Innate Effector Systems in Primary Human Macrophages Sensitize Multidrug-Resistant *Klebsiella pneumoniae* to Antibiotics

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ABSTRACT Infections caused by multidrug-resistant (MDR) *Klebsiella pneumoniae* are difficult to treat with conventional antibiotics. Thus, alternative strategies to control the growth of MDR *Klebsiella* are warranted. We hypothesized that activation of innate effector systems could sensitize MDR *K. pneumoniae* to conventional antibiotics. Thus, human primary macrophages were stimulated with compounds known to activate innate immunity (vitamin D₃, phenylbutyrate [PBA], and the aroylated phenylenediamine HO53) and then infected with MDR *Klebsiella* in the presence or absence of antibiotics. Antibiotics alone were ineffective against MDR *Klebsiella* in the cellular model, whereas vitamin D₃, PBA, and HO53 reduced intracellular growth by up to 70%. The effect was further improved when the innate activators were combined with antibiotics. Vitamin D₃- and PBA-induced bacterial killing was dependent on *CAMP* gene expression, whereas HO53 needed the production of reactive oxygen species (ROS), as shown in cells where the *CYBB* gene was silenced and in cells from a patient with reduced ROS production due to a deletion in the *CYBB* gene and skewed lyonization. The combination of innate effector activation by vitamin D₃, PBA, and HO53 was effective in sensitizing MDR *Klebsiella* to conventional antibiotics in a primary human macrophage model. This study provides new evidence for future treatment options for *K. pneumoniae*.

KEYWORDS *Klebsiella*, antimicrobial peptides, autophagy, innate immunity, reactive oxygen species

Klebsiella pneumoniae is one of the major problems of human health as identified by the World Health Organization (1). *K. pneumoniae* is considered an opportunistic pathogen, colonizing the mucosal surfaces of many individuals without causing infections. However, it is one of the most important Gram-negative bacteria in nosocomial infections. For example, *K. pneumoniae* can cause severe pulmonary infections, urinary tract infections, and sepsis, particularly in very young or elderly patients with compromised immunity (2). Treatment of *K. pneumoniae* infections includes cephalosporins and carbapenems, but resistance against these drugs is emerging. In fact, the frequent use of these antibiotics is driving the growing prevalence of multidrug-resistant (MDR) strains, which leads to increased concerns about present and future treatment options (3). Additionally, *K. pneumoniae* is a known reservoir for antibiotic resistance genes, which can spread to other Gram-negative bacteria. Many of the antibiotic resistance genes commonly found in multidrug-resistant organisms were first identified in *Klebsiella* (4). A recent report from the European Centre for Disease Prevention and Control demonstrated that more than one third of *K. pneumoniae* isolates were resistant to at

Citation Rekha RS, Karadottir H, Ahmed S, Gudmundsson GH, Agerberth B, Bergman P. 2020. Innate effector systems in primary human macrophages sensitize multidrug-resistant *Klebsiella pneumoniae* to antibiotics. *Infect Immun* 88:e00186-20. <https://doi.org/10.1128/IAI.00186-20>.

Editor Manuela Raffatellu, University of California San Diego School of Medicine

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Received 1 June 2020

Accepted 3 June 2020

Accepted manuscript posted online 8 June 2020

Published 21 July 2020

least one antibiotic class. It has been reported that the combined resistance to the third-generation cephalosporins, fluoroquinolones, and aminoglycosides is the most common resistance phenotype in Europe (5). There are few therapeutic options left for patients infected with MDR *K. pneumoniae* with additional resistance to carbapenems and colistin, which are the “last-resort” antibiotic agents to treat MDR *Klebsiella* infections.

Traditionally, *K. pneumoniae* is considered an extracellular bacterium, but recent data clearly show that *K. pneumoniae* survives intracellularly in macrophages by limiting the fusion of lysosomes with the *K. pneumoniae*-containing phagosomes (6). This intracellular reservoir of *K. pneumoniae* bacteria is difficult to treat since several key antibiotics, such as beta-lactams and aminoglycosides, have limited penetration to the phagosomal compartment (6). Given the problems with emerging MDR *K. pneumoniae* together with the lack of new antibiotic drugs, novel and alternative treatment approaches are needed to treat infections caused by this persistent and frequently lethal intracellular pathogen. Host-directed therapies (HDT) against infections have appeared as a promising possibility for adjunctive treatment to modulate immune responses in order to contribute to the elimination of the pathogen in question. This approach may be successful against difficult-to-treat infections, especially together with classic antibiotic drugs. The strategy for HDT is based on small molecules that can induce host immune mechanisms, such as antimicrobial peptides (AMPs), reactive oxygen species (ROS) production, and activation of autophagy in tissues and cells. Our group has shown that vitamin D₃ and phenylbutyrate (PBA) have a strong synergistic effect on induction of AMPs in lung epithelial cell lines and human macrophages (7, 8) and reduce intracellular growth of *Mycobacterium tuberculosis* (7). Recently, we showed that the aroylated phenylenediamine compound HO53 stimulates the expression of the *CAMP* gene, encoding the human cathelicidin LL-37, in human bronchial epithelial cells, improves the barrier function, and induces bactericidal effects in an infection model of *Pseudomonas aeruginosa* (9). Given the capacity of vitamin D₃, PBA, and HO53 to reduce the growth of bacteria via activation of innate effector systems, we hypothesized that these compounds could sensitize MDR *K. pneumoniae* to conventional antibiotics. To test this concept, we designed a study where antibiotics (azithromycin, cefotaxime, ciprofloxacin, and fosfomycin) were combined with three selected inducers of innate immunity (vitamin D₃, PBA, and HO53). The effect against susceptible and MDR *K. pneumoniae* was assessed in primary human macrophages. The mechanistic basis for the observed effects was dissected using knockdown and blocking experiments as well as cells from a patient with a gene deletion in a ROS-producing enzyme.

