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Oral administration of a 2-aminopyrimidine robenidine analogue (NCL195) significantly reduces *Staphylococcus aureus* infection and reduces *Escherichia coli* infection in combination with sub-inhibitory colistin concentrations in a bioluminescent mouse model

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Running title: Antibacterial activity of NCL195-colistin combination

ABSTRACT

We have previously reported promising *in vivo* activity of the first-generation 2-aminopyramidine robenidine analogue NCL195 against Gram-positive bacteria (GPB) when administered via the systemic route. In this study, we examined the efficacy of oral treatment with NCL195 (-/+ low dose colistin) in comparison to oral moxifloxacin in bioluminescent *Staphylococcus aureus* and *Escherichia coli* peritonitis-sepsis models. Four oral doses of 50 mg/kg NCL195, commencing immediately post-infection, were administered at 4 h intervals in the *S. aureus* peritonitis-sepsis model. We used a combination of four oral doses of 50 mg/kg NCL195 and four intraperitoneal doses of colistin at 0.125 mg/kg, 0.25 mg/kg or 0.5 mg/kg in the *E. coli* peritonitis-sepsis model. Subsequently, the dose rates of four intraperitoneal doses of colistin were increased to 0.5 mg/kg, 1 mg/kg, or 2 mg/kg at 4 h intervals to treat a colistin-resistant *E. coli* infection. In the *S. aureus* infection model, oral treatment of mice with NCL195 resulted in significantly reduced *S. aureus* infection loads ($p<0.01$) and longer survival times ($p<0.001$) than vehicle-only treated mice. In the *E. coli* infection model, co-administration of NCL195 and graded doses of colistin resulted in a dose-dependent significant reduction in colistin-susceptible ($p<0.01$) or colistin-resistant ($p<0.05$) *E. coli* loads compared to treatment with colistin alone at similar concentrations. Our results confirm that NCL195 is a potential candidate for further preclinical development as a specific treatment for multidrug-resistant infections either as a stand-alone antibiotic for GPB or in combination with sub-inhibitory concentrations of colistin for Gram-negative bacteria.

Keywords: NCL195; colistin; Gram-positive bacteria; Gram-negative bacteria; multidrug resistance; bioluminescence; synergy

INTRODUCTION

Multidrug-resistant (MDR) infections constitute a serious public health problem worldwide (1-3). It is estimated that deaths caused by MDR bacteria will reach 10 million per year by 2050 unless urgent action is taken (4, 5). While the incidence of Gram-positive bacterial (GPB) infections such as methicillin-resistant *Staphylococcus aureus* (MRSA) infections has slightly decreased, the situation with MDR-Gram-negative bacterial (GNB) infections is more problematic given the limited range of drug classes and ever-increasing resistance. Moreover, the outer membrane of GNB, a largely asymmetric bilayer composed of glycolipid lipopolysaccharides and glycerol phospholipids, serves as a barrier to protect GNB from unwanted compounds and promotes antimicrobial resistance (6). Therefore, comparatively fewer antimicrobial classes (aminoglycosides, polymyxins, tetracyclines, β-lactams and fluoroquinolones) are able to penetrate the outer membrane of GNB, limiting treatment options (7). Additionally, GNB pathogens have quickly acquired resistance to most, and in some cases, to all these antibiotics via multiple mechanisms (2, 8).

Due to the distinctive structure of GNB, no novel antibiotics with a new chemical structure or a new mode of action against GNB infections has been developed and marketed for several decades (9). To date, there are 19 potential antibiotics in clinical development for the treatment of GNB pathogens but none of them has a new mode of action (10). Among currently used antibiotics, polymyxins (such as polymyxin B [PMB] and colistin [polymyxin E]) are highly efficacious against GNB and are considered the last line antimicrobials for the treatment of GNB infections due to their specific targeting of the outer membrane (11, 12); nonetheless, resistance to polymyxins is emerging via different mechanisms (11, 13). In addition, the use of high doses of polymyxins is associated with nephrotoxicity, neurotoxicity and neuromuscular blockade (14, 15). To address the shortfall in effective antibiotics, to

overcome the emerging resistance and outer membrane protection, and to reduce toxicity of polymyxins, an antibiotic combination approach provides an alternative and complementary strategy to effectively and more safely control serious infections caused by MDR-GNB (16-18). While several studies have indicated that combinations of polymyxins with other antibiotics elicit full or partially synergistic activities against MDR-GNB pathogens (19-24), we report here a novel combination approach.

