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EXPERIMENTAL THERAPEUTICS

Prostaglandin E_2 Receptor Antagonist with Antimicrobial Activity against Methicillin-Resistant *Staphylococcus aureus*

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ABSTRACT Polymicrobial intra-abdominal infections (IAI) involving *Candida albicans* and *Staphylococcus aureus* are associated with severe morbidity and mortality (~80%). Our laboratory discovered that the immunomodulatory eicosanoid prostaglandin E_2 (PGE_2) plays a key role in the lethal inflammatory response during polymicrobial IAI using a mouse model of infection. In studies designed to uncover key PGE_2 biosynthesis/signaling components involved in the response, selective eicosanoid enzyme inhibitors and receptor antagonists were selected and prescreened for antimicrobial activity against *C. albicans* or *S. aureus*. Unexpectedly, we found that the EP_4 receptor antagonist L-161,982 had direct growth-inhibitory effects on *S. aureus in vitro* at the physiological concentration required to block the PGE_2 interaction with EP_4. This antimicrobial activity was observed with methicillin-sensitive *S. aureus* and methicillin-resistant *S. aureus* (MRSA) strains, with the MIC and minimum bactericidal concentration values for planktonic cells being 50 μg/ml and 100 μg/ml, respectively. In addition, L-161,982 inhibited *S. aureus* biofilm formation and had activity against preformed mature biofilms. More importantly, treatment of mice with L-161,982 following intraperitoneal inoculation with a lethal dose of MRSA significantly reduced the bioburden and enhanced survival. Furthermore, L-161,982 protected mice against the synergistic lethality induced by coinfection with *C. albicans* and *S. aureus*. The antimicrobial activity of L-161,982 is independent of EP_4 receptor inhibitory activity; an alternative EP_4 receptor antagonist exerted no antimicrobial or protective effects. Taken together, these findings demonstrate that L-161,982 has potent antimicrobial activity against MRSA and may represent a significant therapeutic alternative in improving the prognosis of mono- or polymicrobial infections involving MRSA.

KEYWORDS antimicrobial activity, *Staphylococcus aureus*, MRSA, *Candida albicans*, polymicrobial infection, L-161,982, prostaglandin E_2, EP_4 receptor, antimicrobial activity

Intra-abdominal infection (IAI) is a broad term used to describe infections that occur as a result of the perforation of the gastrointestinal tract. There is currently a 77% mortality rate associated with polymicrobial IAI involving fungal and bacterial species, and this rate far exceeds that of bacterial monomicrobial infections (20%) (1–4). A plethora of microorganisms can cause IAI; however, two microorganisms that are frequently coisolated are the fungal pathogen *Candida albicans* and the pathogenic bacterium *Staphylococcus aureus* (5). In patients with intra-abdominal perforations, isolation of *C. albicans* alone is indicative of a high mortality risk (6). Using animal models, it was demonstrated that coinfection with *S. aureus* raises the mortality rate even further, as a lethal synergistic association exists between these two pathogens

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TABLE 1 Selective and nonselective COX enzyme inhibitors and EP receptor antagonists

Chemical name	Inhibitory target	Concn (mg/kg)
Indomethacin	COX1 and COX2 enzymes	5
SC-560	COX1 enzyme	20
NS-398	COX2 enzyme	10
SC 51322	EP_1 receptor	10
PF 04418948	EP_2 receptor	10
L-798,106	EP_3 receptor	10
L-161,982	EP_4 receptor	10
ONO AE3 208	EP_4 receptor	10

(7, 8). Current research is aimed at understanding the mechanism underlying this lethal synergistic interaction as well as the host immune response to coinfection.

We recently developed a mouse model of IAI with *C. albicans* and/or *S.* aureus and demonstrated that the lethal outcome of coinfection, as opposed to monoinfection, was a result of an amplified host inflammatory response and not dependent on the microbial burden. In addition to proinflammatory cytokines, coinfected mice had significantly higher levels of the immunomodulatory prostanoid prostaglandin E_2 (PGE_2) than monoinfected mice (8, 9). Strikingly, treatment with the nonsteroidal anti-inflammatory drug (NSAID) indomethacin, which reduces PGE_2 synthesis, prevented mortality by resolving the proinflammatory response, demonstrating that the excessive production of PGE_2 is a key mediator of the lethal outcome (8). This finding was further supported by the observation that administration of exogenous PGE_2 to indomethacin-treated mice restored PGE_2 levels, production of proinflammatory cytokines, and mortality (8).

PGE_2 is derived from polyunsaturated arachidonic acid by the enzymatic action of cyclooxygenases (COX; 2 isoforms, COX-1 and COX-2, exist in mammals) and can induce either pro- or anti-inflammatory responses (10, 11). PGE_2 exerts its biological functions by interacting with one of four specific plasma membrane receptors (designated EP_1 through EP_4) coupled to GTP-binding regulatory proteins (G-proteins) (10). While using selective COX enzyme inhibitors and EP receptor antagonists to identify specific components of the prostanoid biosynthetic and signaling pathways involved in PGE_2 production during *C. albicans-S. aureus* IAI, *in vitro* antimicrobial activity against *C. albicans* or *S. aureus* was assessed. Interestingly, the EP_4 receptor antagonist L-161,982 exhibited growth-inhibitory activity toward *S. aureus*. The antimicrobial activity of L-161,982 has not been previously reported; therefore, the goal of these studies was to further investigate the antimicrobial activity of this compound using *in vitro* assays and *in vivo* infection models.