RESULTS

Conventional antibiotics lack activity against MDR *Klebsiella* in primary human macrophages but rapidly kill susceptible *Klebsiella*. Conventional antibiotics can act against phagocytosed susceptible *K. pneumoniae* but lack activity against phagocytosed MDR *K. pneumoniae* (6). We hypothesized that induction of innate effector systems could sensitize phagocytosed MDR *K. pneumoniae* to antibiotics that are not normally active against these isolates. To test our hypothesis, we first selected four conventional antibiotics with different mechanisms (azithromycin, cefotaxime, ciprofloxacin, and fosfomycin) and investigated their effects separately against the selected *K. pneumoniae* isolates. Notably, none of the antibiotic drugs were effective against the MDR isolate (Fig. 1A), whereas the susceptible *K. pneumoniae* isolate was readily killed (Fig. 1B). These results were consistent with the *in vitro* determined MIC values for the isolates and show that the cellular model can be used to test the intracellular effects of antibiotics and potential interactions between antibiotics and innate effector molecules (Table 1).

Activators of innate effector systems enhance antibacterial activity of infected human macrophages. We previously showed that the two FDA-approved drugs vitamin D₃ and PBA activate innate effector systems, including the cathelicidin LL-37 and human beta defensin 1 (8). In addition, we recently characterized a novel aroylated

FIG 1 Conventional antibiotics lack activity against MDR *Klebsiella* inside primary human macrophages but can rapidly kill susceptible *Klebsiella*. MDMs were infected with MDR (AO15200) and wild-type (ATCC 25955) *Klebsiella* strains for 30 min with an MOI of 1:5. The remaining extracellular bacteria were eliminated with gentamicin (25 μ g/ml) treatment for 30 min. The MICs of the antibiotics (azithromycin, 1 mg/liter; cefotaxime, 16 mg/liter; ciprofloxacin, 16 mg/liter; and fosfomycin, 0.0625 g/liter) were administered after bacterial uptake. CFU were measured after 5 h of infection. (A and B) Bacterial survival is presented as CFU/ml of (A) MDR *Klebsiella* and (B) wild-type *Klebsiella*. Each bar represents a median value of 9 independent experiments $\pm$ interquartile range (IRQ). Statistical significance was calculated using the Mann-Whitney U test. **, $P < 0.01$. The untreated control (black) was used for all statistical tests in relation to treatment with the different antibiotics.

phenylene diamine compound (HO53) with potent AMP-inducing capacity (9, 10). Here, we analyzed whether these molecules could have antibacterial effects in a cellular infection model using primary human monocyte-derived macrophages (MDMs). MDMs were treated with PBA, vitamin D₃, and HO53 separately and in combinations for 24 h before infection. Following infection, the remaining extracellular bacteria were eliminated by treatment with gentamicin, and the intracellular bacteria were counted as CFU. Notably, PBA, vitamin D₃, and HO53 alone or in combinations (PBA + vitamin D₃ and HO53 + vitamin D₃) reduced the growth of both MDR *K. pneumoniae* and the susceptible strain in the model (Fig. 2A and B). Bacterial uptake into the MDMs after 1 h

TABLE 1 MICs of ATCC 25955 and AO15200^a

Antibiotic(s)	MIC (mg/liter) for: ^c	
	<i>K. pneumoniae</i> (ATCC 25955)	<i>K. pneumoniae</i> (AO15200)
Amikacin	2 (S)	16 (I)
Cefotaxime	0.12 (S)	>8 (R)
Ceftazidime	0.5 (S)	>16 (R)
Ceftazidime-avibactam	0.25 (S)	>32 (R)
Ceftolozane-tazobactam	1 (S)	>32 (R)
Ciprofloxacin	0.12 (S)	>4 (R)
Colistin	1 (S)	1 (S)
Gentamicin	0.5 (S)	1 (S)
Imipenem	0.5 (S)	16 (R)
Meropenem	0.12 (S)	>16 (R)
Piperacillin-tazobactam	16 (I)	>32 (R)
Tigecycline	0.25 (NI)	1 (NI)
Tobramycin	1 (S)	8 (R)
Azithromycin ^b	16	>256
Trimethoprim-sulfamethoxazole ^b	0.19 (S)	>32 (R)
Doxycycline ^b	8	24
Tetracycline ^b	8	>256
Fosfomycin ^b	12 (S)	24 (S)

^aThe MIC (mg/liter) was determined for both strains (ATCC 25955 and AO15200) with a standard broth microdilution method (BMD panel). For the antibiotics azithromycin, trimethoprim-sulfamethoxazole, doxycycline, tetracycline, and fosfomycin, an Etest was used for MIC determination.

^bNo clinical breakpoint (EUCAST) is registered for this antibiotic on *Enterobacteriales* species.

^cI, susceptible, increased exposure; NI, noninterpretable; R, resistant; S, susceptible, standard exposure.

