J Mol Med
DOI 10.1007/s00109-012-0882-3

ORIGINAL ARTICLE

A new pharmacological agent (AKB-4924) stabilizes hypoxia inducible factor-1 (HIF-1) and increases skin innate defenses against bacterial infection

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Received: 21 November 2011 / Revised: 1 February 2012 / Accepted: 9 February 2012

Abstract Hypoxia inducible factor-1 (HIF-1) is a transcription factor that is a major regulator of energy homeostasis and cellular adaptation to low oxygen stress. HIF-1 is also activated in response to bacterial pathogens and supports the innate immune response of both phagocytes and keratinocytes. In this work, we show that a new pharmacological compound AKB-4924 increases HIF-1 levels and enhances the antibacterial activity of phagocytes and keratinocytes against both methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus* in vitro. AKB-4924 is also effective in stimulating the killing capacity of keratinocytes against the important opportunistic skin pathogens *Pseudomonas aeruginosa* and *Acinetobacter baumanii*. The effect of AKB-4924 is mediated through the activity of host cells, as the compound exerts no direct antimicrobial activity. Administered locally as a single agent, AKB-4924 limits *S. aureus* proliferation and lesion formation in a mouse skin abscess model. This approach to pharmacologically boost the innate immune response via HIF-1 stabilization may serve as a useful adjunctive treatment for antibiotic-resistant bacterial infections.

Keywords Hypoxia inducible factor-1 (HIF-1) · Innate immunity · *Staphylococcus aureus* · Bacterial infection · Antibiotic-resistant bacteria

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Introduction

Treatment of bacterial infections has become increasingly difficult due to the continual emergence of antibiotic-resistant strains. Strikingly, methicillin-resistant *Staphylococcus aureus* (MRSA) infections have reached epidemic proportions in many countries [1] and currently represent the most common cause of skin and soft tissue infections (SSTI) in the USA [2]. Both hospital- and community-associated MRSA may exhibit broad resistance to multiple classes of antibiotics [1, 3]. Drug-resistance in additional species pose problems in the hospital settings, and the acronym "ESKAPE" has been coined to highlight the most problematic strains: *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp. [4]. Although there has been recognition of the need to develop new antibiotics for

Published online: 28 February 2012

these important pathogens, few antibiotics are in phase II or III clinical trials, and no drugs for infection by multidrug-resistant Gram-negative bacilli such as *P. aeruginosa* and *A. baumannii* have reached advanced stages of development [5]. Thus, there is a great medical need for the development of new antibacterial therapies, in particular those that could limit the antibiotic resistance problems common to traditional antibiotics.

The heterodimeric transcription factor hypoxia-inducible factor-1 (HIF-1) is the major regulator of the cellular adaption to hypoxic stress, controlling genes involved in glycolysis, erythropoiesis, and angiogenesis [6]. Under normoxic conditions, the iron- and oxygen-dependent prolyl hydroxylase (PHD) enzymes target the HIF-1α subunit for degradation by the ubiquitin–proteosome pathway [7]. Under hypoxic or iron-restricted conditions, HIF-1α can accumulate, translocate to the nucleus, dimerize with the HIF-1β subunit, and bind to a core DNA sequence called the hypoxic response element (HRE) to induce target gene transcription [8]. Recently, we and other groups have coupled conditional gene targeting and pharmacological strategies to demonstrate that HIF-1 is activated in response to bacteria [9, 10] and functions as a master regulator of the inflammatory and innate immune activity of macrophages, neutrophils, and keratinocytes [9, 11, 12]. In this context, HIF-1 can induce the transcription of genes involved in the inflammatory response such as IL-1β, IL-8, TNFα, iNOS, and cathelicidin (LL-37) [9, 13–15]. Thus, pharmacological agents that allow HIF-1α accumulation to occur could provide a new approach to aid in the treatment of infectious disease [16, 17].

Challenges to the effective control of bacterial infections can arise from factors such as lowered host immunity and antibiotic resistance [12]. A potential advantage to treating host cells to enhance the immune capabilities rather than directly treating the bacteria is to limit selective pressure for resistance. In this study, we show that a new generation HIF-1α stabilizing agent, AKB-4924, can enhance the bactericidal capacity of resident skin cells and phagocytes far more potently than the classical HIF-1 stabilizing agent mimosine, indicating a potential for pharmacological efficacy in infectious disease therapy.

Methods

Cell culture

The human monocytic cell line U937 (ATCC CRL-1593.2) and human skin keratinocyte cell line HaCaT [18] were maintained in RPMI supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen). Neutrophils were isolated from healthy donors (use and procedures approved by the University of California San Diego Human Research Protections Program) using PolyMorphPrep-kit (Fresenius Kabi), and erythrocytes lysed with sterile H_2O as previously described [19]. WT and HIF-1α null mouse embryonic fibroblasts (MEFs) were cultured as previously described [20].

Bacterial strains

Methicillin-sensitive (MSSA, ATCC 25904) and methicillin-resistant (MRSA, ATCC 33591) *S. aureus* strains were maintained as shaking cultures in Todd–Hewitt broth. *S. aureus* growth assays were performed in 96-well plates in RPMI supplemented with 2% 70°C heat-inactivated FBS [21] with addition of 10 or 25 μM AKB-4924 and 250 μM ferric citrate (Sigma) as indicated. An extended panel of hospital associated (HA)- and community associated (CA)-MRSA and additional Gram-positive and Gram-negative bacterial strains was used to exclude potential direct antimicrobial activities of AKB-4924: MSSA strain UAMS-1 (USA200) and MRSA strain UAMS-1182 (USA300), group A *Streptococcus* serotype M1T1 strain 5448 [22], group B *Streptococcus* strain COH1 [23], *Pseudomonas aeruginosa* PA01 (ATCC BAA-47), *Moraxella catarrhalis* (ATCC 25238), and *Haemophilus influenzae* type B (ATCC 10211). Bacterial cultures were maintained according to CSLI guidelines. Minimal inhibitory concentration (MIC) assays were performed in Mueller–Hinton broth supplemented with calcium and magnesium and up to 200 μM AKB-4924.

AKB-4924

AKB-4924 was manufactured in a three-step synthesis with a resulting purity of 98%. Structure and purity were confirmed by NMR followed by mass spectrometry. AKB-4924 was resuspended in dimethyl sulfoxide, pH=4.2–4.4 to 5 mM and used at 10 μM unless otherwise indicated. Alternatively, AKB-4924 was provided at 2 mg/mL in 40% 2-hydroxylpropyl-beta-cyclodextrin (HPβCD) in 50 mM aqueous citrate buffer at pH 4 (used in Fig. 4b, c, Aroz Technologies).