RESULTS

L-161,982 inhibits planktonic growth of *S. aureus*. The antimicrobial efficacy of selective COX inhibitors and EP receptor antagonists (Table 1) against reference strains *C. albicans* DAY185 and *S. aureus* NRS383 was determined as a prerequisite analysis prior to their use in analyzing the role of the prostanoid biosynthetic and signaling pathway during *C. albicans-S. aureus* IAI. The pharmacological inhibitors have been used *in vivo* in animal models with no measurable mammalian cell cytotoxicity (12, 13) and thus were tested *in vitro* in the present study at the relevant physiological concentrations.

The growth of *C. albicans* was not inhibited in the presence of COX inhibitors or EP receptor antagonists (Fig. 1A). Similarly, the growth of *S. aureus* was unaffected by the COX inhibitors as well as EP_1 to EP_3 receptor antagonists (Fig. 1B). Conversely, the EP_4 receptor antagonist L-161,982 had a significant inhibitory effect on *S. aureus* growth (Fig. 1B, gray triangle). Based on this significant finding, we extended the antimicrobial susceptibility screen to include clinical methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) strains isolated from a patient's catheter. In all cases, L-161,982 inhibited growth (Fig. 1C and D).

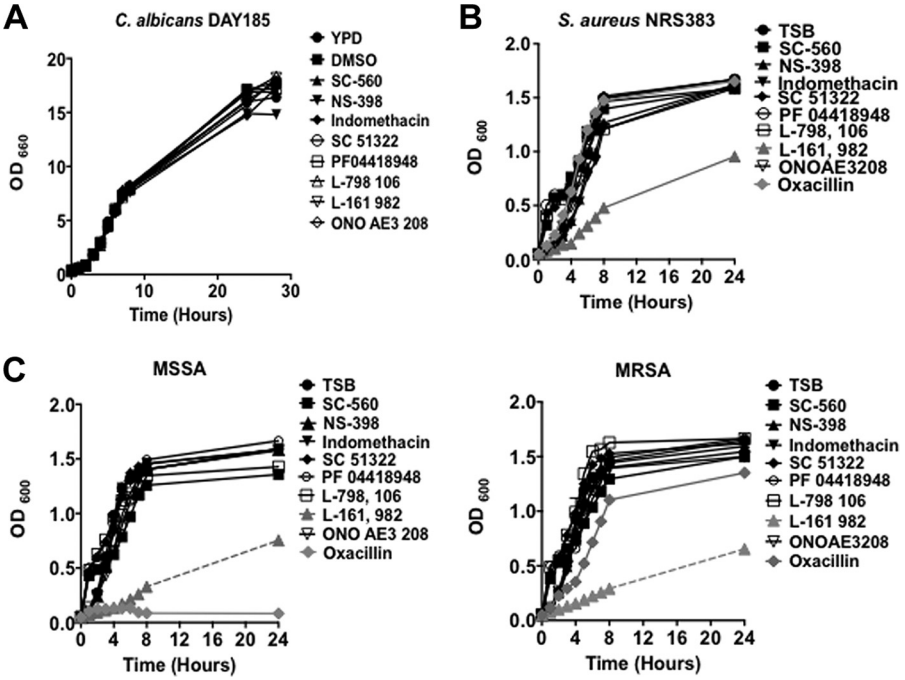

FIG 1 L-161,982 inhibits the growth of *S. aureus* strains. The antimicrobial activity of selective COX enzyme inhibitors or PGE_2 EP receptor antagonists on the growth of *C. albicans* DAY185 (A), *S. aureus* NRS383 (B), and MRSA and MSSA clinical isolates (C) was determined. The growth of *C. albicans* and *S. aureus* in medium alone or in medium supplemented with DMSO, EP receptor antagonists, or COX enzyme inhibitors at physiologically relevant concentrations was monitored for up to 24 h. The data shown are representative of those from three independent experiments. TSB, tryptic soy broth.

Growth inhibition kinetics of L-161,982 against planktonic staphylococcal cells. We next investigated the growth inhibition kinetics of L-161,982 against *S. aureus* and drug stability. The MIC of L-161,982 against planktonic *S. aureus* was 50 μg/ml, while the minimum bactericidal concentration (MBC) was 100 μg/ml. The growth inhibition kinetics of L-161,982 at the MIC of 50 μg/ml revealed that the inhibitory effect of L-161,982 on *S. aureus* was limited to 8 h (Fig. 2, black squares). To address whether the loss of inhibition was due to drug degradation over time (half-life) or to the adaptation of *S. aureus* cells to L-161,982, fresh L-161,982 (50 μg/ml) was added during the coincubation. The results showed that supplementation maintained inhibition over a 24-h period, indicating drug degradation or the drug's half-life (Fig. 2, gray circles).