FIG 2 Activators of innate effector systems enhance antibacterial activity of infected human macrophages. Vitamin D₃, PBA, and HO53 enhance the antimicrobial response in MDMs. MDMs were stimulated with 10 nM vitamin D₃, 2 mM PBA, and 12.5 μM HO53 for 24 h and then infected with the two strains of *Klebsiella* for 30 min. The remaining extracellular bacteria were eliminated with gentamicin (25 μg/ml) treatment for 30 min. CFU were measured after 5 h of infection. Bacterial survival is presented as CFU/ml (A and B). Each bar represents a median value of 9 independent experiments ± interquartile range (IQR). Statistical significance was calculated using the Mann-Whitney U test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The untreated control (black) was used for all statistical tests in relation to the various treatments.

of infection was similar between the treatment groups (see Fig. S1 in the supplemental material), and the growth curves of these two strains were similar (Fig. S2). Control experiments with a *K. pneumoniae* capsule mutant showed a much higher uptake into the MDMs, and a similar level of bacterial killing was observed, suggesting that the capsule protected against uptake but not against intracellular killing (Fig. S3). Thus, PBA, vitamin D₃, and HO53 induced significant bacterial killing of both MDR *K. pneumoniae* and the susceptible strain in MDMs. Notably, the effect was significantly enhanced when vitamin D₃ was combined with PBA ($P \leq 0.0001$) and HO53 ($P \leq 0.0001$). The compounds alone did not reduce the growth of bacteria directly (Fig. S4). Likewise, the compounds were not toxic to the cells at the concentrations used (Fig. S5).

Activation of innate effector systems increases the effects of conventional antibiotics against MDR *Klebsiella*. Next, we investigated the effects of different antibiotics in combination with the innate inducers PBA, vitamin D₃, and HO53. The antibacterial effect of azithromycin (1 mg/liter) was significantly enhanced together with PBA ($P = 0.050$), PBA + vitamin D₃ ($P = 0.028$), HO53 ($P = 0.009$), and HO53 + vitamin D₃ ($P = 0.005$) (Fig. 3A). Cefotaxime (16 mg/liter) showed a significant effect together with PBA + vitamin D₃ ($P = 0.013$), HO53 ($P = 0.010$), and HO53 + vitamin D₃ ($P = 0.050$) (Fig. 3B). Ciprofloxacin (16 mg/liter) exhibited enhanced bactericidal activity together with PBA + vitamin D₃ ($P = 0.028$), HO53 ($P = 0.048$), and HO53 + vitamin D₃ ($P = 0.022$) (Fig. 3C). Fosfomycin (0.0625 g/liter) significantly enhanced the intracellular killing capacity of MDMs together with PBA + vitamin D₃ ($P = 0.017$) and HO53 + vitamin D₃ ($P = 0.045$) (Fig. 3D). Interestingly, these antibiotics were not active alone against MDR *Klebsiella* (Fig. 1A) but gained activity against the MDR strain together with several of the innate inducers. The susceptible strain was also analyzed in the MDM cellular model with antibiotics and innate inducers. Notably, all antibiotics were active alone but also together with the innate inducers (Fig. S4). Combined, these results show that the innate inducers used here can increase susceptibility of MDR *K. pneumoniae* to conventional antibiotics not normally active against this bacterial isolate.

PBA, vitamin D₃, and HO53 induce the expression of antimicrobial peptides and oxidative stress-related genes in human macrophages infected with *Klebsiella*. Next, we set out to define the molecular mechanisms involved in the observed antimicrobial effects exerted by the three inducers. Since antimicrobial peptides (AMPs) have been reported to be involved in the control of intracellular bacterial growth, we

FIG 3 Activation of innate effector systems increases the effects of conventional antibiotics against MDR *Klebsiella*. MDMs were pretreated with 10 nM vitamin D₃, 2 mM PBA, and 12.5 μ M HO53 for 24 h separately or in combination before infection with the MDR *Klebsiella* strain for 30 min. The MICs of the antibiotics (azithromycin, 1 mg/liter; cefotaxime, 16 mg/liter; ciprofloxacin, 16 mg/liter; and fosfomycin, 0.0625 g/liter) were administered after bacterial uptake. The additive effect of antibiotics with the innate inducers was analyzed by comparison with the control group (without antibiotics). Survival of MDR *Klebsiella* is shown in the presence (white) and absence (black) of antibiotics. (A) azithromycin; (B) cefotaxime; (C) ciprofloxacin; (D) fosfomycin. Bacterial survival is presented as CFU/ml. Each bar represents a median value of 9 independent experiments $\pm$ interquartile range (IQR). Statistical significance was calculated using the Mann-Whitney U test. *, $P < 0.05$; **, $P < 0.01$; #, significant compared to untreated control (black bar); ###, $P < 0.001$.

assessed the expression levels of several human AMPs, the cathelicidin LL-37 (encoded by the *CAMP* gene), human beta-defensin 1 (HBD-1), human beta defensin 2 (HBD-2), and human beta defensin 4 (HBD-4). Treatment with PBA, vitamin D₃, and HO53 significantly induced LL-37/*CAMP* and HBD-1 mRNA expression in MDMs with or without MDR *K. pneumoniae* infection (Fig. 4A and B). LL-37 expression remained elevated during the infection, whereas HBD-1 showed a rapid induction followed by a distinct decline in expression at the 5-h time point (Fig. 4B). HBD-2 was significantly induced by vitamin D₃ + PBA, HO53, and HO53 + vitamin D₃, whereas HBD-4 was induced by HO53 and HO53 + vitamin D₃ (Fig. 4C and D). In addition, the expression of oxidative stress-related genes, such as nitric oxide synthase-2 (NOS2), dual oxidase 2 (duox2), the *CAT* gene (encoding catalase), and glutathione peroxidase 3 (GPx-3), was upregulated by the treatment of PBA, PBA + vitamin D₃, and HO53 + vitamin D₃ (Fig. 4E to H). Autophagy is related to control of bacterial viability in MDMs, and autophagy-related genes, *atg5* and *atg12*, were also upregulated by the different inducers (Fig. 4I and J). Finally, the expression profiles of different pro- and anti-inflammatory cytokines were investigated in macrophages with or without MDR *K. pneumoniae* infection. No significant changes of interleukin-8 (IL-8) or IL-17 were observed. However, the expression of IL-23 was significantly increased after MDR *K. pneumoniae* infection in the different treatment groups (Fig. S7). The expression pattern of AMPs and oxidative stress-related genes was similar when MDMs were infected with susceptible strains of *K. pneumoniae* (Fig. S8). To conclude, three different innate effector arms (AMPs, ROS/NOS, and autophagy) with the capacity to be active against *K. pneumoniae* were induced by PBA, vitamin D₃, and HO53 as well as by their combinations.