Western blot analysis of HIF-1α protein levels

MEFs were treated with 100 μM AKB-4924 for 4 h, and U937 monocytes were treated with 10 μM AKB-4924 or vehicle for the indicated times, and nuclei were harvested as described [24]. 30 μg of total protein was run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and detected with 1:1,000 rabbit anti-HIF-1α antibody (Novus Biologicals).

Real-time PCR

MEFs were treated with indicated concentrations of AKB-4924 for 8 h, and U937 monocytes were treated with 10 μM AKB-4924 or vehicle for 24 h prior to total RNA isolation (Qiagen). RNA was DNase treated (Turbo DNase, Ambion), and 1 μg total RNA was reverse transcribed (iScript, Bio-Rad). Real-time PCR was performed using iQ SYBR Green Supermix (BioRad) and primers for PGK, Glut-1 [25], IL-8, and LL-37 [9]. Data were normalized to GAPDH, and relative expression was compared to untreated cells by the ΔΔCt method [26].

Cytotoxicity and antibiotic synergy assays

Cytotoxicity to HeLa, HepG2, and HaCaT cells was tested with up to 100 μM AKB-4924 in culture media (DMEM+ 10% FBS for HeLa and HepG2 cells). Membrane integrity of cells was measured by release of lactate dehydrogenase (CytoTox 96 nonradioactive cytotoxicity assay, Promega) and compared with untreated and vehicle-treated cells. For antibiotic synergy testing, *S. aureus* (5×10^4 cfu) was incubated with 2 μg/mL vancomycin, 4 μg/mL daptomycin, or 1 μg/mL penicillin (4- to 8-fold the previously determined MIC for each antibiotic) and 1:2 dilutions thereof, together with 40 μM AKB-4924 and 1:2 dilutions thereof in RPMI+ 2% (70°C heat-inactivated) FBS in 96-well plates. Plates were incubated overnight at 37°C shaking, and absorbance was read at 600 nm. Bacterial growth was compared with untreated and vehicle-treated controls. No direct bactericidal activity of AKB-4924 was seen (in agreement with Fig. 3). For H_2O_2 synergy testing, *S. aureus* (1×10^7 cfu) was incubated in 96-well plates in RPMI+2% FBS, 10 μM AKB-4924 or vehicle, and 0.05% H_2O_2. Surving bacteria were enumerated on THA plates in 15-min intervals to 45 min and compared with bacterial growth in AKB-4924 or vehicle-treated media in the absence of H_2O_2.

In vitro killing assays

Cell cultures were washed and resuspended in RPMI supplemented with 2% 70°C heat-inactivated FBS [21] to 5×10^5 cells per well in 24-well plates. Cells were incubated with 100 μM mimosine (Sigma) for 4 h or 10 μM AKB-4924 for 1 h prior to bacterial infection. Alternatively, cells were incubated with 10 μM AKB-4924 and the indicated concentrations of 2-oxoglutarate (2OG, Sigma) or 250 μM ferric citrate (Fe) for 1 h prior to bacterial infection. Cells were subsequently infected with log-phase bacterial cultures at a multiplicity of infection (MOI)=1, and plates spun at 500×*g* to initiate bacterial contact with cells. At indicated time points, cells were lysed with 0.025% TritonX-100, and surviving bacteria were enumerated by cfu counts on Todd–Hewitt agar (THA) plates. Bacterial survival was calculated as a percentage of bacteria remaining in treated samples compared with untreated cell samples. Each experiment was performed three independent times with similar results and a representative data set shown.

Mouse infection model

All animal use and procedures were approved by the UCSD Institutional Animal Care and Use Committee. Eight-week-old female CD-1 mice (Charles River Laboratories) were shaved 1 day prior to infection. Mice were injected subcutaneously with 5×10^7CFU of *S. aureus* (MSSA strain) mixed with 1 mg/mL Cytodex beads (Sigma). Mice were treated intralesionally with 50 μL 1 mM AKB-4924 (approximately 1 mg/kg) or vehicle at 0, 6, and 24 h post-infection. Lesion area was monitored daily by photographic measurement using ImageJ software [27]. At 4 days post-infection, mice were killed, lesions were excised and homogenized in PBS, and bacteria recovered from the lesions were enumerated on THA plates. Two independent experiments were performed, and combined data are shown. For histopathological studies, mice were injected subcutaneously with 5 mg/kg AKB-4924 or vehicle. Skin was excised 24 h post-injection, sectioned and stained with hematoxylin and eosin.

Statistical analysis

All data were analyzed as described using GraphPad Prism v.5 (GraphPad Software).

Results

AKB-4924 stabilizes HIF-1α

AKB-4924 was identified in a screen conducted by Proctor and Gamble for potential prolyl-hydroxlase (PHD) inhibitors and found to have a half maximal inhibitory concentration (IC_{50}) of 14 μM for PHD2. It was subsequently in-licensed to Akebia Therapeutics (now Aerpio Therapeutics), where further structural and biochemical analyses were conducted and screened for HIF-1 dependent activity. The generic structure of AKB-4924 is shown in Fig. 1a, where Z is phenyl substituted with one to five halogens chosen from fluorine and chlorine and R_4 is a C_1–C_4 linear alkyl, C_3–C_4 branched alkyl, or a pharmaceutically acceptable salt thereof. AKB-4924 contains an α-hydroxycarbonyl group that can bind iron [28] similar to the known iron chelator and HIF-1 stabilizing compound mimosine (Fig. 1a, [29, 30]). In the discovery process for AKB-4924, structurally related compounds were shown to bind to the iron-binding region