L-161,982 has a narrow spectrum of activity. The spectrum of activity of L-161,982 was investigated by testing several Gram-positive and Gram-negative bacteria. The activity of L-161,982 was restricted to the Gram-positive bacteria *Staphylococcus epidermidis* and *Streptococcus mutans*, as it had no inhibitory effect on another

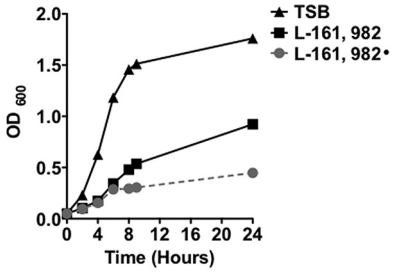

FIG 2 Growth inhibition kinetics of L-161,982. The growth of *S. aureus* NRS383 in medium alone or in medium supplemented with DMSO or 50 μg/ml L-161,982 was monitored for up to 24 h. After 6 h of coincubation, fresh L-161,982 was added to the growth medium (L-161,982●). The data shown are representative of those from three independent experiments.

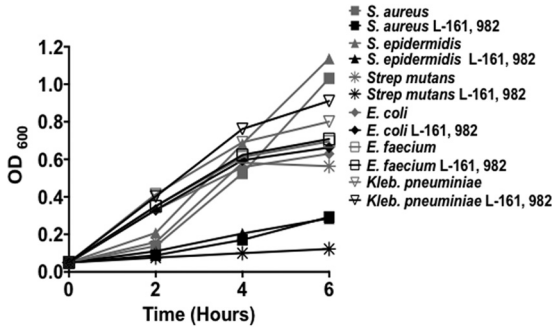

FIG 3 L-161,982 has a narrow spectrum of activity. Bacterial growth was monitored for 6 h in medium alone or in medium supplemented with 50 μg/ml of L-161,982. The data shown are representative of those from three independent experiments.

Gram-positive bacterium, *Enterococcus faecium* (Fig. 3). In addition, no inhibitory effects were observed against the Gram-negative bacteria tested (*Escherichia coli* and *Klebsiella pneumoniae*).

L-161,982 has no potentiating effect on oxacillin. We next tested whether L-161,982 had synergistic or potentiating effects on the antibiotic activity of oxacillin against MRSA using the standard checkerboard broth microdilution assay. The fractional inhibitory concentration (FIC) index of 0.563 indicated indifference (data not shown) (a FIC index of $\leq$0.5 indicates synergy, a FIC index of 0.5 indicates indifference, and a FIC index of $\geq$4 indicates antagonism).

L-161,982 inhibits *S. aureus* biofilm formation. As *S. aureus* and *C. albicans* form biofilms that are resistant to most antimicrobials, we examined the antibiofilm potential of L-161,982 against mono- and dual-species biofilms. For *C. albicans* biofilm formation, no significant inhibition of metabolic activity was observed between treated and untreated monospecies biofilms (Fig. 4A and B). In contrast, the metabolic activity of *S. aureus* was significantly inhibited compared to that of the untreated control at all concentrations tested (Fig. 4A). In addition, the number of CFU of *S. aureus* was significantly reduced by L-161,982 at concentrations ranging from 6.25 to 25 μg/ml, while no viable cells were recovered at concentrations of $>$50 μg/ml (Fig. 4B).

Next we investigated the ability of L-161,982 to disrupt preformed mono- and dual-species biofilms and found no significant inhibitory effects on the metabolic activity of either *C. albicans* or *S. aureus* at any of the concentrations tested (Fig. 5A). Interestingly, though, at the higher concentrations of 100 and 200 μg/ml, no visible biofilm was observed following washing steps, and no CFU were recovered from *S. aureus* mono- and dual-species biofilms, suggesting that treatment with high concentrations of L-161,982 after the formation of the biofilms may have resulted in weak biofilm structures that were easily disrupted mechanically (Fig. 5B).

L-161,982 protects against mono- and polymicrobial infections with MRSA. We next evaluated the efficacy of L-161,982 *in vivo* using an established mouse model of peritonitis/sepsis. Mice were inoculated with three lethal inocula of *S. aureus* and treated with L-161,982 (10 mg/kg of body weight) intraperitoneally (i.p.) at 2 h postinoculation and once daily afterwards through day 4. The control group received the vehicle at the same dose interval.

Results showed that at the intermediate inoculum of 5×10^6 CFU, L-161,982 increased the rate of survival from 25 to 55% by day 2 postinoculation (Fig. 6A). At the lowest inoculum of 4×10^6 CFU, survival increased from 40 to ~85% during the same 2-day period. Continued dosing of L-161,982 sustained survival in the intermediate- and lowest-inoculum groups through day 10, whereas the rate of survival was 20% in the control group. At the high inoculum of 2×10^7 CFU, the 80% mortality by day 2 could not be reversed by L-161,982 treatment. We also investigated the ability of L-161,982 to aid in bacterial clearance in the host by examining changes in the bioburden of *S. aureus* in the peritoneal cavity and spleen after two or three doses of L-161,982 (24 h