FIG 4 Vitamin D₃, PBA, and HO53 induce the expression of antimicrobial peptides, oxidative stress, and autophagy-related genes in human macrophages infected with *Klebsiella*. MDMs were stimulated with (Continued on next page)

FIG 5 Vitamin D₃ alone and in combination with PBA depends on LL-37 for killing of MDR *Klebsiella* in human macrophages. (A) LL-37 mRNA expression and (B) intracellular killing of the MDR-*Klebsiella* in *CAMP*-siRNA-transfected (siRNA-CAMP versus siRNA-mock) macrophages. MDMs were transfected with siRNA (30 pmol) for 24 h before treatment with 10 nM vitamin D₃, 2 mM PBA, and 12.5 μM HO53 for 24 h before infection. The mRNA expression was determined using qRT-PCR and presented as *CAMP* mRNA fold-induction in siRNA-CAMP-transfected macrophages compared to the siRNA-mock-transfected controls. Intracellular killing was determined using CFU/ml counts. Results were obtained from *n* = 4 donors. Data (median ± IQR) are presented in bar graphs. Statistical significance was calculated using the Mann-Whitney U test. *, *P* < 0.05; #, significant compared to untreated control (black bar); ##, *P* < 0.01.

Vitamin D₃ alone and in combination with PBA depends on LL-37 for killing of MDR *Klebsiella* in human macrophages. Since all tested compounds induced the expression of the *CAMP* gene, encoding LL-37, we next sought to assess the contribution of LL-37 in the observed killing of *K. pneumoniae* in the human macrophage infection model. To inhibit expression and function of LL-37, we transfected primary macrophages with small interfering RNA (siRNA) specific for the *CAMP* gene (*CAMP*-siRNA). Transfection with *CAMP*-siRNA and stimulation with PBA, vitamin D₃, and HO53 effectively downregulated *CAMP* mRNA expression in *K. pneumoniae*-infected macrophages (Fig. 5A). The CFU assay showed that PBA-, vitamin D₃-, and vitamin D₃ + PBA-mediated growth inhibition of *K. pneumoniae* was mainly restored by silencing the expression of LL-37 compared to mock-transfected cells (Fig. 5B). Notably, inhibition of the *CAMP* gene expression did not inhibit bacterial killing induced by HO53, which suggested that this compound activates additional bactericidal effector mechanisms in human macrophages.

PBA-, vitamin D₃-, and HO53-mediated killing of *Klebsiella* in human macrophages is inhibited in primary macrophages deficient in reactive oxygen species. Given that ROS-related genes were induced by all tested compounds (Fig. 4), we set out to study the role of ROS by using MDMs from a patient with a mutation in the *CYBB* gene (see Materials and Methods for details). In brief, the disorder was an X-linked trait and presented clinically as recurrent bacterial abscesses in the skin and internal organs. A functional analysis revealed that the patient (female) had a skewed lyonization (inactivation of the X-chromosome) and 18% ROS production in the neutrophilic granulocytes, which explained the clinical presentation of an X-linked disorder in a female patient. In fact, the ROS production of this patient was just under the limit (20%)

FIG 4 Legend (Continued)

10 nM vitamin D₃, 2 mM PBA, and 12.5 μM HO53 for 24 h and then were infected with the MDR *Klebsiella* strain for 30 min. The remaining extracellular bacteria were eliminated with gentamicin (25 μg/ml) treatment for 30 min. The mRNA expression of the different genes was measured before infection and at 1 h and 5 h after bacterial uptake. (A to D) Expression of antimicrobial peptide gene (A) *CAMP*/LL-37 mRNA expression, (B) hBD1 mRNA expression, (C) hBD2 mRNA expression, and (D) hBD4 mRNA expression. (E to H) Expression of oxidative stress-related gene (E) NOS2 mRNA expression, (F) duox2 mRNA expression, (G) catalase mRNA expression, and (H) GPX3 mRNA expression. (I and J) Expression of autophagy-related gene (I) Atg5 mRNA expression and (J) Atg12 mRNA expression (normalized to 18S rRNA expression). Each bar represents a median value of 6 independent experiments ± interquartile range (IQR). Statistical significance was calculated using the Mann-Whitney U test compared with the untreated control. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

FIG 6 Reactive oxygen species (ROS) are essential for HO53-, vitamin D₃-, and PBA-mediated killing of *Klebsiella* in human macrophages. (A) Intracellular killing of the MDR *Klebsiella* in ROS-deficient versus healthy donor macrophages. Data are presented as the median $\pm$ IQR. (B) NOX2 mRNA expression. (C) Intracellular killing of the MDR *Klebsiella* in CYBB-siRNA-transfected (siRNA-mock versus siRNA-CYBB) macrophages. MDMs were transfected with siRNA (30 pmol) for 24 h before treatment with 10 nM vitamin D₃, 2 mM PBA, and 12.5 μ M HO53 for 24 h, followed by the infection. The mRNA expression was determined using qRT-PCR and presented as NOX2 (CYBB) mRNA fold-induction in siRNA-CYBB-transfected macrophages compared to the siRNA-mock-transfected controls. Intracellular killing was determined using CFU/mL. Data were obtained from $n = 1$ donor for panel A and $n = 4$ donors for panels B and C. Statistical significance was calculated using the Mann-Whitney U test. *, $P < 0.05$; **, $P < 0.01$; #, significant compared to untreated control (black bar); ##, $P < 0.01$; ###, $P < 0.001$.