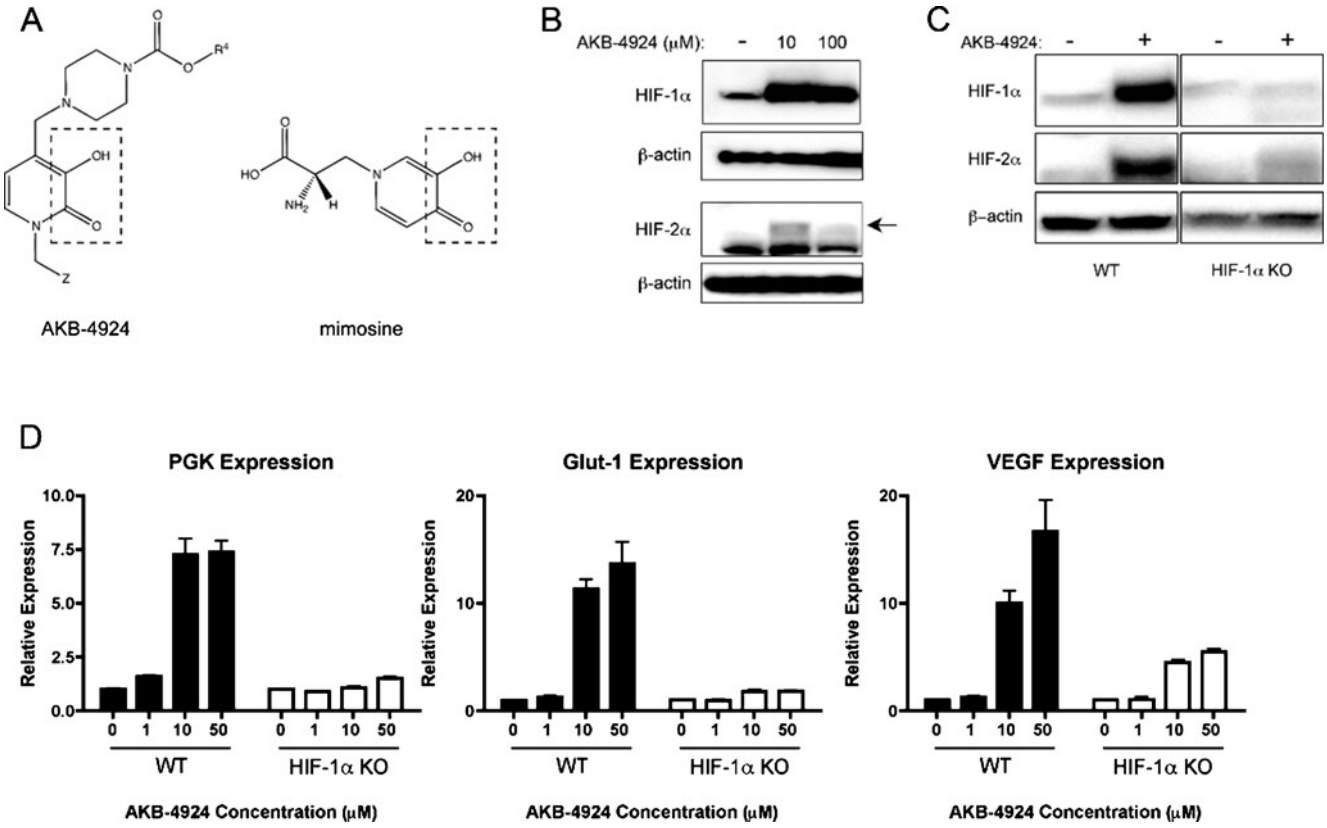

Fig. 1 AKB-4924 stabilizes HIF-1α and induces HIF-1 regulated gene expression. **a** AKB-4924 contains an iron-binding α-hydroxycarbonyl group (boxed) similar to mimosine. **b** Accumulation of HIF-1α and HIF-2α in murine embryonic fibroblasts (MEFs) in response to different concentrations of AKB-4924. *Arrow* indicates HIF-2α protein. **c** AKB-4924 treatment leads to accumulation of HIF-1α protein in wild-type (WT) but not HIF-1α deficient (HIF-1α KO) MEFs. HIF-2α protein accumulates in both WT and HIF-1α KO MEFs with AKB-4924 treatment. **c** AKB-4924 treatment of WT but not HIF-1α-deficient MEFs leads to increased levels of mRNA from known HIF-1 regulated genes PGK, Glut-1 and VEGF

of the PHD2 molecule. Although AKB-4924 was never specifically evaluated for its iron binding capacity, it was demonstrated with many structurally related compounds that the combination of the PHD2 enzyme and the inhibitory molecule would effectively chelate the resident iron molecule [31–33].

In wild-type (WT) mouse embryonic fibroblasts (MEFs), treatment with as little as 10 μM AKB-4924 resulted in robust accumulation of HIF-1α and modest accumulation of HIF-2α (Fig. 1b). Treatment with 100 μM AKB-4924 resulted in accumulation of both HIF-1α and HIF-2α proteins (Fig. 1b, c), whereas HIF-2α protein alone accumulated in MEFs with a targeted deletion of HIF-1α (Fig. 1c). The increased protein levels of both HIF-1α and HIF-2α demonstrate the inhibitory effect of AKB-4924 on PHD2 and perhaps other PHDs, since these enzymes require iron as a cofactor and play roles in regulating the stability of both HIF-1α and HIF-2α [34]. Accumulation of HIF-1α and HIF-2α in MEFs upon treatment with AKB-4924 correspondingly resulted in the induction of HIF-1 regulated mRNA transcripts, including phosphoglycerol kinase (PGK), glucose transporter 1 (Glut-1), and vascular endothelial growth factor (VEGF) (Fig. 1d). Strong induction of the HIF-1α-specific transcript PGK [35] in WT MEFs was observed with as little as 10 μM AKB-4924. Furthermore, only moderate induction of VEGF, a gene controlled by both HIF-1α and HIF-2α [13], was observed upon AKB-4924 treatment of HIF-1α-deficient cells (Fig. 1d), reflecting the modest stabilization of HIF-2α protein by AKB-4924 (Fig. 1b, c). These results demonstrate that although AKB-4924 can stabilize both HIF-1α and HIF-2α, it is a potent and relatively selective inducer of HIF-1α accumulation and subsequent activity.

AKB-4924 enhances the bactericidal activity of phagocytes through HIF-1α

Recent studies have indicated that HIF-1α serves as a key regulator of inflammatory and innate immune activities of phagocytes, including monocytes, macrophages, and neutrophils. Pharmacological stabilization of HIF-1α in these cells therefore represents a potential strategy to enhance host response to bacterial pathogens [9, 16]. We tested the ability

of AKB-4924 to induce HIF-1α accumulation in phagocytes and the consequent effects on their bactericidal capacity. Cultured U937 human monocytes were incubated under normoxic conditions with 10 μM AKB-4924, the minimal concentration we determined was required to boost HIF-1 regulated gene transcription (Fig. 1b, d). U937 cells treated with AKB-4924 for varying times accumulated HIF-1α protein (Fig. 2a), in agreement with the drug effect that we observed in MEFs (Fig. 1b). Transcription of HIF-1 regulated, antimicrobial, and proinflammatory response genes was correspondingly upregulated in these cells (Fig. 2b). A modest effect of AKB-4924 on transcription of glucose transporter 1 (Glut-1) likely reflects the undifferentiated state of the monocyte and is expected to be greater in a differentiated macrophage [36]. The antimicrobial peptide cathelicidin (LL-37) and its mouse homolog CRAMP have been shown in human keratinocytes and mouse phagocytes to be regulated by HIF-1α [9, 11]; we found that LL-37 transcript was increased in U937 monocytes upon AKB-4924 treatment (Fig. 2b). Interleukin-8 (IL-8) is a potent neutrophil chemoattractant and proinflammatory cytokine. An HRE has been identified in the IL-8 promoter [14], and increased IL-8 transcript levels have been reported in hypoxic macrophages [13]. AKB-4924 treatment boosted transcript levels for IL-8 in U937 cells (Fig. 2b). Thus human monocytes can be stimulated by AKB-4924 to upregulate expression of genes important in the innate immune response.