In line with a combination strategy, we previously reported *in vitro* synergistic activity of the anticoccidial aminoguanidine robenidine (NCL812) (25) analogue NCL195 with different adjuvants against clinical MDR-GNB pathogens (26-28). NCL195 showed 100% synergistic activity when combined with sub-inhibitory concentrations of colistin and PMB against clinical MDR-GNB pathogens (including colistin-resistant isolates), with MICs for NCL195 ranging from 0.5–4 mg/L for *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, whereas NCL195 alone had no activity (27, 28). This strongly suggests that NCL195 is a promising candidate as a component of a combination with colistin for the treatment of GNB infections.

Our earlier investigations have shown better toxicity profiles to mammalian cell lines and erythrocytes for NCL195 than NCL812 and it did not elicit observable histological effects in major organs of mice (28, 29). Furthermore, mice that received two intraperitoneal doses of 50 mg/kg NCL195 exhibited significantly reduced *S. aureus* loads compared to untreated mice, but still succumbed to infection (28). Interestingly, we recently showed that mice treated with four 50 mg/kg oral doses of a closely-related robenidine analogue (NCL179) had significant increase in overall survival rate after *S. aureus* challenge compared to the vehicle-only control (30). This provided the opportunity to investigate the efficacy of oral NCL195

and intraperitoneal colistin combination as a proof of concept of *in vivo* antimicrobial activity against GNB in a bioluminescent mouse peritonitis-sepsis model.

RESULTS

Oral administration of NCL195 alone or in combination with intraperitoneally-administered colistin demonstrate systemic safety in mice.

As a prelude to efficacy testing of orally administered NCL195 in a GPB peritonitis-sepsis model, the safety of 4 orally-administered NCL195 at 10 mg/kg or 50 mg/kg, 4 h apart) in comparison with the vehicle was assessed over a 72 h period. We found there were no observable histopathological changes in heart, lung, liver, spleen, stomach, kidneys, small and large intestines of mice treated with either 10 mg/kg or 50 mg/kg NCL195 in comparison with the vehicle at 72 h after initial treatment (Fig. 1A). Subsequent administration of a combination of NCL95 (4 oral doses, 50 mg/kg, 4 h apart) with colistin (4 intraperitoneal doses, 0.125, 0.25, 0.5, 1, 2 or 4 mg/kg, 4 h apart) showed no observed histopathological changes in harvested organs from mice in comparison with vehicle, NCL195 alone or colistin alone at similar concentrations (Fig. 1B and 1C and Fig. S1A and S1B).

Oral treatment of mice with NCL195 reduces *S. aureus* populations and significantly prolongs survival times.

As a proof of concept for efficacy testing of NCL195+colistin combination in GNB peritonitis-sepsis models, the efficacy of orally-administered NCL195 at the safe dose of 50 mg/kg in a bioluminescent *Sa*Xen29 mouse infection model was examined. We observed increasing reduction of *Sa*Xen29 photon intensities after the first ($p<0.05$), second and third NCL195 treatments ($p<0.01$) compared to the vehicle-only group (Fig. 2A). Treatment of mice with four doses of NCL195 resulted in significant increase in median survival time and overall survival rate compared to the vehicle-only control ($p<0.001$; Fig. 2B). As expected,