for when a skewed lyonization presents with symptoms (11). Whole-exome sequencing combined with bioinformatic analysis performed by the Karolinska University Laboratory showed a mutation (deletion of exon 4) in the *CYBB* gene. We determined the intracellular killing capacity in macrophages from the patient treated with PBA, vitamin D₃, PBA + vitamin D₃, HO53, and HO53 + vitamin D₃ and compared that to macrophages from a healthy control. Interestingly, the intracellular bacterial growth was significantly higher in macrophages from the ROS-deficient patient than in those from the healthy control in all treatment conditions, suggesting a crucial role for ROS in the control of intracellular growth of MDR *K. pneumoniae* in human macrophages (Fig. 6A). To validate these findings in an experimental system, MDMs were transfected with siRNA specific for *CYBB* expression (CYBB-siRNA). Transfection with CYBB-siRNA resulted in a blunted expression of NOX2 (CYBB) mRNA expression after stimulation with the innate inducers (Fig. 6B). Accordingly, the CFU assay showed that all inducers tested required intact ROS production to various extents to inhibit bacterial growth, since knockdown of *CYBB* expression resulted in significantly increased bacterial growth under all conditions (Fig. 6C).

Inhibition of autophagy reduces intracellular killing of MDR *Klebsiella* in human macrophages and limits the effects of innate inducers. Autophagy constitutes an important mechanism to control intracellular growth of *K. pneumoniae* in macrophages (12, 13). To study the contribution of autophagy in PBA-, vitamin D₃-, and HO53-mediated intracellular MDR *K. pneumoniae* growth inhibition, bafilomycin A1, a

FIG 7 Vitamin D₃, PBA, and HO53 depend on autophagy to mediate killing of MDR *Klebsiella* in human macrophages. MDMs were treated with 10 nM vitamin D₃, 2 mM PBA, and 12.5 μ M HO53 prior to infection with MDR *Klebsiella*. After infection, MDMs were treated with 0.5 μ M bafilomycin A1. Intracellular killing was determined using CFU/ml. Data were obtained from $n = 5$ donors. Data represent the median $\pm$ IQR. Statistical significance was calculated using the Mann-Whitney U test. **, $P < 0.01$; ***, $P < 0.001$; #, significant compared to untreated control (black bar); ###, $P < 0.001$.

specific inhibitor of the autophagy process, was utilized. This inhibitor inhibits the acidification of the lysosome and blocks the fusion of the autophagosome with the lysosome. *K. pneumoniae*-infected macrophages which were treated with bafilomycin A1 showed enhancement of *K. pneumoniae* growth, suggesting that autophagy is important for physiological control of intracellular *K. pneumoniae* (Fig. 7). Notably, treatment of macrophages with bafilomycin A1 resulted in a significantly reduced effect of PBA ($P = 0.004$), vitamin D₃ ($P = 0.0008$), PBA + vitamin D₃ ($P = 0.0004$), HO53 ($P = 0.0002$), and HO53 + vitamin D₃ ($P = 0.0005$) on bacterial growth (Fig. 7). These results are in line with the upregulation of autophagy-related genes *atg5* and *atg12* in MDMs after different treatments (Fig. 4D). Interestingly, cells from the patient with a deletion in the *CYBB* gene had normal AMP expression (Fig. 8A). In contrast, impaired autophagy was detected, as shown by reduced transcription of *Atg5* and *Atg12* as well as reduced LC3 levels, further supporting a role for autophagy in intracellular control of MDR *K. pneumoniae* (Fig. 8B and C). Thus, our results indicate that autophagy is essential for control of intracellular growth of MDR *K. pneumoniae* and that autophagy contributes to PBA-, vitamin D₃-, and HO53-mediated bactericidal effects against MDR *K. pneumoniae* in macrophages.

DISCUSSION

In this study, we demonstrated that induction of innate effector mechanisms can sensitize MDR *K. pneumoniae* to antibiotics that otherwise are inactive against this isolate. The mechanism behind this observed effect depended on a combination of

FIG 8 Expression of antimicrobial peptides and autophagy-related genes in ROS-deficient human macrophages. (A) Expression of antimicrobial peptide genes (LL-37, hBD1, hBD2, and hBD4). (B) Expression of autophagy-related genes (*Atg5* and *Atg12*) (normalized to 18S rRNA expression). (C) A representative Western blot of autophagy protein LC3-II expression in healthy and ROS-deficient macrophages and the housekeeping protein β -actin. Data were obtained from 6 healthy donors and 1 ROS-deficient patient (samples tested in triplicate). Statistical significance was calculated using the Mann-Whitney U test. *, $P < 0.05$.

effector systems, which were activated differentially depending on the innate inducer drug used. For example, vitamin D₃ and PBA depended on the induction of LL-37, ROS production, and autophagy to reduce intracellular growth of *K. pneumoniae*. In contrast, the recently described compound HO53, an aroylated phenylene diamine, needed ROS to kill intracellular bacteria. Consequently, the interaction between the innate effector systems with antibiotics was also different depending on the activated innate effector pathway and the mechanism employed by the antibiotic drug. Azithromycin, for example, was inactive against the MDR *K. pneumoniae* isolate alone but showed an additive effect together with PBA, PBA + vitamin D₃, HO53, and HO53 + vitamin D₃. Likewise, cefotaxime, ciprofloxacin, and fosfomycin did not kill MDR *K. pneumoniae* alone but exhibited additive effects together with various combinations of the innate inducers. The most striking finding in this study was when macrophages from a patient with reduced ROS production were infected with *K. pneumoniae*. Macrophages derived from this patient were unable to control intracellular growth of this bacterium. Notably, all combinations with vitamin D₃ still reduced the growth of intracellular *K. pneumoniae* in these cells, despite the lack of ROS, which is in line with the fact that vitamin D activates LL-37 expression and most likely other AMPs in macrophages. This is in line with the original results from Robert Lehrer's work in the mid-1970s when it was shown that fungicidal activity in an extract of neutrophilic granulocytes from patients with myeloperoxidase deficiency (ROS-deficient) originated from cationic antimicrobial peptides (14). These AMPs were later shown to be human α -defensins (15). In contrast, we showed here that HO53 exhibited a significantly reduced effect in ROS-deficient patient cells and in cells with an inactivated *CYBB* gene, consistent with a ROS-dependent mechanism for this compound. We also observed that macrophages from the ROS-deficient patient exhibited reduced autophagy, which may contribute to the lack of intracellular growth control of bacteria. In fact, reactive oxygen species have been shown to be essential for LC3-associated phagocytosis, and our data could support such a model for intracellular killing of *K. pneumoniae* (16).