In order to assess the effectiveness of AKB-4924 as a potential innate immune defense booster, we pretreated U937 monocytes with AKB-4924 and subsequently infected the cells with *S. aureus*. AKB-4924 treatment significantly enhanced the bactericidal activity of the monocytes against MRSA compared to control (vehicle alone) cells, with the greatest difference seen at 3 h post-infection (Fig. 2c). The increased bactericidal activity of monocytes by AKB-4924 at the time of maximal killing was seen with additional strains of *S. aureus*, including an MSSA strain, a community-associated MRSA strain (USA300), and a hospital-associated MSSA strain (USA200, Fig. 2d). Treatment with AKB-4924 also

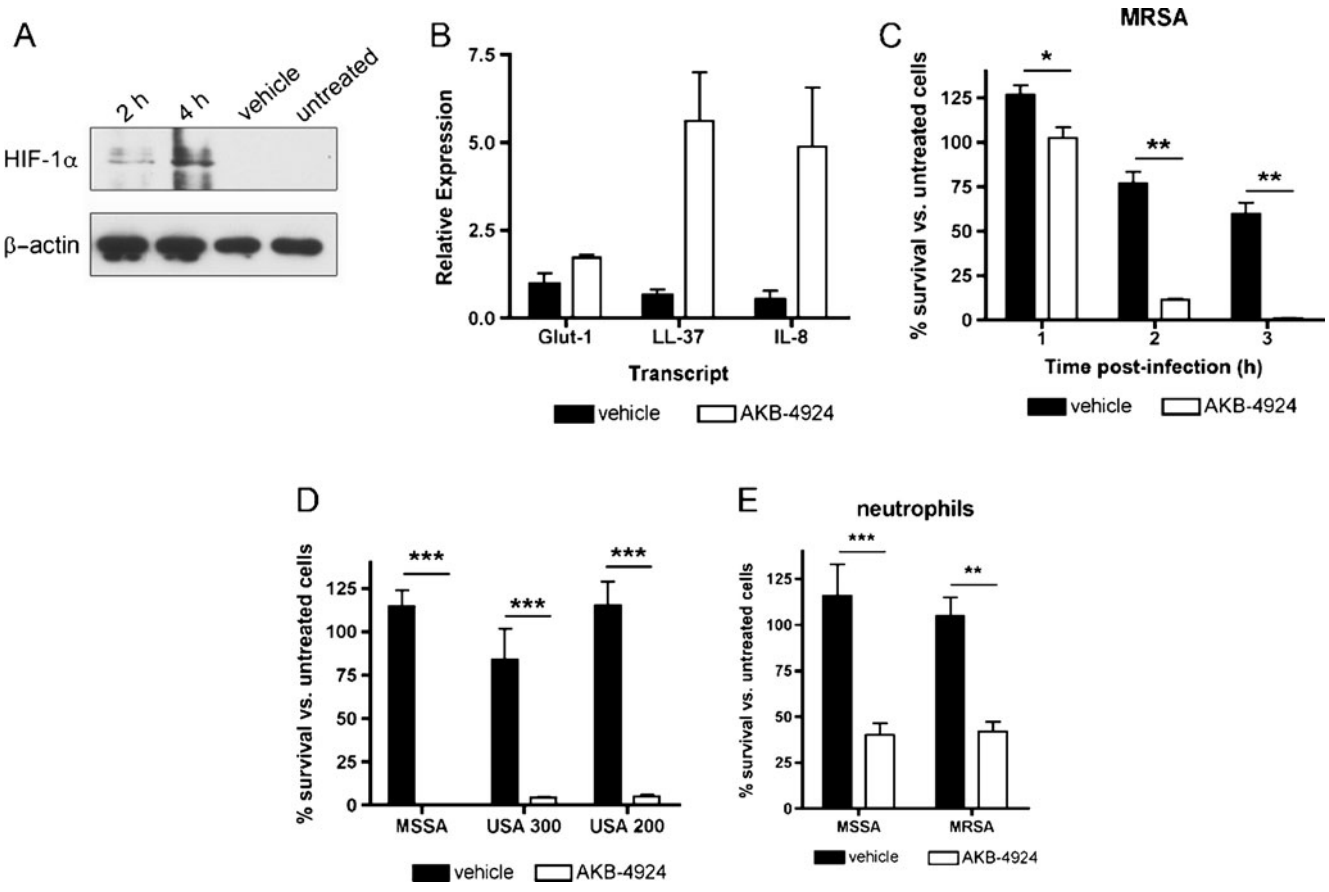

Fig. 2 AKB-4924 stabilizes HIF-1α and enhances the bactericidal activity of phagocytes. **a** U937 human monocytes incubated with 10 μM AKB-4924 for the indicated times accumulate HIF-1α protein. **b** The transcripts for HIF-1 target gene Glut-1 and the genes encoding cathelicidin antimicrobial peptide LL-37 and neutrophil chemokine/activating cytokine IL-8 are upregulated upon AKB-4924 treatment. **c** U937 monocytes treated with AKB-4924 display increased bactericidal activity against MRSA over time. **d** AKB-4924 increases bactericidal activity of U937 monocytes against both antibiotic-sensitive and -resistant strains of *S. aureus*. **e** AKB-4924 increases the bactericidal capacity of human neutrophils. For each panel, mean±SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$ by unpaired *t* test with Welch's correction

enhanced the killing of both MSSA and MRSA by freshly isolated human neutrophils (Fig. 2e). Thus, AKB-4924 is a potent booster of the phagocyte antimicrobial response through its ability to stabilize HIF-1α and induce antimicrobial effectors such as the cathelicidin antimicrobial peptide LL-37.

