Pentobra: A Potent Antibiotic with Multiple Layers of Selective Antimicrobial Mechanisms against *Propionibacterium Acnes*

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Although antibiotics are a common treatment for acne, the difficulties inherent to effective antimicrobial penetration in sebum and selective antimicrobial action in the skin are compounded by increasing resistance of *Propionibacterium acnes* clinical isolates. To address these problems, we engineered Pentobra, a peptide–aminoglycoside molecule that has multiple mechanisms of antibacterial action and investigated whether it can be a potential candidate for the treatment of acne. Pentobra combines the potent ribosomal activity of aminoglycosides with the bacteria-selective membrane-permeabilizing abilities of antimicrobial peptides. Pentobra demonstrated potent and selective killing of *P. acnes* but not against human skin cells *in vitro*. In direct comparison, Pentobra demonstrated bactericidal activity and drastically outperformed free tobramycin (by 5–7 logs) against multiple *P. acnes* clinical strains. Moreover, electron microscopic studies showed that Pentobra had robust membrane activity, as treatment with Pentobra killed *P. acnes* cells and caused leakage of intracellular contents. Pentobra may also have potential anti-inflammatory effects as demonstrated by suppression of some *P. acnes*–induced chemokines. Importantly, the killing activity was maintained in sebaceous environments as Pentobra was bactericidal against clinical isolates in comedones extracts isolated from human donors. Our work demonstrates that equipping aminoglycosides with selective membrane activity is a viable approach for developing antibiotics against *P. acnes* that are effective in cutaneous environments.

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INTRODUCTION

The commensal bacterium *Propionbacterium acnes* is a major etiological factor in acne vulgaris (Ross *et al.*, 2003; McInturff *et al.*, 2005; Williams *et al.*, 2012). Although topical antibiotic therapies are used (Eady *et al.*, 2003), increasing resistance have made acne treatment challenging. The incidence of *P. acnes* antibiotic resistance increased from 20% in 1978 to 72.5% in 1995 (Humphrey, 2012); widespread resistance has become a major dermatological issue (Cooper, 1998; Eady *et al.*, 2003; Humphrey, 2012; Williams *et al.*, 2012). Antibiotic resistance is compounded by the difficulties inherent to effective antimicrobial penetration in the skin. Many antibiotics are charged and do not penetrate into the largely hydrophobic sebaceous environmental niche of *P. acnes*. Clearly, antibiotic therapies that are more tailored to these problems are needed.

It is difficult for an organism to simultaneously evolve resistance against multiple antimicrobial mechanisms. This observation has motivated the development of multidrug strategies (antibiotic cocktails). Although this approach has been effective for some diseases (i.e., leprosy), it has led to multidrug resistant strains in others (i.e., tuberculosis), in part due to inherent problems from the use of multiple drugs. Rather than using a complex cocktail, we aim to engineer a *P. acnes* antibiotic by combining the potent ribosomal activity of aminoglycosides with the bacteria-selective membrane-permeabilizing abilities of antimicrobial peptides (AMPs), which can perforate prokaryotic membranes but not eukaryotic membranes. Aminoglycoside antibiotics target the 16S rRNA component of the bacterial ribosome leading to mistranslation, inhibition, and cell death (Fourmy *et al.*, 1996; Vicens and Westhof, 2002). *In vitro* studies have shown that, although aminoglycosides are usually potent antimicrobials, *P. acnes* is not strongly susceptible to them (Wang *et al.*, 1977). As *P. acnes* is an anaerobic bacterium, it is hypothesized that its intrinsic resistance is a result of poor

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Abbreviations: AMP, antimicrobial peptide; CFU, colony-forming unit

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aminoglycoside uptake, not a lack of ribosomal activity (Davis, 1987; Taber *et al.*, 1987). AMPs can selectively permeabilize bacterial membranes (Zasloff, 2002; Brogden, 2005; Hancock and Sahl, 2006; Schmidt and Wong, 2013). Although many AMPs kill via lysis, many others combine membrane activity with additional mechanisms such as inhibition of metabolic functions by binding intracellular targets (Brogden, 2005). Aminoglycosides equipped with cell-penetrating abilities can have activity against slow-growing bacteria like *P. acnes,* which have little uptake. Moreover, the addition of AMP-like membrane activity will add an extra dimension of selectivity to the specific mechanisms inherent to aminoglycosides.

Here we report an aminoglycoside-based compound with bactericidal activity against *P. acnes*. Our design is informed by recent work that elucidates the roles of cationic and hydrophobic residues in AMP sequences (Schmidt *et al.*, 2011, 2012b), cell-penetrating peptide sequences (Mishra *et al.*, 2011), and non-peptidic AMP mimetic compositions (Schmidt *et al.*, 2012a; Hu *et al.*, 2013) and relates them to the geometric requirements of membrane permeabilization. Tobramycin, a potent aminoglycoside, is conjugated to a short 12AA peptide to equip the composite molecule, Pentobra (Figure 1a), with preferential activity against bacterial membranes like an AMP, so that the composite molecule has multiple levels of selectivity and multiple mechanisms of killing (Schmidt *et al.*, 2014). The work presented herein demonstrates that equipping aminoglycosides with selective membrane activity is a viable approach for developing antibiotics against *P. acnes* that are effective in cutaneous environments.