Clearly, macrophages are important cells in the defense against *K. pneumoniae*. Recently, it was shown that *Bacteroidetes* spp. in the normal flora are essential for the protection against *K. pneumoniae* by a mechanism involving IL-36 signaling and macrophages (17). In addition, the key role of endogenous ROS production against *K. pneumoniae* has also been established (18). Further, patients with chronic granulomatous disease (CGD), lacking or having severely impaired ROS production, have been shown to suffer from invasive *K. pneumoniae* infections (19), which underscores that ROS is, indeed, important for physiological growth control of *K. pneumoniae* in macrophages. In fact, our own results are consistent with these results and show clearly that ROS is needed for bacterial control in normal cells but also that the novel inducer studied here (HO53) is dependent on this pathway in macrophages.

It is relevant to consider the role of ROS in relation to antibiotic treatment. For example, ciprofloxacin, a topoisomerase inhibitor, is known to activate ROS in order to kill bacteria (20, 21). Notably, we showed that ciprofloxacin exhibited an additive effect together with HO53, which could indicate that endogenous and bacterial ROS production could play a synchronized role in bacterial killing.

Fosfomycin was also studied here by virtue of its clinical use against MDR Gram-negative bacteria. Besides its known target in the bacterial cell wall (MurA), fosfomycin has been shown to activate the ROS pathway in target cells, which resulted in increased intracellular killing of *Staphylococcus aureus* (22). In line with these results, we found that fosfomycin exhibited an additive effect together with HO53, similar to ciprofloxacin, suggesting that under these conditions also, endogenous and bacterial ROS production might cooperate in bacterial killing.

It should be noted that ROS production has been implicated in the development of bacterial resistance by promoting DNA-damage and the selection of resistant mutants (23). Thus, ROS is indeed a double-edged sword, and potential ROS induction as a therapeutic intervention should be used with caution in the context of host microbial interactions.

Here, we also observed that enhanced transcription of the *CAMP* gene, encoding the human cathelicidin LL-37, was important for vitamin D₃- and PBA-mediated inhibition of *Klebsiella* growth. The precise mechanism for the involvement of LL-37 in bacterial killing in this model is not entirely clear. It is known that LL-37 is active against *Klebsiella in vitro* (24), which indicates that there could be a direct antimicrobial killing mechanism exerted by this peptide in our cellular model. In the case that LL-37 interacts directly with bacteria, our results are in line with data from Sakoulas and Nizet, who showed that subinhibitory concentrations of LL-37 sensitized several MDR bacterial species to azithromycin (25). The proposed mechanism was that LL-37 damaged the bacterial membrane, which facilitated the transport of azithromycin to its ribosomal target inside the bacterium. It is possible that this mechanism can explain our findings that azithromycin had additive effects together with several of the innate inducing drugs tested.

It has been shown that *K. pneumoniae* can inhibit the autophagy process and survive in macrophages by blocking the fusion of bacterium-containing vesicles with lysosomes (6). PBA and vitamin D₃ have the capacity to activate autophagy through LL-37-dependent pathways and kill intracellular bacteria, such as *Mycobacterium tuberculosis* (7). Notably, we observed that blocking autophagy with bafilomycin A1 reduced the effect of all inducers, suggesting that autophagy is involved in the observed effects.

The concept of sensitization of MDR bacteria has been gaining increased interest given the limited pipeline of new antibiotic drugs. Numerous approaches are being explored, including CRISPR/Cas9-mediated deletion of extended-spectrum beta-lactamase (ESBL) enzymes in plasmids, blocking efflux channels, and applying membrane-active drugs in combination with antibiotics (26–28). Here, we have demonstrated that the concept of activating innate effector systems, including induction of AMP expression, ROS production, and autophagy in combination with antibiotics, known to interact with cellular pathways counteracts resistance. Our results show that activation of innate effector systems leads to additive effects against MDR *Klebsiella* in a primary macrophage infection model. Notably, different inducers activated specific pathways and consequently exhibited differential interactions with the antibiotic drugs. For example, vitamin D₃ + PBA-mediated bacterial killing relied on the *CAMP* gene and exhibited an additive effect together with the cell wall-active antibiotic cefotaxime. In contrast, the novel innate inducer HO53 depended more on the ROS pathway and had a significant additive effect with ciprofloxacin, an antibiotic drug known to activate the ROS pathway. Importantly, the activation of several effector mechanisms at the same time appears to be very effective against MDR bacteria. The potential resistance development against these simultaneous attacks of innate effectors is unlikely given the broad bacterial targets used by AMPs, ROS, and autophagy.

Our data suggest that the concept of sensitizing MDR bacteria to conventional antibiotics holds great promise for future treatment of infections caused by MDR *K. pneumoniae* and other bacteria that are difficult to treat. However, it is expected that the innate immune system employs a specific repertoire against individual bacteria, and therefore a detailed understanding of these mechanisms will be essential to fully exploit this concept in the treatment of human infections.