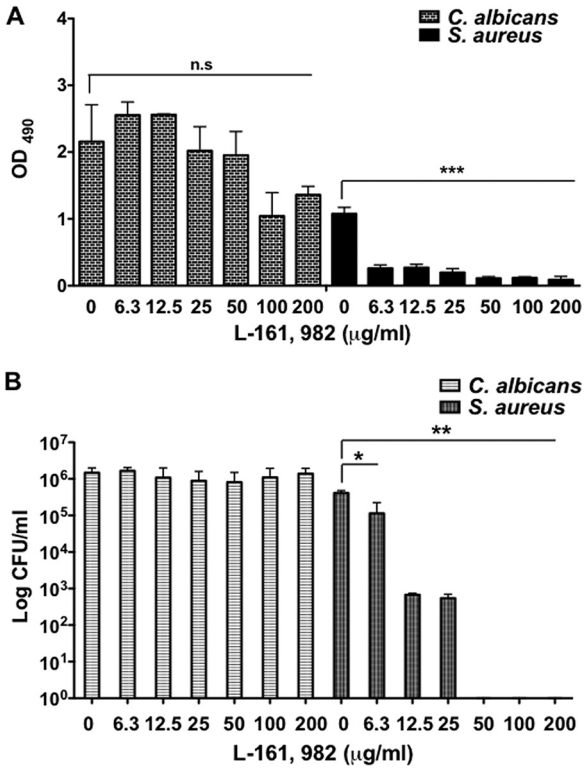

FIG 4 L-161,982 prevents MRSA biofilm formation. Mono- and dual-species *C. albicans* and/or *S. aureus* biofilms were grown on 96-well plates for 24 h with the indicated concentration of L-161,982. After incubation, the metabolic activity of the biofilms was quantified by the XTT assay (A), and then the biofilms were disrupted to determine the number of live CFU (B). The results are cumulative results from three independent experiments with duplicate samples. Statistical analysis was performed using one-way ANOVA with the Tukey *post hoc* test for the XTT assay. The Mann-Whitney U test was used to analyze the CFU data. Significance is indicated as follows: n.s, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars represent standard deviations.

or 48 h postinoculation, respectively). Compared to no treatment, treatment with L-161,982 significantly reduced the *S. aureus* burden in the peritoneal cavity and spleen by 3 logs at 24 h, and an additional 3-log reduction was achieved after a second dose of L-161,982 (48 h postinfection) (Fig. 6B).

In a final set of experiments, we evaluated the ability of L-161,982 to reduce lethality in coinfected mice. Mice coinoculated with the standard inocula of 7×10^6 *C. albicans* CFU and 8×10^7 *S. aureus* CFU (8.7×10^7 CFU total) were treated with L-161,982 (10 mg/kg) at 2 h postinfection and once daily afterwards until day 5. To ensure that the beneficial effect of L-161,982 was not a result of blocking of PGE_2-EP_4 receptor signaling, we included an alternative antagonist, ONO AE3 208, which was confirmed to have no antimicrobial effect on *S. aureus in vitro* and *in vivo* (Fig. 1 and 7). Compared to the vehicle control group, the rate of survival in mice given L-161,982 improved from 10 to 50%, while ONO AE3 208 had no effect (Fig. 7).

DISCUSSION

In studies designed to uncover the specific pathways involved in PGE_2 synthesis and downstream signaling via EP receptors, we discovered that the EP_4 receptor antagonist L-161,982 had direct antimicrobial activity against *S. aureus*. This is the first report of a PGE_2 receptor antagonist having antimicrobial activity, although some NSAIDs have been known to have antimicrobial activity, in addition to anti-inflammatory activity (14–17). Therefore, this novel activity represents a major finding that could be exploited as a therapeutic alternative. It is noteworthy that the *S. aureus* reference strain used in this study (NRS383) is methicillin resistant, which makes this finding more clinically

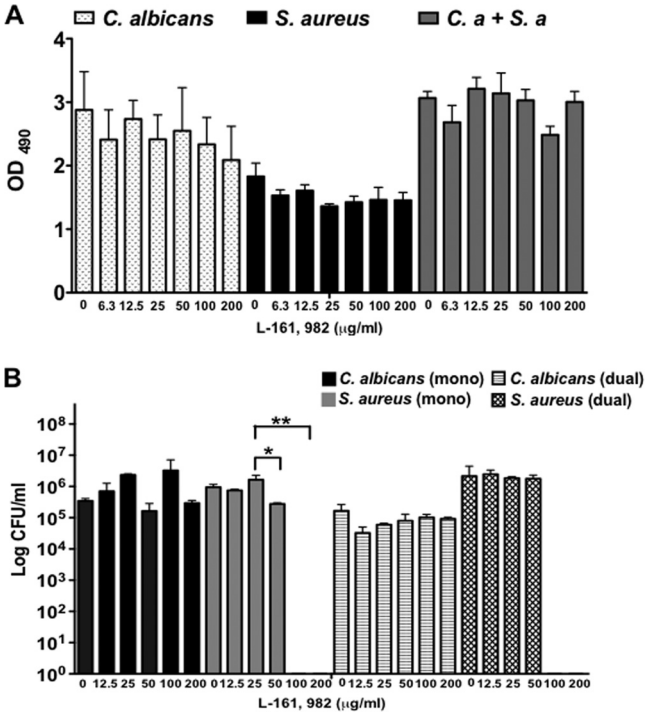

FIG 5 Effect of L-161,982 on preformed mono- and dual-species biofilms. The effect of L-161,982 on preformed mature biofilms was tested. Mono- and dual-species *C. albicans* (C.a) and/or *S. aureus* (S.a) biofilms were grown on 96-well plates for 24 h before being treated with the indicated concentration of L-161,982 for a further 24 h. After treatment, the metabolic activity of the biofilms was quantified by the XTT assay (A), and then the biofilms were disrupted to determine the number of live CFU (B). The results are cumulative results from three independent experiments with duplicate samples. Statistical analysis was performed using one-way ANOVA with the Tukey *post hoc* test for the XTT assay. The Mann-Whitney U test was used to analyze the CFU data. Significance is indicated as follows: ns, not significant; *, $P < 0.05$; **, $P < 0.01$. Error bars represent standard deviations.