128 treatment with moxifloxacin (control drug) showed progressively significant reduction in
129 *Sa*Xen29 photon intensities, increase in survival time and overall survival rate (Fig. 2A-C).
130
131 **Low dose colistin is unable to clear *Ec*Xen14 or col^{R}-*Ec*Xen14 infection in mice.**
132
133 Preliminary investigations to determine the appropriate sub-inhibitory concentrations of
134 colistin to test the efficacy of NCL195+colistin combination in a bioluminescent *Ec*Xen14
135 mouse model have been reported previously (27). To ensure that a NCL195+colistin
136 combination will work against a colistin-resistant GNB *in vivo*, a stable col^{R}-*Ec*Xen14 strain
137 with MIC of 32 mg/L was generated which produced a similar bioluminescence emission
138 profile as the parent *Ec*Xen14 (Fig. S2). Whole genome sequence comparison of the col^{R}-
139 *Ec*Xen14 strain with the parent revealed an amino acid substitution ($Gly_{53}\rightarrow Val_{53}$) in PmrA
140 (Fig. S3) of the PmrAB two-component system that remodels the composition and charge of
141 lipid A and the barrier properties of the outer membrane (31, 32). Additionally, agar well
142 diffusion results confirm that NCL195 and colistin retain their antimicrobial activity when
143 formulated (Fig. S4).
144
145 The optimal sub-inhibitory concentration of colistin to be used for efficacy testing of
146 NCL195+colistin combinations in a col^{R}-*Ec*Xen14 mouse challenge model was then
147 explored. We found 4 intraperitoneal doses of colistin 0.5 or 2 mg/kg were unable to clear the
148 infection up to 12 h post-infection, whereas almost complete bacterial clearance was
149 observed after 4 intraperitoneal doses of colistin 4 mg/kg, with complete bacterial clearance
150 after 2 intraperitoneal doses of colistin 8 mg/kg by 12 h post-infection (Fig. S5A and S5B).
151 Therefore, colistin at 0.5, 1 mg/kg or 2 mg/kg were used in subsequent efficacy testing of
152 NCL195+colistin combinations against col^{R}-*Ec*Xen14 intraperitoneal challenge.

Treatment of mice with NCL195+colistin combination reduces colistin-susceptible and colistin-resistant *Ec*Xen14 populations and significantly prolongs survival times.

The efficacy of orally-administered NCL195 (4 doses, 50 mg/kg, 4 h apart) was tested against both *Ec*Xen14 and col^{R}-*Ec*Xen14 using colistin 0.5 mg/kg and 8 mg/kg as drug controls (Fig. S6). We previously demonstrated that NCL195 has no *in vitro* activity against GNB except for *Neisseria meningitidis* and *N. gonorrhoeae* (MIC, 32 μg/mL) (27, 28). Here, NCL195-treated mice succumbed as rapidly as vehicle only-treated mice to *Ec*Xen14 (Fig. S6A and S6B) or col^{R}-*Ec*Xen14 (Fig. S6C and S6D) challenge, confirming NCL195 has no activity against *E. coli*.

The results of efficacy experiments with 50 mg/kg oral NCL195 (4 doses, 4 h apart) in combination with colistin 0.125, 0.25 or 0.5 mg/kg (4 intraperitoneal doses, 4 h apart) in a *Ec*Xen14 peritonitis-sepsis model are shown in Fig. 3 and Fig. S7. Overall, NCL195+colistin combinations cleared bacteria faster than colistin alone at similar concentrations. All NCL195+colistin combination treatments showed statistically significant reduction in *Ec*Xen14 photon signals from 4 to 12 h in comparison with NCL195 alone ($p<0.01$ to $p<0.001$) (Fig. S7A). Furthermore, all NCL195+colistin combination treatments resulted in a significant increase in median survival times for mice compared to NCL195 alone ($p<0.0001$) (Fig. S7B). Specifically, the NCL195 (50 mg/kg)+colistin (0.125 mg/kg) combination showed statistically significant reduction in *Ec*Xen14 photon intensities at 10, 12 and 24 h ($p<0.01$) in comparison with colistin alone at the same concentration (Fig. 3A). Additionally, treatment with 4 doses of a combination of NCL195 (50 mg/kg)+colistin (0.125 mg/kg) 4 h apart resulted in a significant increase in median survival time compared to treatment with 4

doses of NCL195 50 mg/kg (p<0.0001) or treatment with 4 doses of colistin 0.125 mg/kg (p<0.05, Log-rank (Mantel-Cox) test) (Fig. 3B). The bacterial reduction caused by different treatments could be clearly observed in bioluminescent images of mice (Fig. 3C and Fig. S7C).