MATERIALS AND METHODS

Primary human macrophages. Monocyte-derived macrophages (MDMs) were obtained from buffy coats from healthy donors at the Karolinska Hospital Blood Bank, Stockholm, Sweden. Briefly, monocytes were isolated from peripheral blood by density gradient sedimentation using Ficoll-Paque Premium (GE Healthcare Life Sciences, USA). Cells were allowed to adhere in culture plates (Sarstedt AG & Co., KG, Germany) for 2 to 3 h at 37°C in serum-free RPMI 1640 medium (Thermo Fisher Scientific, USA). The nonadherent cells were removed by washing with phosphate-buffered saline (PBS), and macrophages were generated from the retrieved monocytes by culturing for 6 days in RPMI medium supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, USA), 2 mM L-glutamine, and 1 mM Na-pyruvate (Thermo Fisher Scientific, USA) containing 50 ng/ml human macrophage colony-stimulating factor (M-CSF) (Sigma-Aldrich, USA). The recovered cells were >95% monocytes as determined by flow cytometric analysis. The viability of primary macrophages was determined with trypan blue (Invitrogen, USA) staining.

Reagents. Human MDMs were treated with 10 nM $1,25(\text{OH})_2\text{D}_3$ (vitamin D_3) (Sigma) and/or 12.5 μM HO53 (Recipharm, Sweden) or 2 mM 4-phenylbutyrate (PBA) (Cayman Chemical Company, USA) in the presence or absence of azithromycin (1 mg/liter), fosfomycin (0.0625 g/liter), cefotaxime (16 mg/liter), or ciprofloxacin (16 mg/liter) (Sigma-Aldrich) for 4 h. Bafilomycin A1 (0.5 μM) (Sigma) was used to block the autophagy pathway.

Patient material. Heparin blood was collected from a patient with a deletion in the *CYBB* gene, encoding a component of the NADPH-oxidase. The patient is a 37-year-old woman with a history of systemic lupus erythematosus, which first presented during adolescence. On this occasion, she presented to the clinic with unknown fever of a few weeks duration. A clinical workup revealed the presence of a liver abscess. Extensive immunological investigations showed that the patient exhibited severely reduced capacity to produce ROS in neutrophilic granulocytes. In fact, two pools of cells were detected in the analysis, one devoid of ROS production and the other, constituting approximately 15% of the cells, with intact ROS production, suggesting skewed lyonization. A subsequent genetic analysis revealed an 8-kb-long inversion in the *CYBB* gene, which resulted in a 4.6-kb deletion comprising the entire exon 4 of the gene. Real-time PCR showed that the patient expressed 18% of the *CYBB* transcript levels compared to a healthy control. Combined, the results indicated that an X-linked trait resulted in clinical disease in this female patient due to skewed lyonization of the X-chromosome (11).

Study approval. The study was approved by the regional ethical review board in Stockholm (the FUNGEN-study, registration number 2011/116-31/4), and written informed consent was collected prior to the study.

***Klebsiella pneumoniae* culture, infection, and CFU assay.** Three *Klebsiella pneumoniae* strains were utilized in this study, a wild-type isolate (ATCC 25955), a VIM-1-producing multidrug-resistant (MDR) isolate (AO 15200), and a capsule mutant isolate (52145- $\Delta\text{wca}_{\text{K2}}$). *Klebsiella* isolates were cultured in LB medium for 1.5 to 2 h. Bacteria were washed twice with PBS, and the bacterial concentration was determined from the optical density (OD) of the culture at 600 nm as a function of CFU per milliliter. MDMs were cultured in 6-well plates (1×10^6 cells/well) and infected with the bacteria (multiplicity of infection [MOI], 1:5) for 30 min before the cells were washed to remove extracellular bacteria with gentamicin (25 $\mu\text{g}/\text{ml}$) and incubated for 30 min. Gentamicin was washed off, followed by washing with PBS and lysis of cells with 1% Triton X-100 for 15 min. Lysates were then plated in 10-fold serial dilutions on blood agar plates. Samples used to study the induced killing effect (with or without antibiotics) were incubated further for 4 h in the medium, allowing intracellular bacterial replication or killing. After washing, cells were lysed as described above. Plates were incubated overnight at 37°C, and bacterial viability was calculated by determining the CFU count.

CFU assay. The survival of the *Klebsiella* strains in the presence of the inducers was evaluated with a CFU assay. The two *Klebsiella* strains were cultured in LB medium to the exponential phase (OD, 0.5), the optical density was measured at OD₆₀₀, and the culture was diluted to an OD of 0.05. The inducers were diluted to a working concentration in RPMI cell culture medium, and 5 μl of bacteria at an OD of 0.05 (10^5 bacteria) was added to a final concentration of 100 μl in a 96-well round-bottom plate (Corning, USA). The samples were incubated for 1 and 5 h in a 37°C rotating incubator. The samples were diluted 10-fold, and all dilutions were plated on a blood agar plate and incubated overnight at 37°C. Next, the colonies were counted, and the total bacteria count was evaluated.

Bioscreen growth curve analysis. Bacterial colonies from a culture grown overnight on blood agar plates were picked and cultured to the exponential phase (OD at 600 nm of 0.3 to 0.5) in Luria broth (LB) (pH 7.5) at 37°C on a shaking incubator. For each well, the total starting inoculum was fixed to 5 μl from an OD of 0.05 (10^5 CFU) in a final volume of 150 μl in a honeycomb plate (Bioscreen C; Oy Growth Curves Ab Ltd., Finland). At OD₆₀₀, the Bioscreen instrument registered measurements every 10 min for 24 h at 37°C, thus producing a growth curve from each well for the two *Klebsiella* strains.