significant, given that MRSA is currently among the most common multidrug-resistant pathogens worldwide and the need to identify novel antibiotics to combat infections with these pathogens is urgent. The equivalent activity of L-161,982 against other clinical MRSA and MSSA isolates obtained from contaminated catheters enhances the potential that L-161,982 could be a formidable alternative therapeutic against any *S. aureus* strains.

The MIC for the reference strain was 50 μg/ml, while the MBC was 100 μg/ml. Continued dosing of L-161,982 was required to maintain the growth-inhibitory effect, and the need for continued dosing could have been due to either drug degradation (half-life) or depletion. Interestingly, the spectrum of activity of L-161,982 extended to other Gram-positive bacteria, including *S. epidermidis* and *S. mutans*, but it had no activity against *E. faecium* or several Gram-negative bacterial species. While *E. faecium* is classified within the phylum *Firmicutes*, order *Bacilli*, similar to staphylococci and streptococci, it belongs to a different bacterial family (*Enterococcaceae*). This suggests that it has a spectrum of activity even narrower than that of other drug classes targeting Gram-positive bacteria, such as glycopeptides (18). However, further testing is needed to confirm whether this range of activity holds true for all Gram-positive versus Gram-negative species. We also explored the possible synergistic activity of L-161,982 with oxacillin and the potential for L-161,982 to lower the MIC of oxacillin against MRSA; however, we found the activity of L-161,982 to be mutually exclusive of that of oxacillin against MRSA strains.

Both *C. albicans* and *S. aureus* are notorious for forming biofilms on medical devices that are extremely resistant to antimicrobial agents and host defense mechanisms, and

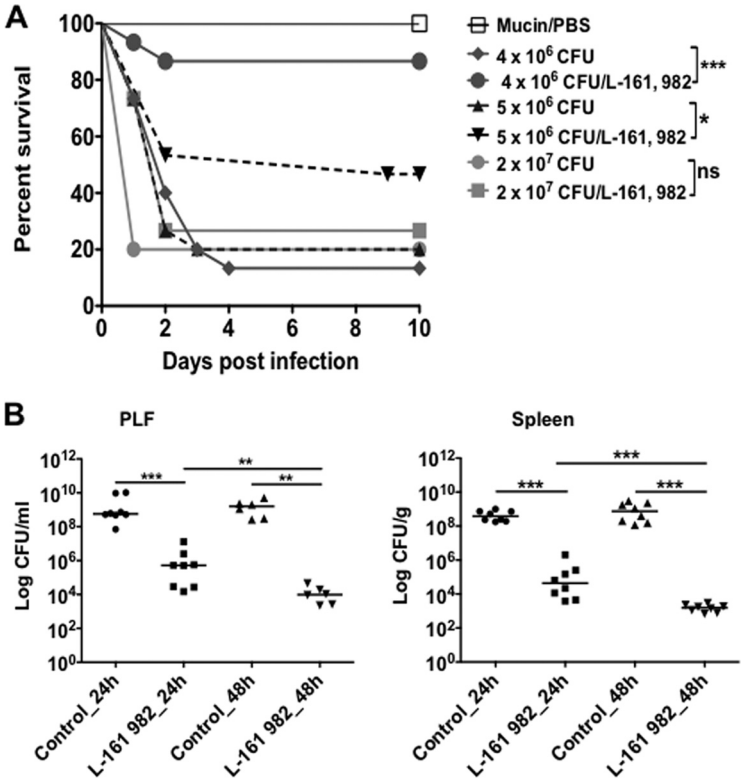

FIG 6 Effect of L-161,982 on survival and microbial burden. Mice were infected intraperitoneally with 0.2 ml of the indicated inoculum of *S. aureus* NRS383 in 3% hog gastric mucin–PBS. One group of mice per inoculum received either the vehicle or L-161,982 at a dose of 10 mg/kg by i.p. injection, as indicated in Materials and Methods. (A) Mortality was assessed using the Kaplan-Meier test and was compared to that of the control groups (*, $P < 0.05$; ***, $P < 0.001$; ns, not significant; $n = 15$/group). (B) The microbial burden in peritoneal lavage fluid (PLF) and spleen between the untreated (control) and treated (L-161,982) groups was assessed at 24 and 48 h postinoculation ($n = 6$ to 8/group). Values are plotted as the median number of CFU and were compared using the Mann-Whitney U test (**, $P < 0.01$; ***, $P < 0.001$). Data shown are representative of those from three replicate experiments.

this becomes more pronounced with mixed-species biofilms. We show here that concentrations of L-161,982 below the MIC (50 μg/ml) for planktonic growth inhibited *S. aureus* monospecies growth during biofilm formation, whereas the MBC of 100 μg/ml was required to weaken preformed mature biofilms. Unfortunately, L-161,982 showed