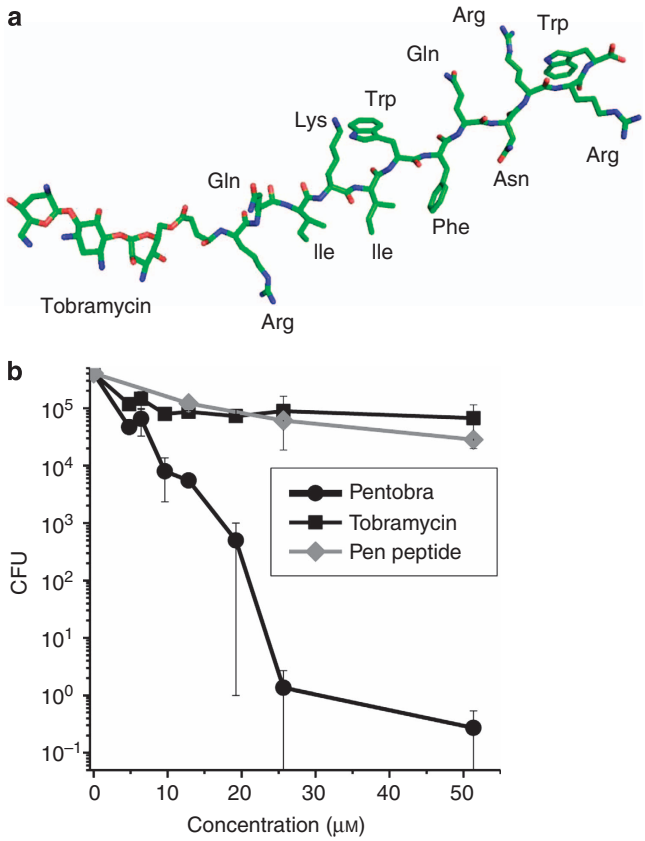

Figure 1. Pentobra is bactericidal against *Propionibacterium acnes*. (**a**) Representation of Pentobra. (**b**) *P. acnes* ATCC 6919 was incubated with different concentrations of Pentobra, tobramycin, or pen peptide (0–52 μM) for 3 hours and tested for bactericidal activity using the colony-forming unit (CFU) assay. Data show average CFU from three independent experiments ($n = 3$), error bars are ± SEM.

RESULTS

Pentobra has potent and selective antimicrobial activity against *P. acnes* but not against human skin cells

As Pentobra is designed to permeabilize membranes, we hypothesized that Pentobra should be bactericidal against *P. acnes*. We tested the killing potency of Pentobra against *P. acnes* using colony-forming unit (CFU) assays (Figure 1b). Pentobra displayed dose-dependent killing activity against *P. acnes* laboratory strain ATCC 6919. Concentrations as low as 8 μM Pentobra produced a 10-fold reduction in viable colonies, and 26 μM Pentobra led to a 5-log reduction in CFU. In contrast, tobramycin was not strongly bactericidal, as concentrations as high as 52 μM led to less than 10-fold reduction in CFU. These data show that membrane-active aminoglycosides can kill the *P. acnes* lab strain, whereas neither tobramycin nor the free pen peptides were effective. Importantly, Pentobra is not toxic to human skin cells as treatment did not affect the viability of human peripheral blood mononuclear cells, keratinoctyes, or sebocytes over 72 hours (Supplementary Figure S1A and B online).

Pentobra is active against a wide variety of *P. acnes* clinical strains

The predominant microbe found in the microcomedone content is *P. acnes*, which accounts for ~90% of the microbiota (Fitz-Gibbon *et al.*, 2013). Moreover, microcomedones from healthy vs. diseased skin harbor *P. acnes* strains from distinct lineages and possess distinct nucleopeptide signatures of 16S rDNA sequences. Although some *P. acnes* strains are found on healthy skin (phylotype III and ribotype 6), others are associated with acne disease (ribotypes 4, 5, 8, and phylotype IC) and with diseases such as medical device infections (phylotype II) (McDowell *et al.*, 2013). To examine the antimicrobial activity of Pentobra against different *P. acnes* strains (Table 1), we conducted CFU assays on clinical isolates. In general, Pentobra exhibited robust bactericidal activity against all tested *P. acnes* strains (Figure 2). Against *P. acnes* clinical isolates HL063PA2 (healthy) and HL005PA1 (healthy) (Figure 2a and b), greater than 5-log reductions in CFU were observed at 26 μM Pentobra. Although strain HL110PA4 (healthy) was less susceptible (Figure 2c), a 2-log reduction occurred at the highest concentration tested. Interestingly, this differential activity may allow Pentobra to shift slightly the ecology of *P. acnes* toward strains associated with healthy skin. Pentobra also killed *P. acnes* strains HL053PA2, HL043PA1, and HL110PA1 that are associated with acne skin (Figure 2d–f), as 13 μM Pentobra was sufficient to reduce CFU by greater than 5-log units for the first two strains and 3-log units for the third one. Similar to ATCC 6919, tobramycin did not exhibit significant antimicrobial activity against most of these clinical isolates, whereas the free pen peptide typically demonstrated moderate 2–3-log reductions

in CFU. However, tobramycin was strongly bactericidal against strain HL005PA1, suggesting that aminoglycosides may be effective against certain strains of *P. acnes*. Our results demonstrate that Pentobra has potent activity against clinically relevant strains.