To examine the mechanism of AKB-4924-induced HIF-1α stabilization, we incubated AKB-4924-treated U937 cells in the presence of PHD cofactors 2-oxoglutarate (2OG) or iron and examined the effects on bactericidal activity. AKB-4924 treatment could still markedly enhance bactericidal activity in the presence of increasing concentrations of 2OG, whereas the addition of iron abolished the effect of AKB-4924 (Fig. 3a), thus supporting its activity as an iron chelator. The enhancement of bactericidal activity of U937 monocytes by treatment for 1 h with 10 μM AKB-4924 was significantly greater for the MSSA and the MRSA strains than treatment for 4 h with 100 μM mimosine, an iron chelator and HIF-1 stabilizing agent previously shown to boost phagocyte activity more effectively than the other established iron blocking agonists desferroxamine, cobalt chloride, and hydroxypyridone [16] (Fig. 3b). Thus, AKB-4924 most likely exerts its effects on PHDs and subsequent HIF-1α stabilization and bactericidal activity by limiting iron.

AKB-4924 does not exhibit direct antimicrobial activity

In our in vitro assays, augmentation of monocytes by AKB-4924 to kill *S. aureus* was equal (MSSA) or even more potent (MRSA) than direct treatment with 1 μg/mL of the antibiotic vancomycin (Fig. 4a). Because of the superior antibacterial response of phagocytes in our *in vitro* assays, we examined whether AKB-4924 might exert any direct antimicrobial effects. Coincidently, AKB-4924 was originally identified in a screen for the inhibition of bacterial methionine aminopeptidase (bMAP) in a search for potential antimicrobial agent. Since bMAPs require iron as a cofactor [37], the iron-chelating properties of AKB-4924 could potentially contribute to its activity against bMAPs. Although AKB-4924 displayed 2 μM activity against recombinant bMAP enzyme, the concentration required to achieve adequate

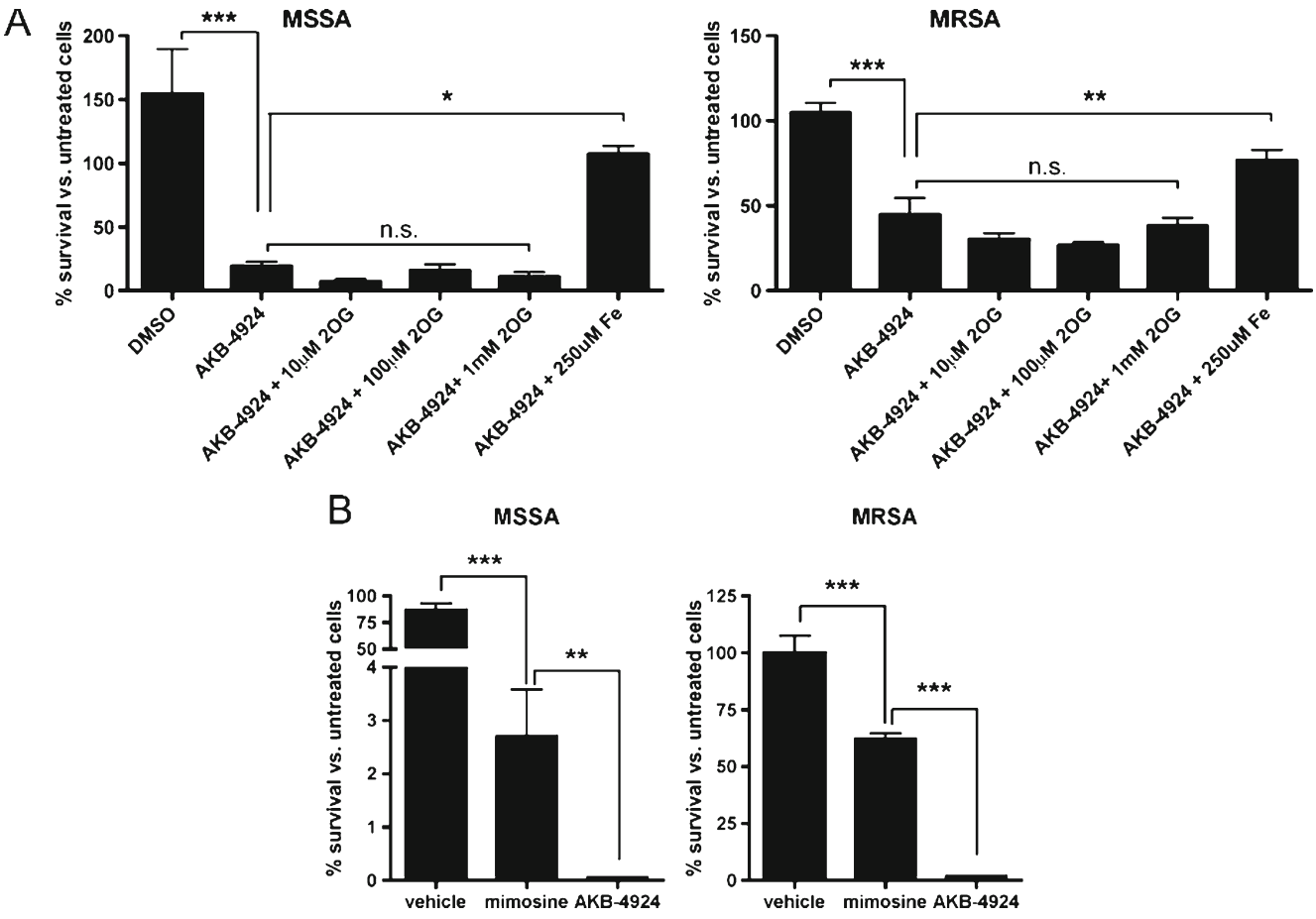

Fig. 3 AKB-4924 is a potent iron chelator. **a** AKB-4924 boosting of bactericidal activity is inhibited by iron, but not by 2-oxoglutarate. **b** AKB-4924 boosting of monocyte bactericidal activity is more robust than that observed with the classical HIF-1 agonist and iron chelator mimosine. For each panel, mean±SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$ by one-way ANOVA with Tukey's multiple comparison post-test

bMAP inhibition in a whole bacterial cell assay was extremely high, similar to other compounds that selectively inhibit the Fe (II)-form of bMAP [38]. We found that in iron-restricted cell culture media, log-phase growth of *S. aureus* was not affected in media containing the 10 μM dose of AKB-4924 used in our in vitro cell-based assays (Fig. 4b) and modestly slowed by 25 μM AKB-4924 during the first 5 h, with no significant differences seen at later time points (Fig. 4c). Supplementing the media with excess iron rescued the growth inhibition seen with 25 μM AKB-4924, supporting the mechanism of action of AKB-4924 on bacteria as an iron chelator (Fig. 4d).