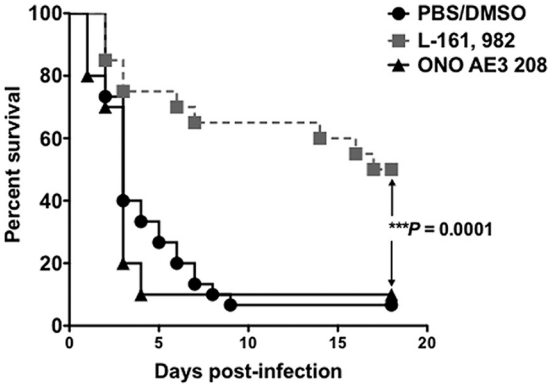

FIG 7 Antibacterial effect of L-161,982 on MRSA enhances survival in a mouse model of *C. albicans-S. aureus* polymicrobial intra-abdominal infection. The effect of inhibition of EP_4 receptor signaling on survival is shown. Mice received either vehicle or an EP_4 receptor antagonist at a dose of 10 mg/kg by i.p. injection, as indicated in Materials and Methods. Mortality compared to that in the control groups was assessed using the Kaplan-Meier test (***, $P < 0.001$; $n = 20$/group). The data shown are representative of those from three replicate experiments.

no inhibitory effect in a dual-species biofilm with *C. albicans*, suggesting that the presence of *C. albicans* might have prevented L-161,982 accessibility. This finding was not surprising, as the resulting extracellular matrix (ECM) produced by *C. albicans*, which is composed of secreted β-1,3-glucan molecules, has been shown to coat bacterial cells within the mixed-species biofilm, thereby preventing drug penetration (19–21). Nevertheless, the antimicrobial activity of L-161,982 against *S. aureus* or other biofilm-forming bacteria in a mixed-species biofilm could enhance the efficacy of other antimicrobials.

Our *in vivo* studies also highlight the potential use of L-161,982 against mono- and polymicrobial IAI with MRSA. In a mouse model of MRSA peritonitis, L-161,982 significantly decreased the bioburden in the peritoneal cavity and spleen with just one dose administered at 2 h postinoculation. We also found that, depending on the infectious dose of *S. aureus*, one treatment with L-161,982 raised the survival rate by at least 50%. Consistent with the results of the *in vitro* study, sustained survival required continued dosing. There was an upper limit to the activity, as L-161,982 was ineffective when the inoculum was very high (2×10^7 CFU), despite continued dosing. The protective role of L-161,982 was further demonstrated in a mouse model of polymicrobial intra-abdominal infection with *C. albicans*, where survival was enhanced by 40% (50% survival), which was likely due to the antimicrobial effect of L-161,982 on *S. aureus*.

The antimicrobial activity of L-161,982 was independent of effects on PGE_2 signaling, as treatment of mice with a different EP_4 receptor antagonist (ONO AE3 208) had no effect on enhancing survival. If it is presumed that the antagonist effectively blocked PGE_2 signaling, it is unlikely that PGE_2 promotes lethal inflammation via EP_4 in our model. Further studies will be aimed at evaluating the pharmacodynamics and pharmacokinetics of L-161,982 as an antimicrobial agent, as well higher dose-responses to determine its upper limits of activity. Although extensive toxicological studies have not been carried out, the toxic dose low (TD_{LO}) values reported for L-161,982 subcutaneous administrations in mice are 100 μg/kg to 333.33 μg/kg (Tocris). In our studies using peritoneal administration, L-161,982 was given at 10 mg/kg of body weight on the basis of previous reports with no apparent toxic effects (22, 23). Further studies are required to establish the full range of toxicological properties for L-161,982.

In summary, this study provides the first evidence of L-161,982 antibacterial activity via several *in vitro* study designs and *in vivo* protection against *S. aureus* mono- and polymicrobial IAI. In addition, the results of the preliminary studies presented here indicate that it has *in vitro* activity against two other clinically relevant Gram-positive bacteria. Further studies are required to uncover the growth-inhibitory mechanism of L-161,982 and determine its safety as a therapeutic agent in clinical trials. If successful, L-161,982 could be used as an alternative preventive or therapeutic agent with activity against mono- and polymicrobial infections with *S. aureus*, including MRSA.

MATERIALS AND METHODS

Strains and growth conditions. The methicillin-resistant *S. aureus* strain NRS383 used in all experiments was obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA) data bank. NRS383 is positive for the toxic shock syndrome toxin (*tst*) and δ-toxin genes. SJ-MRSA 6 and SJ-SA5 are clinical methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) strains, respectively, isolated from a patient's catheter. Other bacterial species used in this study included *Escherichia coli* (ATCC 25922, clinical isolate), *S. epidermidis* (ATCC 12228), *Klebsiella pneumoniae* (ATCC 43816), *Enterococcus faecalis* (ATCC 51299, clinical isolate), and *Streptococcus mutans* (UA159) (24). The *S. mutans* strain was maintained in brain heart infusion (BHI) broth, while the other bacterial species were maintained in Luria-Bertani broth. Frozen stocks were obtained at −80°C and streaked onto agar plates at 37°C prior to use. A single colony was transferred to 10 ml of culture broth and grown at 37°C overnight with aeration at 200 rpm. Overnight cultures were diluted 1:100 in fresh growth medium and shaken at 37°C for 3 h to obtain cells in mid-log-growth phase, with the exception of *S. mutans* cultures, which were grown without agitation in the presence of 5% CO_2.