Pentobra alters cell surface morphology, permeates, and lyses *P. acnes*

Pentobra is designed to have selective membrane permeation activity similar to AMPs. To determine its effects on the cell envelope, we examined *P. acnes* treated with Pentobra using electron microscopy (Figure 3). Transmission EM micrographs of untreated *P. acnes* illustrate their normal pleomorphic structure (Figure 3a). After a 3-hour treatment with Pentobra, different types of cell envelope disruptions are observed (Figure 3b). Cell surfaces appear ruffled from blebbing, and complete breaches in the envelope are observed, with externalized cytoplasmic contents characteristic of lysis. These morphological changes are consistent with those from membrane active molecules. The observed robust permeabilization of *P. acnes* membranes corresponds well with previous x-ray studies, which showed that Pentobra generates disruptive membrane curvatures in model bacterial cell membranes and permeabilizes *E. coli* inner membranes (Schmidt *et al.*, 2014). To see how the different components of Pentobra impact *P. acnes*, EM studies were performed with free pen peptide and tobramycin. The pen peptide is lytic (Figure 3c), which is consistent with its strong membrane permeabilization profiles on *E. coli* cells (Schmidt *et al.*, 2014). *P. acnes* treated with tobramycin are indistinguishable from control (Figure 3d), in line with our antibacterial assays and with the reduced uptake of aminoglycosides into anaerobic bacteria cells.

Table 1. *P. acnes* clinical isolates used in the study

Clinical isolate	*Phylotype*	*Ribotype (RT)*	*Disease association*
ATCC 6919	IA-1	RT1	Neutral/commensal[a]
HL005PA1	IA-1	RT1	Healthy[b]
HL043PA1	IA-2	RT5	Acne[c]
HL053PA2	IB-1	RT8	Acne
HL063PA1	IA-1	RT1	Healthy
HL110PA1	IB-1	RT8	Acne
HL110PA4	II	RT6	Healthy

[a]*P. acnes* laboratory strain.
[b]Clinical isolates associated with healthy skin.
[c]Clinical isolates associated with acne skin.

Pentobra maintains antimicrobial activity in human comedone extracts

Acne therapeutics must maintain activity in lipid-rich environments. To assay the effectiveness of Pentobra against

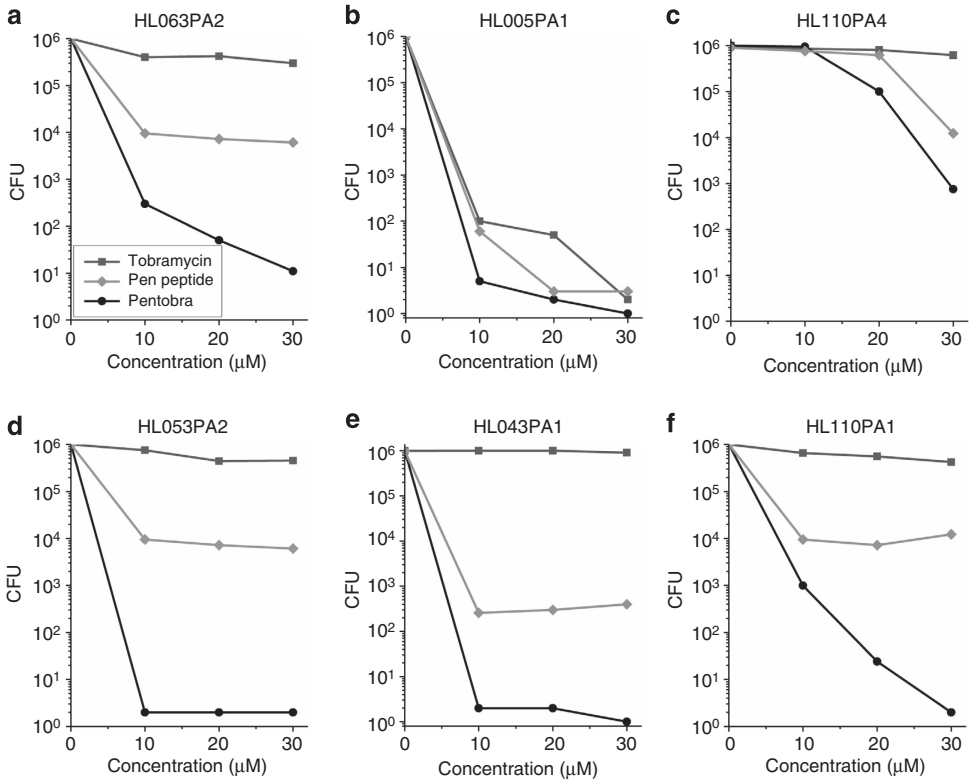

Figure 2. Pentobra is bactericidal against a broad range of *P. acnes* strains. Colony-forming unit (CFU) assay results for Pentobra, pen peptide, and tobramycin at varying concentrations (0–26 μM) incubated with *P. acnes* clinical isolates (**a**) HL063PA2 (health-associated), (**b**) HL005PA1 (health-associated), (**c**) HL110PA4 (health-associated), (**d**) HL053PA2 (acne-associated), (**e**) HL043PA1 (acne-associated), and (**f**) HL110PA1 (acne-associated) for 3 hours. Data from one experiment are shown, and the trends in antimicrobial activity of the compounds and activity differences between compounds are representative of three independent experiments.