In iron-rich bacteriologic media, growth of *S. aureus* was not inhibited in the presence of any concentration of AKB-4924 (Table 1). Furthermore, no minimal inhibitory concentration (MIC) could be established for AKB-4924 in bacteriologic media for a panel of Gram-positive and Gram-negative bacterial pathogens (Table 1). In other words, no significant direct antibacterial activity could be identified for AKB-4924 alone. To test whether AKB-4924 rendered the bacteria more susceptible to other antibiotics, we performed synergy assays with the cell wall active agents, penicillin, vancomycin, and daptomycin. AKB-4924 did not enhance bacterial susceptibility to killing with any of these antibiotics (Table 1). Furthermore, no synergy was seen with AKB-4924 and peroxide, a product of the neutrophil oxidative burst response that contributes to bacterial killing [39, 40] (Table 1). Finally, AKB-4924 itself did not exhibit cytotoxicity to a variety of eukaryotic cells, including liver and skin cells (Table 1). Thus, the effect of AKB-4924 is solely restricted to modulating the bactericidal response of the host cells and demonstrates a very favorable therapeutic index at its effective concentrations with only very limited cytotoxicity toward target host cells at high concentrations.

AKB-4924 enhances the bactericidal capacity of keratinocytes against skin pathogens

We have previously shown that HIF-1α is critical in keratinocyte defense against bacterial infection [11]. Given the ability of AKB-4924 to stabilize HIF-1α and enhance the immune response of phagocytes, we also tested the ability of the compound to enhance the bactericidal capacity of keratinocytes. Keratinocytes were pretreated with AKB-4924 to induce the HIF-1 regulated antimicrobial response and subsequently

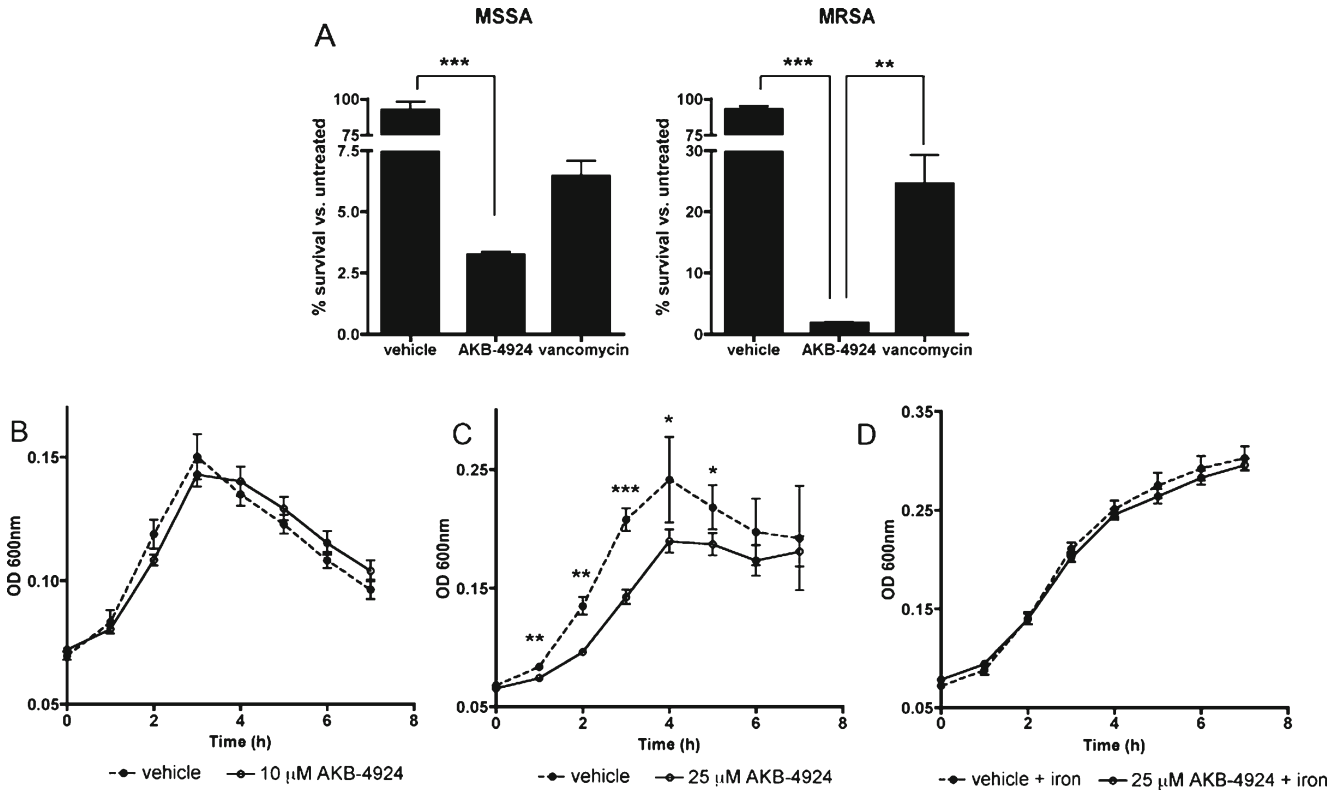

Fig. 4 AKB-4924 boosts immune cell bactericidal activity without exerting direct antibiotic action. **a** Incubation of U937 monocytes with 10 μM AKB-4924 led to equivalent (MSSA, *left*) or superior (MRSA, *right*) bacterial killing in vitro than observed with addition of 1 μg/mL vancomycin. **b** AKB-4924 does not have direct bactericidal activity (see Table 1) and no effect on bacterial growth at the concentration used in killing assays (10 μM). **c**, **d** Partial effects of AKB-4924 on bacterial growth at 25 μM are reversed by iron supplementation. For each panel, mean±SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$ by one-way ANOVA with Tukey's multiple comparison post-test (**a**) or by unpaired *t* test (**c**)

Table 1 AKB-4924 has no direct antimicrobial activity or cytotoxic effects on host cells

Test	Result
Growth inhibition in bacteriologic media (Todd–Hewitt broth)	None observed for MSSA, GAS, GBS
MIC assays in bacteriologic media (Meuller–Hinton Broth)	>200 μM for MSSA, MRSA, GAS, GBS, *P. aeruginosa*, *M. catarrhalis*, *H. influenza*
Antibiotic synergy	None with penicillin, vancomycin, daptomycin
Synergy with peroxide (H_2O_2)	None
Host cell cytotoxicity	HeLa cells: none to 100 μM AKB-4924
	HepG2 cells: none up to 50 μM AKB-4924
	HaCaT cells: none up to 50 μM AKB-4924

All tests were conducted in 10 μM AKB-4924 unless otherwise indicated and compared to vehicle and untreated controls

infected with *S. aureus*. Treatment with AKB-4924 significantly enhanced the ability of keratinocytes to kill both MSSA and MRSA, with the greatest bacterial killing achieved 3 h post-infection (Fig. 5a). The enhanced bactericidal activity of treated keratinocytes may be in part due to HIF-1 regulation of antimicrobial peptides (Fig. 2b and [11]). In addition to *S. aureus*, AKB-4924-treated keratinocytes were more effective at killing *P. aeruginosa* and *A. baumanii*, two Gram-negative opportunistic skin pathogens (Fig. 5b). Thus, AKB-4924 is an effective agent for enhancing the antimicrobial response in a variety of cell types against different medically important pathogens.