Antibiotic susceptibility testing. For antibiotic susceptibility testing with broth microdilution (BMD), the commercial protocol "Instructions for Use – Manual Read of MIC Susceptibility Plates for Gram Negative Non-fastidious Isolates" provided by Thermo Scientific Sensititre was followed. For the antibiotics tested with Etests (azithromycin, trimethoprim-sulfamethoxazole, doxycycline, tetracycline, and fosfomycin), a bacterial lawn was made on a Mueller-Hinton broth (MHB) agar plate (from a bacterial suspension concentration of MacFarland 0.5), and Etest strips were applied on the lawn. Plates were incubated at 37°C for 24 h, and corresponding MIC values (mg/liter) were measured for each antibiotic.

Quantitative real-time PCR (qRT-PCR). RNA was extracted from MDMs utilizing an Isolate II RNA minikit as described by the manufacturer (Bioline Reagents Limited, UK). RNA was eluted in RNase-free elution solution, and cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, USA). Transcripts of the *CAMP* gene encoding LL-37, *DEFB1* encoding human beta-defensin-1 (hBD-1), nitric oxide synthase-2 (NOS2) encoding iNOS, *NOX2*/*CYBB* encoding NADPH oxidase-2, *duox2* encoding dual oxidase 2, *CAT* gene encoding catalase, glutathione peroxidase 3 (GPx-3), interleukin-8 (IL-8), IL-17, IL-23, autophagy-related gene 5 (Atg5) and Atg12, and the housekeeping 18S rRNA (reference gene), were measured in triplicate from the cDNA samples using quantitative real-time PCR (qRT-PCR) (CFX96 real-time PCR detection systems; Bio-Rad). The results were analyzed using the relative standard method. Data are presented as the fold change of mRNA.

Lipofectamine transfection of primary macrophages with hCAP-18/LL-37 and *CYBB* siRNAs. MDMs were seeded in 6-well plates at a density of 1×10^6 cells/well and cultured in 2 ml of opti-MEM reduced serum medium (Thermo Fisher Scientific, USA). On day 7 of culturing, cells were transfected with 30 pmol of siRNA for Exon-1 of the *CAMP* gene (s2374-siRNA or siRNA-*CAMP*), encoding LL-37, siRNA for Exon-4 of the *CYBB* gene (s3789), or a negative-control vector (nonspecific siRNA, siRNA-mock) according

to the manufacturer's protocol (Invitrogen). After 24 h, cell culture medium was removed, and cells were treated with vitamin D₃ and/or HO53 and PBA before being infected with the MDR *Klebsiella* strain.

Cell viability assay (WST-1). Macrophages were cultured in a 96-well plate (Sarstedt AG & Co. KG, Germany), differentiated, and stimulated with different inducers. To measure cell viability, WST-1 reagent (Sigma-Aldrich, Germany) was mixed (1:10) with fresh RPMI cell culture medium for a final working concentration. The medium was removed from the cells, the cells were washed twice with PBS, and WST-1 reagent in cell culture medium was placed on the cells for 10 min at 37°C. The supernatant was removed and transferred to a new clear-bottom 96-well plate, and the absorbance was measured at 450 nm with 620 nm reference wavelength in a Tecan plate reader.

Western blot analysis. Human monocyte-derived macrophages were lysed with RIPA buffer (Sigma) together with a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktail. The SDS-PAGE and Western blotting transfer were carried out using NuPAGE Novex 4 to 12% Bis-Tris gels and the iBlot 2 gel transfer device (Thermo Fisher Scientific). After the membranes were incubated with 5% fat-free milk (Sigma-Aldrich) for 2 h at room temperature, the membranes were incubated with primary antibodies (rabbit anti-LC3 [Sigma] and mouse anti- β -actin [Proteintech Europe, UK]) overnight at 4°C, followed by anti-rabbit or anti-mouse IgG (GE Healthcare, UK) for 1 h. The Western blot images were acquired with a ChemiDoc XRS⁺ system (Bio-Rad, USA).

Statistics. The statistical analyses were done in GraphPad Prism 8 (GraphPad Software, Inc., USA). Data distribution, normality, and homogeneity of variances were checked using descriptive statistics. Data are presented as the median and IQR (interquartile range). Depending on the data distribution, we used Student's *t* test or the Mann-Whitney U test for comparisons between groups; we used ANOVA or the Kruskal-Wallis test for comparisons among more than two groups. *P* ≤ 0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

We thank José Bengoechea for providing the capsule mutant, Inga Fröding for help with the MIC determinations, Christian G. Giske for advice, the nurses at the immunodeficiency unit for help with blood samples, and the patient for giving blood to the study.

Rokeya Sultana Rekha was supported by Karolinska Institutet research grant 2018-01568 and the Lars Hierta Memorial Foundation (FO2018-0233). Harpa Karadottir received a Ph.D. grant from the Karolinska Institutet (KID-2016). Sultan Ahmed was supported by the Karolinska Institutet. Gudmundur H. Gudmundsson was supported by the Icelandic Centre for Research and Birgitta Agerberth, by the Swedish Research Council and Swedish Heart and Lung Foundation. Peter Bergman was supported by the Swedish Research Council, the Swedish Heart and Lung Foundation, the Scandinavian Society for Antimicrobial Chemotherapy, The Foundation to Prevent Antibiotic Resistance, KI, and grants provided by Region Stockholm (ALF project).

P.B., B.A., R.S.R., S.A., G.H.G., and H.K. contributed to the design of the research study. R.S.R., S.A., and H.K. conducted experiments and acquired data. P.B. provided patient material. R.S.R. and S.A. conducted formal analysis. R.S.R. and P.B. wrote the original draft of the manuscript. All authors contributed to the review and editing of the manuscript.

We declare that no conflict of interest exists.

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