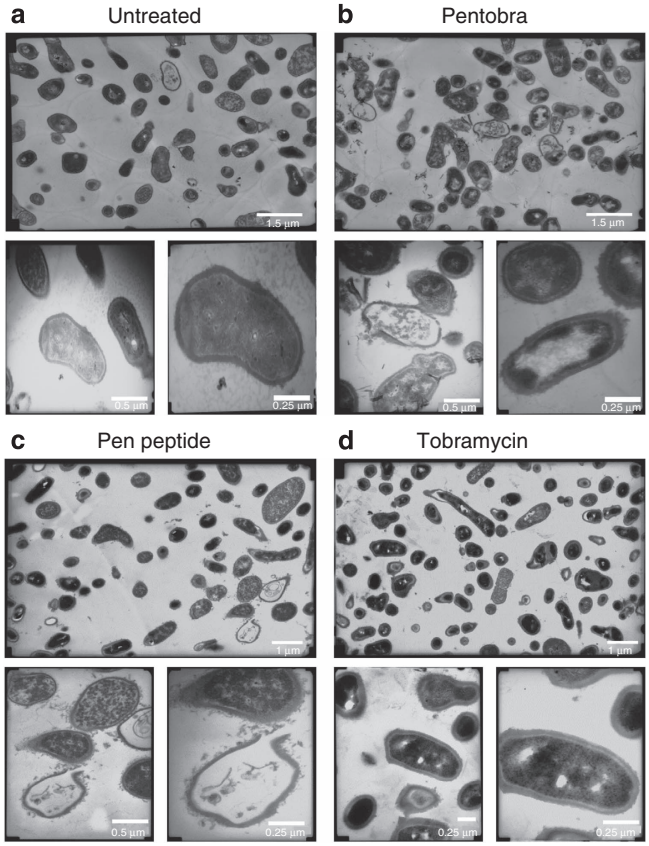

Figure 3. Electron microscopy studies show that Pentobra acts on *P. acnes* cell membranes. Representative transmission electron microscopy (TEM) micrographs of (**a**) untreated *P. acnes* control, (**b**) *P. acnes* after 3-hour incubation with 25.7 μM Pentobra, (**c**) *P. acnes* after 3-hour incubation with 25.7 μM pen peptide, and (**d**) *P. acnes* after 3-hour incubation with 25.7 μM tobramycin. TEM were imaged at 10K magnification (top), 36K (bottom left), and 72K (bottom right). Compared with the untreated control, the bacteria exposed to Pentobra and pen peptide exhibit cellular differences indicative of stresses on the membrane. Complete lysis of the cell membrane occurs, and the envelope boundary is now decorated with numerous blebbing events (bottom images in **b** and **c**). Scale bar = 5 nm.

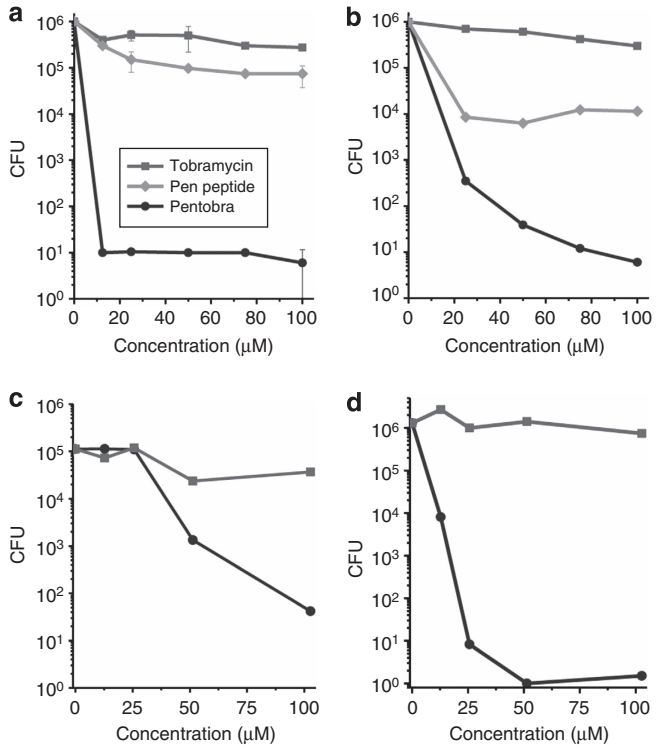

Figure 4. Pentobra is bactericidal in the sebaceous microcomedone environment. Antimicrobial activity against *P. acnes* in the microcomedones was determined using a colony-forming unit (CFU) assay. Pentobra, pen peptide, or tobramycin (0–102 μM) were incubated for 3 hours in collected lipid-rich microcomedones isolated from (**a**–**d**) four donors' faces using deep cleaning pore strips. Pentobra exhibits strong bactericidal activity, whereas tobramycin and pen peptide did not. Data in **a** represent mean ± SD of three experiments using microcomedones extracted on different days from the same donor. Data in **b**, **c**, and **d** are each from a different donor.

clinical isolates in sebaceous environments, we used comedone extracts isolated from human donors as an *in vitro* model (Figure 4). Pentobra showed dose-dependent bactericidal activity against *P. acnes* in comedone extracts from four donors, producing 5-log (Figure 4a and b), 3-log (Figure 4c), and 6-log (Figure 4d) reductions in CFUs. In contrast, tobramycin and the pen peptide alone were significantly less effective. That Pentobra is amphiphilic may contribute significantly to the enhanced activity in strongly hydrophobic environments. The amphiphilic peptide moiety of Pentobra has at least two beneficial effects: it drastically increases the activity of aminoglycoside antibiotics against *P. acnes*, and the lipophilicity aspect of Pentobra allows it to maintain activity in sebaceous environments.