Next, we assessed the efficacy of NCL195 (4 oral doses, 50 mg/kg, 4 h apart) in combination with colistin (4 intraperitoneal doses, 0.5, 1 or 2 mg/kg, 4 h apart) in a mouse col^{R}-*Ec*Xen14 peritonitis-sepsis challenge model. Overall, the NCL195+colistin combinations cleared bacteria faster than colistin alone at the same concentrations. NCL195+colistin at 0.5 mg/kg combination showed statistically significant reduction in col^{R}-*Ec*Xen14 photon signals from 8 h and significant reduction in photon signals from 4 h when NCL195 was combined with 1 or 2 mg/kg colistin in comparison with NCL195 alone (p<0.01 to p<0.001; Fig. S8A). Furthermore, all NCL195+colistin combination treatments resulted in significant increase in median survival time for mice compared with NCL195 treatment alone (p<0.0001; Fig. S8B).

NCL195 (50 mg/kg)+colistin (1 mg/kg) combination showed statistically significant reduction in col^{R}-*Ec*Xen14 photon signals at 12 and 24 h (p<0.05) in comparison with a similar concentration of colistin alone (Fig. 4A). Treatment with NCL195 (50 mg/kg)+colistin (1 mg/kg) combination resulted in significant increase in median survival time compared to colistin alone at 1 mg/kg (p<0.01; Fig. 4B). NCL195 (50 mg/kg)+colistin (2 mg/kg) combination showed statistically significant reduction in col^{R}-*Ec*Xen14 populations at 8, 12 and 24 h (p<0.05) compared to treatment with 2 mg/kg colistin alone (Fig. S8A). Treatment with NCL195 (50 mg/kg)+colistin (2 mg/kg) combination resulted in significant increase in median survival time compared to colistin alone at 2 mg/kg (p<0.01; Fig. S8B), clearly observed in bioluminescent images of mice (Fig. 4C and Fig. S8C).

203

DISCUSSION

In this study, we show that four oral doses of 50 mg/kg NCL195 and combination with four intraperitoneal doses of two-fold increasing colistin concentrations (0.125–4 mg/kg) at 4 h intervals could be administered to mice without any observable histopathological changes in comparison to vehicle only, NCL195 alone or colistin alone at similar concentrations. We also demonstrate that administration of four oral doses of NCL195 after an otherwise lethal *S. aureus* systemic challenge significantly increased overall mouse survival. Furthermore, we show that co-administration of four oral NCL195 doses with four intraperitoneal sub-inhibitory colistin doses resulted in a significant dose-dependent reduction in colistin-susceptible and colistin-resistant *E. coli* infection loads and significantly increased survival of mice compared to treatment with colistin alone at similar concentrations. The *in vivo* efficacy was correlated with previous *in vitro* time-kill kinetics showing that NCL195+colistin combinations killed bacteria faster than colistin alone at similar concentrations and was associated with ultrastructural damage of the outer and inner membrane of cells as assessed by transmission electron microscopy (27, 33).

Robenidine is an oral antibiotic used to control coccidiosis in poultry (25), but chemically modified to yield NCL195 to enhance potency and systemic delivery (26, 29). However, while intraperitoneally-administered NCL195 led to significantly reduced *S. aureus* loads compared to untreated mice, this did not result in increased overall survival (28). Our recent finding that oral delivery of a closely-related robenidine analogue (NCL179) resulted in increased overall survival after *S. aureus* challenge (30) provided the impetus for testing oral administration of NCL195 in this study, which showed a significantly increased overall mouse survival. The reason for the superior efficacy of oral administration over the systemic route is yet uncertain, but will be investigated.

The use of colistin therapy alone or in combination with other antibiotics for GNB infections is still controversial (16, 17). Although polymyxins (including colistin) are considered last line drugs for the treatment of GNB infections, the high risk of nephrotoxicity (34), neurotoxicity and neuromuscular blockade (14, 15) are a major reason for debate and concern about use. Our results agree with other studies reporting that colistin combination therapy demonstrated superiority in safety and efficacy profiles compared with monotherapy against GNB infections (34-39). Of note, the sub-inhibitory concentration of colistin used in the combination was as effective as a higher concentration of colistin alone, and the combination of colistin at 1 mg/kg with NCL195 successfully treated col^{R}-*Ec*Xen14 infection in mice whereas colistin alone at the same concentration showed no effect. Therefore, NCL195 having a proposed site of action on the bacterial inner membrane is a promising strategy to address the shortfall in antibiotics for GNB infections when combined with low concentration of colistin, particularly in the environment of increasing colistin-resistance among GNB.