The *C. albicans* strain used in these experiments was DAY185, a prototrophic control strain of strain BWP17 (an auxotrophic derivative of strain SC5314) into which the *HIS1*, *URA3*, and *ARG4* genes were reinserted (25). Frozen stocks were obtained at −80°C and streaked onto yeast extract-peptone-dextrose (YPD) agar prior to use. A single colony was transferred to 20 ml of YPD broth and grown at 30°C for 18 h with aeration at 200 rpm. Prior to inoculation into mice, both *C. albicans* and *S. aureus* were rinsed 3

times by centrifugation in sterile phosphate-buffered saline (PBS; pH 7.4), counted on a hemocytometer, and diluted in sterile PBS to prepare standardized inocula.

Planktonic antimicrobial assay. *C. albicans* and *S. aureus* were grown as described above. On the following day, cultures were diluted 1:100 in fresh medium containing the following chemical inhibitors at the indicated concentrations: indomethacin (50 μg/ml; a nonselective COX inhibitor; Cayman Chemicals), SC-560 (200 μg/ml; a selective COX1 inhibitor; Cayman Chemicals), NS-398 (100 μg/ml; a selective COX2 inhibitor; Cayman Chemicals), SC 51322 (100 μg/ml; an EP_1 receptor antagonist; Tocris), PF 04418948 (100 μg/ml; an EP_2 receptor antagonist; Tocris), L-798,106 (100 μg/ml; an EP_3 receptor antagonist; Tocris), L-161,982 (100 μg/ml; an EP_4 receptor antagonist; Tocris), and ONO AE3 208 (100 μg/ml; an EP_4 receptor antagonist; Tocris) in 0.28% dimethyl sulfoxide (DMSO) or 0.28% DMSO alone to serve as a control (Table 1). Because the average peritoneal volume of a Swiss Webster mouse is approximately 2 ml, the concentration of each inhibitor and DMSO was chosen to reflect the physiologically delivered final concentrations. *C. albicans* and *S. aureus* cells were grown with shaking as described above, aliquots were removed each hour in duplicate and diluted 1:100, and the optical density at 600 nm (OD_{600}) and OD_{660} were measured with a spectrophotometer (Biomate 3S; Thermo Scientific).

MIC/MBC assay. The CLSI reference method for antimicrobial susceptibility testing of bacteria was used to determine the MIC of L-161,982 (26). Cells were diluted in sterile Mueller-Hinton broth (Sigma) to a final concentration of 5.0×10^5 CFU/ml, and 200-μl aliquots (1×10^5 CFU/well) were added to the wells of sterile round-bottom 96-well plates. A stock solution of L-161,982 was diluted in sterile Mueller-Hinton broth prior to addition to the cell suspension. The drug concentrations tested ranged from 1 to 100 μg/ml and were tested in triplicate. The plates were incubated at 37°C for 24 h, and the MIC was determined to be the lowest drug concentration that inhibited visible growth. Bacteriostatic or bactericidal activity was assessed by plating 20 μl of broth from all wells with no visible growth on Mueller-Hinton agar plates, and the plates were incubated at 37°C for 24 to 48 h. The MBC was the lowest drug concentration that prevented growth. MIC and MBC determinations were done in triplicate, and oxacillin was used as a positive control.

Test for synergy between L-161,982 and oxacillin. Due to the newfound antimicrobial activity of L-161,982 against *S. aureus*, the checkerboard method (27) with the broth microdilution method was used to test for synergy between L-161,982 and oxacillin, a drug similar to methicillin used more commonly in the clinical setting. Synergy was determined using the fractional inhibitory concentration (FIC) index method (27, 28). With this method, the MIC of the antibiotic compound in the combination is divided by the MIC of the compound alone, giving the fractional contribution of each drug component in the combination. Quotients for all compounds in combination are summed, and drug interactions are scored using the formula FIC index = $[(\text{MIC } A_{\text{combA + B}})/\text{MIC}_A] + [(\text{MIC } B_{\text{combA + B}})/\text{MIC}_B]$, where MIC $A_{\text{combA + B}}$ is the MIC of compound A in the combination of compounds A and B, MIC_A is the MIC of compound A, MIC $B_{\text{combA + B}}$ is the MIC of compound B in the combination of compounds A and B, and MIC_B is the MIC of compound B.

The stock solutions and serial 2-fold dilutions of oxacillin ranged from 8× the MIC to 32× below the MIC, and those of L-161,982 ranged from 8× the MIC to 2,000× below the MIC. Oxacillin was serially diluted along the ordinate, while L-161,982 was diluted along the abscissa. The resulting checkerboard contained each combination of two drugs, with the wells that contained the highest concentration of each antibiotic being at opposite corners. Each well of the microtiter plate was inoculated with 1×10^5 CFU. The plates were incubated at 37°C for 24 h, and the OD_{600} was measured using a microplate spectrophotometer. A FIC index of ≤0.5 indicates synergy, a FIC index of between 0.5 and 4 indicates indifference, and a FIC index of >4 indicates antagonism (28, 29).