Immunomodulatory effect of Pentobra and tobramycin in human monocytes

To determine whether Pentobra has an immunomodulatory effect on host innate immune response, we isolated and co-treated human monocytes and keratinocytes with *P. acnes* in the presence of either Pentobra or tobramycin and measured cytokine and chemokine expression levels by ELISA and real-time PCR. *P. acnes* induced the expression of *MCP-1* (monocyte chemoattractant protein 1), *CCL22* (C-C motif chemokine 22), *IP-10* (interferon gamma-induced protein 10), and *IL-12p40* in both cell types (Figure 5), which in monocytes could be inhibited (40-100%) by both Pentobra and tobramycin except for *IL-12p40* gene expression, where both antibiotics demonstrated little to no inhibition of *IL-12p40* by *P. acnes*-stimulated monocytes. Tobramycin was more effective in inhibiting *MCP-1* expression in monocytes compared with Pentobra. Both antibiotics completely abrogated *P. acnes*–induced *IP-10* expression ($P < 0.001$) in monocytes and keratinocytes. In contrast, no significant change in IL-6, IL-8, tumor necrosis factor-α, and IL-12p40 cytokine production was found in *P. acnes*–activated monocytes in the presence of both antibiotics (Supplementary Figure S2 online and data not shown). Therefore, both Pentobra and tobramycin appear to modulate specific cytokine and chemokine production induced by *P. acnes* but not all cytokines and chemokines that have been reported to have a role in the formation of inflammatory acne lesions (Vowels *et al.*, 1995a,b; McInturff *et al.*, 2005).

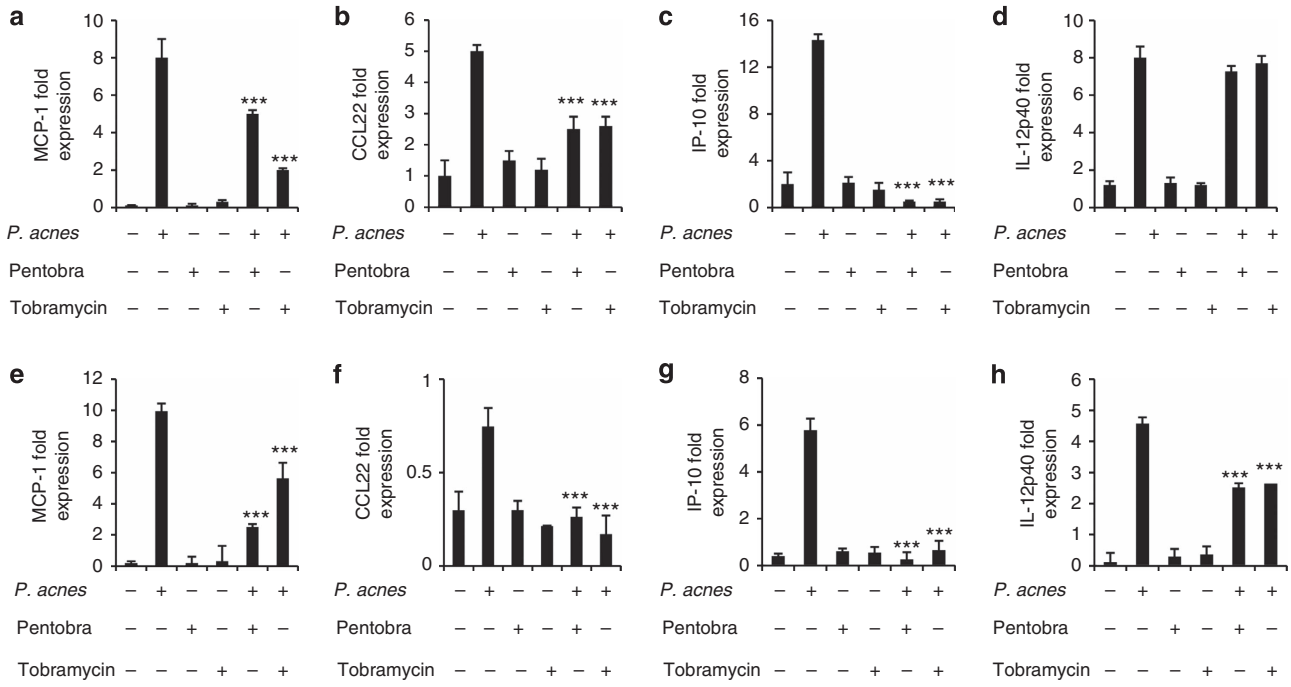

Figure 5. Immunomodulatory effects of Pentobra on human monocytes and the keratinocyte cell line stimulated with *P. acnes*. (**a**–**d**) Adherent monocytes and (**e**–**h**) HaCaT cells were cultured ($2–3 \times 10^6$ ml^{-1}) with *P. acnes* in the presence of Pentobra (26 μM) and tobramycin (26 μM)). *MCP-1* (**a** and **e**), *CCL22* (**b** and **f**), *IP-10* (**c** and **g**), and *IL-12p40* (**d** and **h**) mRNA expression was analyzed 24 hours following *P. acnes* stimulation. Gene expression was normalized to the housekeeping genes *GAPDH* and quantified by the comparative method $2^{-\Delta\Delta CT}$. Each panel is representative of three independent donors and experiments. Data represent mean ± SD (***$P \leq 0.001$). Primers used in the study are listed in Supplementary Table S1.