We demonstrated previously that NCL195 has low propensity to select for resistance in *S. aureus* (29), a desirable characteristic for further investigation as a novel antimicrobial class to treat acute bacterial infections. Our current results add to those findings on the antibacterial efficacy of NCL195 and provide further support for combination therapy with colistin in the presence of colistin resistance among MDR-GNB pathogens by administering colistin as an adjuvant to permeabilize the outer membrane allowing increased exposure to NCL195. Together, our findings demonstrate that the new antibacterial class represented by NCL195 provides a promising new scaffold. Pre-clinical studies to optimise pharmacokinetic/pharmacodynamic profiles and dose regimens of NCL195+colistin

253 combinations as well as cumulative toxicity testing in appropriate animal models will allow
254 refinement of dosing schedules.
255

MATERIALS AND METHODS

Ethics.

Outbred 5 to 6-week-old male CD1 Arc:Arc(S) (Swiss) mice (25–30 g), obtained from the Laboratory Animal Services breeding facility of the University of Adelaide, were used for safety and efficacy assessments of NCL195, colistin and NCL195+colistin administration. Mice had access to food and water *ad libitum*. The Animal Ethics Committee of The University of Adelaide (approval number S-2015-151) reviewed and approved all animal experiments. The study was conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8^{th} Edition 2013) and the South Australian Animal Welfare Act 1985.

Antibiotics and chemicals.

NCL195, an analogue of robenidine NCL812 (26, 29) (Fig. 5) was synthesised at the University of Newcastle, NSW, Australia. Colistin sulfate, daptomycin and kanamycin were purchased from Sigma-Aldrich (Australia). Stock solutions containing 25.6 g/L of each compound (NCL195 and daptomycin dissolved in DMSO; colistin and kanamycin dissolved in water) were stored in 1 mL aliquots at -20 °C away from direct light. Moxifloxacin was used as a control drug in the GPB mouse model and was prepared in almond oil (BovaVet, Australia).

Organisms and growth conditions.

A bioluminescent derivative of *Staphylococcus aureus* ATCC 12600 (*Sa*Xen29) and bioluminescent *E. coli* (*Ec*Xen14; derived from the parental strain *E. coli* WS2572) were purchased from PerkinElmer. Colistin-resistant *Ec*Xen14 (col^{R}-*Ec*Xen14) was generated by daily serial passages in increasing concentrations of colistin from 0.06 mg/L to 256 mg/L over 15 days, as described previously (29). All bacteria were stored at -80 ^{o}C in Luria Bertani (LB) broth with 50% (vol/vol) glycerol at the Microbiology Laboratory, Health and Biomedical Innovation, Clinical and Health Sciences, University of South Australia, Australia. Bacteria were routinely grown on horse blood agar (HBA) and LB broth. *Sa*Xen29 was grown on HBA containing 200 mg/L kanamycin while *Ec*Xen14 and col^{R}-*Ec*Xen14 were grown on HBA containing 30 mg/L kanamycin for selection.

DNA extraction and whole genome sequencing.

Genomic DNA of *Ec*Xen14 and col^{R}-*Ec*Xen14 were extracted using the PureLink® Genomic DNA Kit (Invitrogen, Australia). Whole genome sequencing was performed at Public Health and Epidemiology, Microbiology and Infectious Diseases, SA Pathology, Australia using the Illumina NextSeq 500/550 Mid-Output kit v2.5 (300 cycles) (Illumina Inc., USA). Raw paired-end reads were assembled and annotated using the TORMES pipeline v.1.3.0 (40). Amino acid sequence alignments were generated using CLUSTAL OMEGA version 1.2.4 (41) to assess the presence of mutations and visualised using ESPript v3.0 (42).

Safety testing of NCL195 alone and in combination with colistin and histopathological examination.

To ascertain the safety of a regimen of 4 consecutive oral doses of 10 mg/kg or 50 mg/kg NCL195 at 4 h intervals of NCL195 in mice, a safety study was conducted, using the vehicle (20% (v/v) DMSO in PEG400) as a control agent. Subsequently, safety of 4 oral doses (4 h apart) of NCL195 (50 mg/kg) combined with 4 intraperitoneal doses (4 h apart) colistin at 0.125, 0.25 or 0.5 mg/kg was conducted. Later, safety of oral NCL195 (50 mg/kg) combined with intraperitoneal colistin at 1, 2 or 4 mg/kg was assessed. The group that received NCL195+colistin was compared with the group that received NCL195 alone or colistin alone at the same concentrations or stand-alone colistin at 8 mg/kg.

