~10⁻⁶) in the range of those generated by rifampicin. The novel peptide deformylase inhibitors, which are currently in late-stage preclinical development, show similar resistance rates for staphylococci²⁰. These resistance frequencies will probably restrict the use of ADEPs to combination therapy, as it is the case for rifampicin. It is noteworthy in this respect that we did not observe a single ADEP-resistant strain among 200 recent staphylococcal isolates from US hospitals, half of which were methicillin-resistant *S. aureus* strains (data not shown). Furthermore, there is growing evidence that proteins of the Clp family have a crucial role in survival and virulence of pathogens during host infection²¹⁻²³, warranting further *in vivo* studies to investigate whether ADEP-resistant isolates have also pathogenicity deficiencies. In conclusion, because of their unprecedented target, ADEPs show no cross-resistance to any antibiotic classes that are currently on the market or in development, which makes them ideally suited as combination partner for the often life-threatening infections caused by (multidrug)-resistant bacterial isolates.

METHODS

Determination of antibacterial activity. We determined MIC values by broth microdilution according to the recommendations of the National Committee for Clinical Laboratory Standards.

Bacterial infection models. We intraperitoneally inoculated female CFW-1 mice or female Wistar rats with bacterial loads that exceeded the previously determined lethal infective doses. For the treatment of the *S. aureus* and *E. faecalis* bacteremia, we treated mice once intraperitoneally 30 min after infection. For the treatment of the *S. pneumoniae* sepsis, we treated rats twice intraperitoneally or intravenously 30 min and 24 h after infection. We used five animals per treatment group and monitored survival for 5 d. For determination of colony-forming units (CFU), we removed organs 6 h after infection, homogenized them and counted bacteria on agar plates after overnight incubation. We performed statistical analysis in Graph Pad Prism using the Mann-Whitney test (CFU determination) or the log-rank test (survival models). Animal experiments were performed according to German laws for animal welfare and were approved by the District President of Düsseldorf.

Microscopy of ADEP-treated *B. subtilis*. We treated exponentially growing *B. subtilis* 168 trpC2 (ref. 24) with 0.4 µg/ml ADEP 1 and analyzed it by microscopy on agar-coated microscope slides (2% agar).

Determination of the ADEP resistance determinant in a genomic library of *E. coli*. We spread *E. coli* HN818 on Isosensitest agar plates supplemented with

50 µg/ml of ADEP 1 and 25 µg/ml of polymyxin B nonapeptide (Sigma-Aldrich). We extracted the DNA of an ADEP-resistant colony, partially digested it, and ligated fragments of 2–4 kb into pQE70 (Qiagen). We transformed wild-type HN818 with the vector and isolated several resistant colonies from ADEP-containing agar plates. We purified their plasmids, transformed them back into wild-type HN818 to ensure that the resistance was indeed plasmid mediated, and sequenced them.

Determination of resistance rates. We spread bacterial isolates (10^8 CFU) on Mueller-Hinton agar (for *S. pneumoniae*, isolates were supplemented with 20% bovine serum) containing ADEP 1, ADEP 2 or ADEP 4 in concentrations representing tenfold the MIC. We counted resistant colonies after incubation at 37 °C for 24 h for *E. faecalis* and 48 h for *S. pneumoniae*.

Overproduction and purification of ClpP. We expressed His₆-tagged ClpP of *B. subtilis* 168 in *E. coli* BL21 (DE3)pLys (Invitrogen) and purified it using standard procedures. We cloned native ClpP from *B. subtilis* into pET11a (Qiagen) and expressed it in *E. coli* BL21 (DE3)pLys. We performed purification as previously described for *E. coli* ClpP by polyethyleneimine precipitation and anion-exchange chromatography on Q-Sepharose and Mono-Q⁹.

Peptidase activity of *B. subtilis* ClpP. We measured hydrolysis of *N*-succinyl-L-tyr-amidomethylcoumarin (2 mM) by His-tagged ClpP (16 µM) or native ClpP (2 µM) in ClpP activity buffer (50 mM Tris/HCl pH 8, 25 mM MgCl₂, 100 mM KCl, 2 mM dithiothreitol) as previously described¹⁴.

Autoproteolysis of His-tagged *B. subtilis* ClpP. We incubated His-tagged ClpP (3.9 µM) with ADEP 2 for 15 min up to 24 h at 37 °C. We stopped reactions by addition of SDS-PAGE sample buffer and heating to 100 °C.

Protease activity of ClpP. We incubated 15 µM bovine β-casein (Sigma-Aldrich) with either 5 µM His-tagged ClpP or 0.42 µM native ClpP (both from *B. subtilis*) in 100 µl ClpP activity buffer. We chose this high amount of His-tagged ClpP in order to detect the protease band on the gel and follow autoproteolysis in parallel with casein degradation. We incubated samples at 37 °C (5 min to 24 h) and analyzed them using SDS-PAGE. We N-terminally sequenced degradation products of casein after blotting. In an alternative assay format, ClpP released fluorescent peptides from fluorogenic α-casein. We degraded 15 µg/ml α-casein BODIPY FL conjugate (EnzChek protease assay kit green fluorescence, Mobitec) using 2.5 µM native *B. subtilis* ClpP from *B. subtilis* in 100 µl 20 mM Tris/HCl pH 7.8, 25 mM MgCl₂, 100 mM KCl, 1 mM dithiothreitol. After incubation at 37 °C protected from light, we determined fluorescence at 485 nm (excitation) and 535 nm (emission).

Proteome analysis. We grew *B. subtilis* 168 in Belitzky defined minimal medium in the presence of ADEP 1 (1 µg/ml) or puromycin (9 µg/ml, representing the MIC). We pulse-labeled the samples with [³⁵S]-L-methionine 10 min after addition of the antibiotic. We performed proteome analysis for cytoplasmic proteins in the pI range of 4–7. We performed cell culturing, labeling, two-dimensional PAGE, spot identification by in-gel digestion, peptide mass fingerprinting and data analysis as previously described¹⁶. For N-terminal sequencing of the newly appearing protein fragments by Edman degradation, we separated an unlabeled culture aliquot by two-dimensional PAGE, blotted and stained it with Coomassie blue.

Accession codes. BIND identifiers (<http://bind.ca>): 334403 and 334404.

URL. Clinical and Laboratory Standards Institute, <http://www.nccls.org>.

Note: Supplementary information is available at the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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