Reduction in *S. aureus* disease severity by AKB-4924 treatment

As AKB-4924 showed remarkable boosting of host cell bactericial activity in vitro, we sought to provide proof-of-principle of the potential of this compound to serve as a therapeutic agent in an in vivo infection. Outbred CD-1

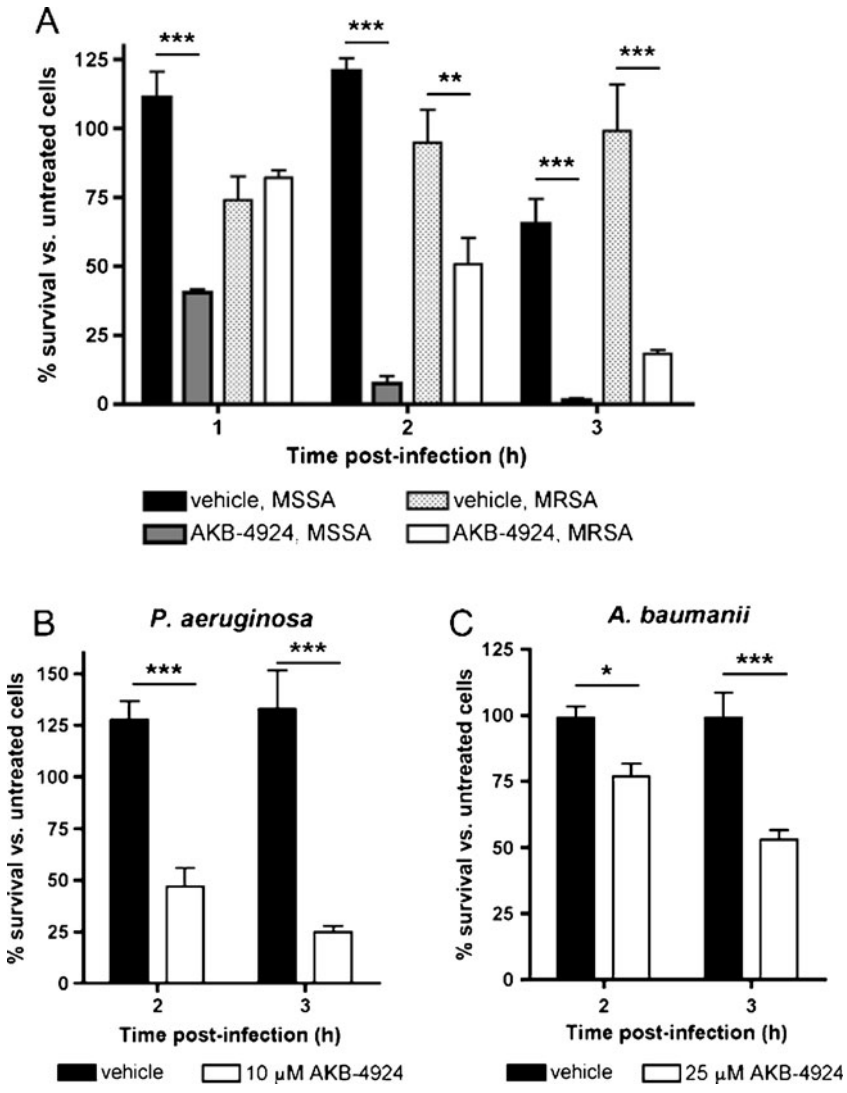

Fig. 5 AKB-4924 augments the ability of keratinocytes to kill bacterial pathogens. **a** Human HaCaT keratinocytes treated with 10 μM of AKB-4924 show increased bactericidal activity against both MSSA and MRSA strains over time. **b**, **c** HaCaT keratinocytes treated with the indicated concentrations of AKB-4924 boosts killing of Gram-negative pathogens *P. aeruginosa* and *A. baumanii*. A representative experiment is shown for each panel with mean±SD. **P*<0.05, ***P*<0.01, ****P*<0.001 by one-way ANOVA with Tukey's multiple comparison post-test (**a**) or by unpaired *t* test (**b**, **c**)

mice were infected subcutaneously with *S. aureus* and treated intralesionally with 1 mM AKB-4924 or vehicle at 0, 6, and 24 h post-infection. At 4 days post-infection, the average lesion size of AKB-4924-treated mice was 70% smaller than control-treated mice (Fig. 6a, b). Correspondingly, bacterial loads in the lesions were significantly lower in the AKB-4924-treated mice (Fig. 6c). In histopathological sections of uninfected skin, overall neutrophil recruitment was observed to be similar for mice treated with either AKB-4924 or vehicle alone (Fig. 6d). Thus, our in vitro and in vivo experiments collectively suggest that AKB-4924 can enhance of the bactericidal activities of host cells present at the site of infection to restrict bacterial spread.

Discussion

Skin and soft-tissue infections (SSTI), especially those caused by MRSA, are on the rise in the USA and several other countries and place a considerable burden on public health [41–43]. Challenges in the treatment of bacterial SSTI include factors such as antibiotic-resistant bacteria, compromised host immunity, and compromised barrier function. MRSA SSTIs are becoming an increasing problem in the community at large in addition to the hospital setting [42]. *P. aeruginosa*, normally considered commensal skin bacteria, can cause SSTI in immunocompromised or burn patients [44]. *A. baumanii* SSTIs are an emerging problem in some hospital settings and in combat wound patients [43, 45]. Effective treatments of these antibiotic-resistant pathogens must include tissue-penetrating drugs that have rapid bactericidal kinetics and low risk of developing resistance [46]. Critical cells in defense against SSTI infections include resident keratinocytes and neutrophils that rapidly influx to the site of infection. In our studies, AKB-4924 demonstrated strong enhancement of the bactericial activity of these cell types (Figs. 2e and 5a–c) and is rapid and equivalent or better than direct antibiotic treatment of bacteria (Fig. 4a).