Mice were monitored for clinical signs of adverse effects and observations recorded every 4 h for the first 24 h, then at 48 h and 72 h on a Clinical Record Sheet approved by the Animal Ethics Committee of The University of Adelaide. At the conclusion of the experiment (72 h after the initial treatment), mice were humanely killed by carbon dioxide asphyxiation. Mouse organs (heart, lung, liver, kidneys, spleen, stomach, small intestines and large intestines) were collected, fixed in 10% neutral-buffered formalin and processed routinely. The specimens were embedded in paraffin blocks and sections of 4 μm thickness were cut using a microtome. Hematoxylin staining of the sections was performed and the slides were observed and recorded under light microscopy.

Agar well diffusion method.

All formulations of NCL195 and colistin used for safety and efficacy assessments were tested for antibacterial activity using the agar well diffusion method to ensure that the drugs were released from the vehicle as a reference for the interpretation of *in vivo* activity in mice. NCL195 was prepared as a 50 mg/mL solution in 20% (v/v) DMSO in PEG400 (vehicle).

Colistin was prepared as 0.037, 0.075, 0.15, 0.3, 0.6, 1.2 and 2.4 mg/mL in water corresponding to 0.125, 0.25, 0.5, 1, 2, 4 and 8 mg/kg, respectively, based on consideration of administration to a 30 g mouse for the mouse safety and efficacy studies. For this assay, colonies from an overnight HBA culture of *Sa*Xen29, *Ec*Xen14 and col^{R}-*Ec*Xen14 were suspended in saline equivalent to 0.5 McFarland Standard ($A_{600\ nm}$ = 0.1). A sterile swab was then dipped in the 0.5 McFarland Standard bacterial suspension and then streaked over the entire surface of a sterile plate count agar. Duplicate holes (on each of two separate occasions of safety and efficacy trials) were then punched on the agar plates using an 8 mm diameter biopsy punch (Livingstone International Pty Ltd, NSW, Australia). Each well contained 0.03 mL of each formulation. The antimicrobial activity of each drug was then determined by measuring and comparing the zone of inhibition with that of vehicle only after 20 h incubation at 37 °C in air.

Efficacy testing of NCL195 oral administration following systemic challenge with *Sa*Xen29.

To test the efficacy of 50 mg/kg NCL195 (four oral doses, 4 h apart) against *S. aureus*, mouse-passaged *Sa*Xen29 was used. Oral 6 mg/kg moxifloxacin (four doses, 4 h apart) suspension in almond oil (prepared by BovaVet, Australia, 6 mg/mL) was used as drug control. Three groups of mice (n=6 mice per group) were challenged intraperitoneally with approx. 3×10^7 CFU of *Sa*Xen29 in 200 µl saline containing 3% porcine stomach mucin type III (Sigma Aldrich), then immediately subjected to bioluminescence imaging in both ventral and dorsal positions on the IVIS Lumina XRMS Series III system. Subsequently, group 1 received the drug vehicle only, group 2 received oral NCL195 at 50 mg/kg, while group 3 received oral moxifloxacin 6 mg/kg. At 2 h post-infection, all mice were imaged as above and their clinical conditions closely monitored and recorded. At 4 h post-infection, all mice

were similarly imaged and received the second dose. At 6 h post-infection, all mice were imaged again and the clinical conditions monitored and recorded. At 8 h post-infection, all surviving mice in each group were imaged and given a third identical dose. At 10 h post-infection, all surviving animals in each group were imaged followed by an identical treatment regimen at 12 h as described above. Mice were further monitored frequently for signs of distress at 18, 24, 28, 36, 48 and 72 h post-infection, their clinical conditions recorded, imaged and those that had become moribund or showed any evidence of distress were humanely killed by CO2 asphyxiation. In all experiments, signals were collected from a defined region of interest and total flux intensities (photons/s) analysed using Living Image Software 4.7.2. Differences in median survival times (time to moribund) for mice between groups were analysed by the Log-rank (Mantel-Cox) tests. Differences in luminescence signals between groups were compared by Mann-Whitney *U*-tests, two-tailed.