Biofilm formation on polystyrene. Following growth as described above, the microbes were washed in phosphate-buffered saline (PBS) by centrifugation, counted on a hemocytometer, and adjusted to 2×10^7 CFU/ml in RPMI buffered with MOPS (morpholinepropanesulfonic acid; 165 mM). For monomicrobial biofilms, 50 μl of adjusted *C. albicans* or *S. aureus* culture was added to each well of a sterile 96-well cell culture polystyrene microtiter plate (1×10^6 CFU per well); to this, 50 μl of sterile RPMI plus MOPS was added. For polymicrobial biofilms, 50 μl of each organism was added per well (1×10^6 CFU of each organism per well). The plates were incubated for 24 h at 37°C to induce biofilm formation. Following confirmation of mature biofilm growth by macro- and microscopic examination, the plates were washed once with 200 μl sterile saline, and then 200 μl RPMI plus MOPS containing L-161,982 at various concentrations (6.25 to 200 μg/ml) was added to each well. L-161,982-free RPMI plus MOPS was added to wells with and without microbes to serve as positive and negative controls, respectively. The plates were incubated at 37°C for 24 h. After incubation, the plates were washed once with PBS and processed for the sodium 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide inner salt (XTT) metabolic activity assay, followed by CFU enumeration. To dislodge the biofilms, the plates were sonicated for 5 to 10 s and suspensions were pipetted up and down prior to serial dilutions, which were done with constant vortexing (5 to 10 s) in between dilutions and prior to plating out.

The XTT reduction assay was used to determine the metabolic activity of biofilms following treatment with L-161,982 (30). Briefly, the wells were washed once in PBS, the plates were incubated at 37°C for 2 h with 200 μl XTT working reagent (0.5 mg/ml XTT, 1 μM menadione), and the resulting absorbance at 490 nm was measured on a microplate spectrophotometer. After L-161,982 treatment and metabolic activity measurement, enumeration of CFU was carried out as previously described (31).

Mouse model of infection. All animals were housed and handled according to institutionally recommended guidelines. All animal protocols were reviewed and approved by the Institutional Animal

Care and Use Committee (IACUC) of the LSU Health Sciences Center, New Orleans, LA. Mice were given access to food and water *ad libitum*. In all experiments, 5- to 7-week-old female outbred NIH Swiss mice, purchased from Charles Rivers, Frederick, MD, were used.

The mouse peritonitis/intra-abdominal infection model was performed as previously described (8). In brief, for MRSA peritonitis, mice were injected intraperitoneally (i.p.) with 4×10^6 CFU, 5×10^6 CFU, or 2×10^7 CFU of *S. aureus* in 0.2 ml of PBS containing 3% hog gastric mucin type III (Sigma-Aldrich). Mice injected i.p. with 0.2 ml of mucin-PBS served as a negative infection control. For intra-abdominal infection with *C. albicans* and *S. aureus*, mice were injected i.p. with 7×10^6 CFU of *C. albicans* and 8×10^7 CFU of *S. aureus* (8.7×10^7 CFU total) in 0.2 ml of PBS. After inoculation, the mice were observed over 10 days for signs of morbidity (hunched posture, inactivity, lethargy, and ruffled fur) and mortality. Mice that were significantly moribund were euthanized according to institutionally recommended guidelines. In the MRSA peritonitis experiments, mice were sacrificed at 24 or 48 h postinoculation to assess the microbial burden. The peritoneal cavities were lavaged by injection of 2 ml of sterile PBS, followed by gentle massaging of the peritoneal cavity. Peritoneal lavage fluid was then removed using a pipette inserted into a small incision in the abdominal cavity. The spleens were removed, weighed, and mechanically homogenized prior to analysis of the number of CFU. The microbial burdens in the peritoneal lavage fluid and spleen were enumerated as described above.

Inhibitor treatment. Each inhibitor was prepared fresh as a concentrated stock (20 to 25 mg/ml) in DMSO and diluted to a working concentration of 10 mg/ml in sterile PBS (final DMSO concentration, 2.3%). A sham treatment containing only 2.3% DMSO in PBS was also prepared. Groups of 5 to 10 mice were intraperitoneally administered 0.1 ml of inhibitor (as indicated in Table 1). For the MRSA peritonitis study, the EP_4 receptor antagonist was administered 2 h after *S. aureus* inoculation and once daily thereafter until day 4 postinoculation. For the IAI coinfection study, a COX inhibitor or vehicle control was administered 4 h prior to and 4 and 8 h after inoculation with *C. albicans* and *S. aureus*, while the EP receptor antagonists were administered daily starting 1 day prior to and 5 days after infection. All other procedures were performed as described above. Control groups received the vehicle at the same dose interval.

Statistical analysis. All experiments used groups of 5 to 10 mice and were repeated in duplicate, except where noted. All assays were repeated in triplicate, and the results were averaged. Survival data were analyzed using the Kaplan-Meier test. The metabolic activities of the treated biofilms were compared to those of the controls using one-way analysis of variance (ANOVA) with the Tukey *post hoc* test. The Mann-Whitney U test was used to analyze all CFU data. In all tests, differences were considered significant at a P value of <0.05. All statistical analyses were performed with GraphPad Prism software.

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