DISCUSSION

The increasing antibiotic resistance in clinical isolates of *P. acnes* highlights the need for new therapeutic strategies. The performance of Pentobra against *P. acnes* suggests that equipping aminoglycosides with the ability to permeate membranes in the manner of AMPs can be broadly enabling. Aminoglycosides are known to generally target bacterial ribosomes by binding the decoding aminoacyl site on the 16S rRNA component of the 30S ribosomal subunit, leading to faulty protein translation and inhibition (Fourmy *et al.*, 1996; Vicens and Westhof, 2002). This mechanism of action is potent and highly bactericidal against many types of clinically relevant bacteria. Empirically, aminoglycosides are known to have little effectiveness on *P. acnes* (Wang *et al.*, 1977; Dréno *et al.*, 2004; Williams *et al.*, 2012) and other anaerobic bacteria. Aminoglycosides bind the ribosomes of anaerobic bacteria and bacteria that use oxygen with similar affinities (Bryan *et al.*, 1979). It is therefore hypothesized that anaerobic bacteria are intrinsically resistant to aminoglycosides because the molecules cannot cross the cytoplasmic membranes of anaerobic cells to reach the bacterial ribosome (Taber *et al.*, 1987; Allison *et al.*, 2011). The lower proton-motive force (Davis *et al.*, 1986; Magnet and Blanchard, 2004) across the membranes of anaerobic bacteria in comparison with oxygen-using bacteria impairs internalization, as aminoglycoside uptake is proposed to be energy-dependent from reliance on a threshold proton-motive force (Davis, 1987; Taber *et al.*, 1987; Magnet and Blanchard, 2004; Allison *et al.*, 2011). Although the general AMP membrane permeabilization mechanism is broad spectrum (Schmidt and Wong, 2013), and their activities are less sensitive to the metabolic status of the cell (Hurdle *et al.*, 2011), AMPs often only display moderate potency. When juxtaposed, the distinct mechanisms of action of aminoglycosides and AMPs become complementary. Hybrid membrane-active aminoglycosides can kill bacteria by membrane disruption and can also enter cells and interfere with ribosome translation. The direct killing of all tested *P. acnes* clinical isolates by Pentobra shows that our approach has promising general therapeutic value.

Bacteria must simultaneously evade both of pentobra's killing mechanisms to develop resistance, similar to drug combination therapies used to minimize chances of bacterial resistance (Walsh, 2000; Fischbach, 2011; Worthington and Melander, 2013). Although bacteria have well-developed pathways to decrease susceptibility to AMPs through phenotypic changes (Guo *et al.*, 1997; Li *et al.*, 2007; Koprivnjak and Peschel, 2011), these mechanisms do not usually confer complete resistance (Hancock and Sahl, 2006). This is believed to be a result of the ability of AMPs to target generic properties in the lipid composition of bacterial membranes (Zasloff, 2002; Hancock and Sahl, 2006; Schmidt and Wong, 2013). In fact, to evolve AMP resistance by eliminating these generic lipid properties of bacterial membranes amounts to a lethal mutation (Yang *et al.*, 2008). By multiplexing the

specificity of ribosomal activity from tobramycin with the general susceptibility of bacterial membranes to AMPs, Pentobra provides potent antimicrobial activity with reduced likelihood of *P. acnes* resistance.

A topical antimicrobial therapy must penetrate lipid-rich media and maintain activity in cutaneous environments. The initial stage of acne infection is a microcomedone, and previous work has used extracted microcomedone contents to determine the effectiveness of antimicrobial therapies for acne (Piérard-Franchimont *et al.*, 2002). We performed similar microcomedone experiments to determine whether Pentobra, which is amphipathic, can penetrate hydrophobic environments and remain active when presented with lipidic material from the skin. Hydrophilic drugs like aminoglycosides may not be able to penetrate through the layers of fatty acids in human skin, whereas overly hydrophobic drugs may become irreversibly sequestered with fatty acids and will be unavailable to act on *P. acnes*. Our assays on *P. acnes* isolated from microcomedones show that Pentobra can kill *P. acnes* in a sebum-rich environment. Human skin widely expresses cathelicidin LL37 (Dürr *et al.*, 2006), as well as defensins HBD-2 (Harder *et al.*, 1997) and HBD-3 (Harder *et al.*, 2001), which suggests that AMPs can offer protection in the skin against microbial colonizers, and AMP gene knockout studies have shown that these animals are more vulnerable to infection (Bowdish *et al.*, 2006; Dürr *et al.*, 2006). Many membrane-permeabilizing AMPs including HBD2 (Bals *et al.*, 1998) and LL37 (Dürr *et al.*, 2006) show loss of antimicrobial activity in elevated salt concentrations *in vitro*. Similar to these AMPs, the bactericidal activity of Pentobra against *P. acnes* is attenuated with increasing sodium chloride concentrations (Supplementary Figure S1C online). This affords future engineering possibilities: Although plate killing assays provide important measures of drug potency, it does not always mirror the performance of a drug *in vivo*. In principle, it is possible to engineer membrane-active aminoglycosides that are not as salt sensitive because some AMPs have *in vitro* salt tolerant antibacterial profiles (Harder *et al.*, 2001). Furthermore, drug activities can be modulated by formulation and method of delivery. Overall, the microcomedone experiments highlight the efficacy of Pentobra in lipid-rich cutaneous environments, which is suggestive of its utility as a topical antimicrobial agent.