Determination of the lowest colistin dose unable to clear col^{R}-*Ec*Xen14 in mice.

We have previously determined the lowest colistin dose that was unable to clear *Ec*Xen14 infection in a mouse model (27). In this study, we extended our investigation to determine the lowest colistin dose unable to clear col^{R}-*Ec*Xen14 in an identical mouse peritonitis-sepsis model.

Col^{R}-*Ec*Xen14 cells were grown in LB broth at 37 °C to $A_{600\ nm}$ of 0.5 (equivalent to approx. 5×10^{8} CFU/mL) and four groups of mice (n=3) were challenged intraperitoneally with approx. 1.0×10^{8} CFU of the col^{R}-*Ec*Xen14 in 200 µL saline containing 3% porcine stomach mucin type III (Sigma Aldrich, Australia). All mice were then subjected to bioluminescence imaging in both ventral and dorsal positions on the IVIS Lumina XRMS Series III system. Immediately thereafter, group 1 received the drug vehicle only, groups 2, 3 and 4 received

colistin at 0.5, 2 and 4 mg/kg intraperitoneally, respectively, at 0, 4, 8 and 12 h while group 5 received colistin at 8 mg/kg at 0 and 4 h and mice further subjected to bioluminescence imaging at 4, 6, 10, 12, 24, 48 and 72 h post-infection. Mice were monitored frequently for signs of distress and those that had become moribund or showed any evidence of distress were humanely killed by CO_2 asphyxiation. In all groups, signals were collected from a defined region of interest and total flux intensities (photons/s) analysed using Living Image Software 4.7.2.

Efficacy testing of oral NCL195+intraperitoneal colistin combination following systemic challenge with *Ec*Xen14 or col^R-*Ec*Xen14.

*Ec*Xen14 and col^R-*Ec*Xen14 were grown in LB broth at 37 °C to $A_{600\ nm}$ of 0.5 (equivalent to approx. 5×10^8 CFU/mL) and each mouse was challenged intraperitoneally with approx. 1×10^8 CFU in 200 µL PBS containing 3% porcine stomach mucin type III (Sigma Aldrich).

Efficacy testing of NCL195 (4 oral doses, 4 h apart)+colistin (4 intraperitoneal doses, 4 h apart) in a *Ec*Xen14 peritonitis-sepsis mouse model was performed using the following treatment groups: (i) NCL195 50 mg/kg; (ii) NCL195 50 mg/kg+colistin 0.125 mg/kg; (iii) colistin 0.125 mg/kg; (iv) NCL195 50 mg/kg+colistin 0.25 mg/kg; (v) colistin 0.25 mg/kg; (vi) NCL195 50 mg/kg+colistin 0.5 mg/kg; (vii) colistin 0.5 mg/kg. The col^R-*Ec*Xen14 peritonitis-sepsis challenge model enrolled the following treatment groups: (i) NCL195 50 mg/kg; (ii) NCL195 50 mg/kg+colistin 0.5 mg/kg; (iii) colistin 1 mg/kg; (iv) NCL195 50 mg/kg+colistin 2 mg/kg; (v) colistin 2 mg/kg; (v) colistin 8 mg/kg (two doses at 0 and 4 h post-infection). Bioluminescence imaging, monitoring, treatment regimen and analysis followed the procedure described above.

405

406 **Figure legends**

407 **FIG 1.** Selected histological images of organs from control and treated mice. No
408 morphological abnormalities or no apparent changes were observed in mice orally treated
409 with NCL195 (10 or 50 mg/kg, 4 doses, 4 h apart) alone (**a**); treated with NCL195 (4 oral
410 doses, 50 mg/kg, 4 h apart) combined with colistin (4 intraperitoneal doses, 0.125, 0.25, 0.5
411 or 1 mg/kg, 4 h apart) in comparison with vehicle, NCL195 (50 mg/kg) and colistin at the
412 same concentrations after 72 h post-treatment (**b** & **c**). Scale bar: 200 µm.