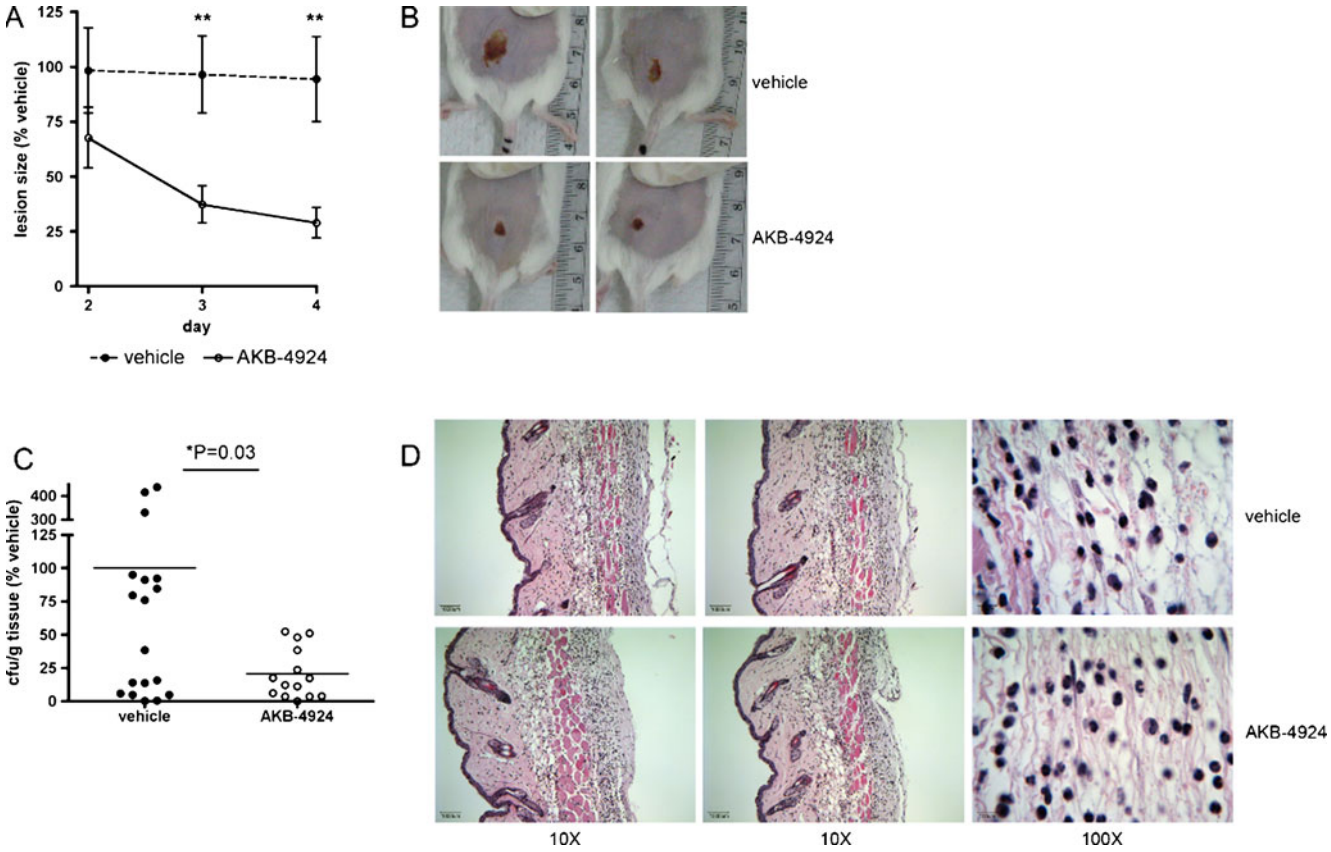

Fig. 6 AKB-4924 reduces disease severity in a *S. aureus* skin infection model. **a** Lesion size in AKB-4924 treated mice is significantly reduced compared to vehicle-treated mice. **b** Representative photographs of necrotic skin lesions on day 4 post-infection in control and AKB-4924-treated groups. **c** Bacterial loads recovered from the lesions are significantly reduced in AKB-4924 treated mice compared to vehicle-treated controls. Data were pooled from two independent experiments (n=14 for AKB-4924-treated mice, n=18 for vehicle treated mice) and shown with mean±SE. *P<0.05, **P<0.01 by unpaired t test with Welch's correction. **d** Histopathological sections (hematoxylin–eosin; original magnification, 10× and 100×) of skin treated with vehicle or AKB-4924 show similar levels of neutrophil recruitment. Neutrophils in the dermis were identified as cells containing lobulated nuclei at 100×. Image color levels were adjusted to set the white balance

Furthermore, because AKB-4924 has no direct antimicrobial activity (Fig. 4b, c and Table 1), its use as a therapeutic agent would have a low risk of developing resistance mechanisms in bacteria. AKB-4924 is furthermore not cytotoxic to host cells (Table 1). Thus, AKB-4924 possesses many properties that make it an attractive drug candidate as a potent adjunctive therapy to use alongside currently approved antibiotics for the treatment of multidrug SSTI infections.

Although AKB-4924 did not have direct antibacterial effects in our in vitro culture systems (Fig. 4b, c and Table 1), the compound belongs to a class of drugs with potential to exert antimicrobial activity. Furthermore, iron acquisition in *S. aureus* is important for full virulence of the pathogen and resistance to neutrophil killing [47, 48]. Future derivatization of AKB-4924 to enhance direct effects on bacterial growth and iron sequestration may therefore serve a dual purpose to both enhance host cell immunity by stabilizing HIF-1α and to directly inhibit bacterial growth and virulence. Finally, while the predominant effect of AKB-4924 is on HIF-1α, there is also an effect to increase HIF-2α (Fig. 1b, c). In the future, it will also be important to probe the differential effects of AKB-4924 on different PHD enzymes for optimization of AKB-4924 to target different aspects of HIF-1 function and potentially other targets of PHDs such as IKKβ.

In summary, we have discovered a new pharmacological compound that has potent HIF-1 inducing activity and can efficiently boost the host immune response of resident skin cells against important bacterial pathogens both in vitro and in vivo. This novel pharmacological approach may serve as a useful adjunctive therapy against a variety of bacterial infections, including those complicated by resistance to classical antibiotics.

Acknowledgements We would like to thank Katja Branitzki-Heinemann, Yohan Penny, and Sabina Anik for technical assistance, Drs. Laura Crotty-Alexander and Mary Hensler for advice regarding experimental design, and Dr. Charlotte Hartman for assistance with project planning. Support for this work was provided by NIH grant AI090863 (V.N., R.S.J., R.S.) and USAMRAA award (R.S., V.N.). C.Y. M.O. was supported through the UCSD/SDSU IRACDA Postdoctoral Fellowship Program (GM06852).

Conflicts of interest R.A. Shalwitz and A. Kotsakis are employees of Aerpio Therapeutics.

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