For a clear understanding of the immunomodulation by Pentobra, we explored its function at the transcriptional and protein levels. Many mammalian AMPs have immunomodulatory functions (Bowdish *et al.*, 2006). For example, human cathelicidin LL37 can decrease cytokine production in response to lipopolysaccharide (Scott *et al.*, 2002), which is a ligand for Toll-like receptor-4 (TLR4). Previous studies have proposed that the antiendotoxin abilities of implicated cathelicidins are partly due to direct binding between the cationic peptides and anionic lipopolysaccharide (Scott *et al.*, 2000; Rosenfeld and Shai, 2006), as the neutralized lipopolysaccharide is unavailable to associate with pattern recognition receptors. The 12AA pen peptide is derived from the protein transduction domain of the *Drosophila* antennapedia homeodomain (Joliot *et al.*, 1991; Derossi *et al.*, 1994). The polycationic nature of both Pentobra and tobramycin can lead to strong electrostatic interactions with anionic components of *P. acnes*. It is possible that the partial anti-inflammatory effects of Pentobra are derived from interference with aspects of the TLR2 pathway, such as nuclear factor κB activation, as *P. acnes* stimulates cells through TLR2 (Kim *et al.*, 2002). Although the ligands from *P. acnes* that activate TLR2 remain under investigation, the interaction of Pentobra with these molecules may reduce inflammation. Furthermore, the polyanionic charge of cell envelope components like peptidoglycan can promote complexation with Pentobra, which may prevent detection of peptidoglycan by intracellular sensors Nod1 and Nod2. As inflammation is a key clinical feature of acne, the partial anti-inflammatory nature of Pentobra combined with its antibacterial activity increases its therapeutic potential.

The immunomodulatory capabilities of membrane-active aminoglycosides suggest potential clinical applications. Constraints on the amino acid content of AMPs under-determine the full peptide sequence (Schmidt *et al.*, 2011; Schmidt and Wong, 2013); hence, other functions may be written into these peptide–aminoglycoside composites. LL37 is a notable natural example as it permeabilizes bacterial membranes and also has multiple immunomodulatory functions. In the same spirit, it should be possible to construct future generations of Pentobra that are not only potent bactericides against *P. acnes* but also complex strongly with *P. acnes* ligands and thereby modulate immune responses.

In conclusion, we have demonstrated the efficacy of a hybrid antibiotic for *P. acnes*, Pentobra, by synergistically combining the antimicrobial functions and specificity mechanisms of two different classes of antimicrobials, aminoglycosides and AMPs. Pentobra exhibited bactericidal activity against a wide range of *P. acnes* clinical isolates and effectively killed *P. acnes* from human donors in microcomedone sebum-rich environment in an *in vitro* assay. Furthermore, various skin cells treated with Pentobra showed no adverse effects, suggesting that Pentobra is not only an effective antimicrobial but may also be a safe and less irritating topical agent. Pentobra displayed anti-inflammatory activity by suppressing the expression of inflammatory chemokines from monocytes and keratinocytes stimulated with *P. acnes*. The broad bactericidal activity against clinical strains of *P. acnes* in the lipid-rich cutaneous environment, in concert with their potential as anti-inflammmatory agents, shows that peptide–aminoglycoside antibiotics designed using rules for AMPs are promising therapeutics for infections caused by drug-resistant *P. acnes*.

MATERIALS AND METHODS

Pentobra

Pentobra is a composite peptide+aminoglycoside compound consisting of a 12AA peptide named the pen peptide, which is conjugated to tobramycin. (Schmidt *et al.*, 2014). The five amine groups of tobramycin were Boc (tert-butyloxycarbonyl) protected, then the C6′′ primary hydroxyl of tobramycin-Boc5 was selectively reacted with succinic anhydride to produce a terminal carboxyl function. Pen

peptide was synthesized manually by solid phase synthesis. The carboxyl function extending from tobramycin was coupled with the N-terminal group of the fully protected resin-anchored pen peptide. The resulting Pentobra was cleaved from resin and fully deprotected by cleaving the side-chain protecting groups, as well as Boc groups on tobramycin with a trifluoroacetic acid mixture containing scavengers. Pentobra was finally purified by preparative HPLC and characterized by matrix assisted laser desorption ionization time-of-flight mass spectrometry.

The pen peptide, sequence RQIKIWFQNRRW, was designed with the cationic and hydrophobic motif shared by AMPs. It is cationic (+3 from 2 Arg and 1 Lys) and hydrophobic (2 Ile, 2 Trp, and 1 Phe, it is >30% hydrophobic). We designed Pentobra with the ability to disrupt bacterial membranes and cross them so that it can bind ribosomes through its tobramycin part. To ensure that Pentobra permeates cell membranes, the pen peptide sequence was derived from the 17AA sequence of antennapedia (ANTP) penetratin cell-penetrating peptide (Derossi *et al.*, 1994), such that the amounts of cationicity and hydrophobicity of the composite molecule (pen peptide+tobramycin) are consistent with design principles of cell-penetrating peptide and AMP sequences (Mishra *et al.*, 2011; Schmidt *et al.*, 2011). Therefore, the sequence of the pen peptide was chosen such that the composite pen peptide plus tobramycin would be able to permeabilize and penetrate bacterial membranes, thereby allowing it to enter bacteria cells and bind ribosomes using the mechanism of action from tobramycin.

P. acnes and clinical isolates

P. acnes strain ATCC 6919 was obtained from American Type Culture Collections (Manassas, VA). Clinical isolates were obtained from Biodefense and Emerging Infections Research Resources Repository (BEI Resources) and from nasal skin microcomedones from patients attending the Division of Dermatology outpatient clinic at UCLA, after signed written informed consent as approved by the Institutional Review Board at UCLA in accordance with the Declaration of Helsinki Principles. The level of endotoxin contaminating the *P. acnes* was quantified with a *Limulus* Amoebocyte Lysate assay (BioWhittaker, Radnor, PA) and found to be <0.1 ng-ml^{-1}. *P. acnes* cultures were grown as previously described (Agak *et al.*, 2014). *P. acnes* strains and clinical isolates used in the study are summarized in Table 1.

CFU assay

The CFU assay was performed as described previously (McInturff *et al.*, 2005). *P. acnes* strains (Table 1) were grown under anaerobic conditions in Reinforced Clostridial Medium (Oxoid, Basingstroke, England) and collected in mid-log phase. The bacteria were washed with the assay buffer (10 mM Tris pH 7.4, supplemented with 1% volume Trypticase soy broth, Tris-TSB) and enumerated by applying a conversion factor of 7.5×10^7 bacteria per ml = 1 OD unit at 600 nm. Various concentrations of Pentobra, pen peptide, or tobramycin were incubated with 3.75×10^5 bacteria in a final volume of 100 µl at 37 °C for 3 hours. After incubation, 10^3–10^4-fold dilutions were prepared and plated on solid media comprised of Brucella broth (BD Biosciences, San Diego, CA) with 5% sheep red blood cells (Remel, Lenexa, KS). Plates were incubated for 4 days at 37 °C under anaerobic conditions, then individual colonies were counted and the number of CFU per tube was calculated.

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay

Three cell types, peripheral blood mononuclear cells, keratinocytes (HaCaT), and sebocytes cell lines were tested. Cell proliferation experiments were performed in 96-well plates (five replicates). Pentobra, pen peptide, and tobramycin treatments (all 26 µM) were initiated at 24 hours postseeding for 3 days, and assay developed as previously described (Agak *et al* 2014).

Electron microscopy

P. acnes ATCC 6919 at 3×10^8CFU ml^{-1} was incubated untreated or with 25.7 µM of Pentobra, pen peptide, or tobramycin for 3 hours, washed twice with phosphate-buffered saline, and resuspended in phosphate-buffered saline with 2% glutaraldehyde. The bacteria were fixed for 5 minutes with 0.05% OsO_4, dehydrated in graded ethanol, and then embedded in Eponate 12 (Ted Pella, Redding, CA). A total of 60–70 nm slices were cut with a Reichert-Jung (Reichert, Depew, NY) Ultracut E ultramicrotome, which were picked up on formvar-coated copper grids. Samples were stained with uranyl acetate and Reynolds lead citrate and visualized at 80 kV on a JEOL (Peabody, MA) 100CX electron microscope.

Microcomedone assay

After informed consent was obtained, comedones were collected from human volunteers as previously described (McInturff *et al.*, 2005). Briefly, individual plugs were removed from the strips with a fine point tweezer and pooled in a microfuge tube. These were then resuspended in 200 µl of assay medium (Tris-TSB) until the particulate material was broken up into a colloidal suspension. Then, 30 µl of this suspension was combined with either 30 µl of drug (Pentobra, pen peptide, tobramycin,) or 30 µl of clostridium medium (positive control). These were then incubated for 3 hours at 37 °C in an ambient air incubator. A serial dilution of each sample was prepared and 30 µl of each dilution was spotted on Brucella blood agar plates and incubated as previously described. The CFU per ml was determined by counting.

Monocyte isolation, stimulation, and cytokine ELISAs

Monocytes were isolated as previously described (Agak *et al.*, 2014; Qin *et al.*, 2014). Adherent monocytes were stimulated (co-treated) with media or *P. acnes* in the presence of tobramycin or Pentobra and cytokine levels measured by ELISA as previously described (Agak *et al.*, 2014; Qin *et al.*, 2014) Samples were assayed in triplicates. Results are expressed as mean ± SD of at least three independent experiments, with monocytes obtained from three independent donors.

RNA isolation, cDNA synthesis, and real-time PCR

Monocytes and keratinocytes (HaCaT) were stimulated with media or *P. acnes* in the presence of tobramycin or Pentobra. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and cDNA synthesis and real-time PCR reactions were carried out as previously described (Agak *et al.*, 2014; Qin *et al.*, 2014). The list of primers used in the study are summarized in Supplementary Table S1.

Statistical analysis

Results are expressed as the means ± SD for the number of separate experiments indicated in each case ($n\geqslant3$). One-way analysis of variance was used to compare variances within groups and among

them. *Post hoc* two-tailed Student's *t*-test was used for comparison between two groups. Significant differences were considered for those probabilities $\leqslant 5\%$ ($P \leqslant 0.05$).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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