413

414 **FIG 2.** Oral efficacy of NCL195 in a bioluminescent *Sa*Xen29 mouse peritonitis-sepsis
415 model. (**a**) Comparison of luminescence signals between groups of CD1 mice (n=6)
416 challenged intraperitoneally with *Sa*Xen29 and orally treated with vehicle only, 50 mg/kg
417 NCL195 or 6 mg/kg moxifloxacin at 0, 4, 8 and 12 h post-infection. Mice were subjected to
418 bioluminescence imaging on IVIS Lumina XRMS Series III system at the indicated times
419 (ns, not significant; *, p<0.05; **, p< 0.01; ***, p<0.001, ****, p<0.0001, Mann-Whitney U-
420 test, two-tailed). Broken segment on y-axis represents limit of detection (LOD). (**b**) Survival
421 analysis for mice orally treated with NCL195, moxifloxacin and vehicle (***, p<0.001; Log-
422 rank (Mantel-Cox) test). (**c**) Ventral and dorsal images of representative CD1 mice
423 challenged with approx. 3×10^7 CFU of bioluminescent *Sa*Xen29.

424

425 **FIG 3.** Efficacy of NCL195+colistin combination in a bioluminescent *Ec*Xen14 peritonitis-
426 sepsis mouse model**.** (**a**) Luminescence signal comparisons between groups of CD1 mice
427 (n=6) challenged intraperitoneally with bioluminescent *Ec*Xen14 and treated at 0, 4, 8 and 12
428 h with the indicated drug concentrations. Mice were subjected to bioluminescence imaging
429 on IVIS Lumina XRMS Series III system at the indicated times (ns, not significant; **,
430 p<0.01; ***, p<0.001, Mann-Whitney U-test, two-tailed). Broken segment on y-axis

represents limit of detection (LOD). (**b**) Survival analysis for mice treated with the indicated drugs (*, $p<0.05$; ****, $p<0.0001$; Log-rank (Mantel-Cox test); "X" denotes no surviving mice. (**c**) Ventral and dorsal images of representative CD1 mice challenged with approx. 1×10^8 CFU of bioluminescent *Ec*Xen14. Col, colistin.

FIG 4. Efficacy of NCL195+colistin combination data in bioluminescent col^R-*Ec*Xen14 peritonitis-sepsis mouse model. (**a**) Luminescence signal comparisons between groups of CD1 mice ($n=6$) challenged intraperitoneally with bioluminescent col^R-*Ec*Xen14 and treated at 0, 4, 8 and 12 h with the indicated drug concentrations. The drug control group received two doses of 8 mg/kg colistin at 0 and 4 h. Mice were subjected to bioluminescence imaging on IVIS Lumina XRMS Series III system at the indicated times (ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ****, $p<0.0001$, Mann-Whitney *U*-test, two-tailed). Broken segment on *y*-axis represents limit of detection (LOD). (**b**) Survival analysis for mice treated with the indicated drugs (**, $p<0.01$, ****, $p<0.0001$; Log-rank (Mantel-Cox test); "X" denotes no surviving mice. (**c**) Ventral and dorsal images of representative CD1 mice challenged with approx. 1×10^8 CFU of bioluminescent col^R-*Ec*Xen14. Col, colistin.

FIG 5. Chemical structures of NCL812 (2,2′-bis[(4-chlorophenyl)methylene]carbonimidic dihydrazide) and NCL195 (4,6-*bis*-(2-((E)-4-methylbenzylidene)hydrazinyl)pyrimidin-2-amine). Red colour shows the structural changes in NCL195 relative to NCL812.

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AUTHOR CONTRIBUTIONS

Hang Thi Nguyen contributed to the study design, performed experiments, wrote the preliminary manuscript and editing. Henrietta Venter contributed to study design, editing and discussion. Lucy Woolford contributed for histopathology examination, editing and discussion. Sylvia Sapula contributed to whole genome sequencing, editing and discussion. Kelly Young and Adam McCluskey were responsible for synthesizing NCL195 and contributed to editing of the manuscript. Sanjay Garg contributed to formulations. Stephen W. Page contributed to editing, discussion and provided financial support for the study. Abiodun David Ogunniyi contributed to study design, experiments, technical guidance, supervision, editing, and discussion. Darren Trott contributed to study design, supervision, editing, discussion, and provided financial support for the study. All authors read and approved the submitted version of the manuscript, in addition to contributing to manuscript revision.

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Conflicts of interest

Stephen W. Page is a director of Neoculi Pty. Ltd. Darren J. Trott and Adam McCluskey have received research funding from Neoculi Pty. Ltd.

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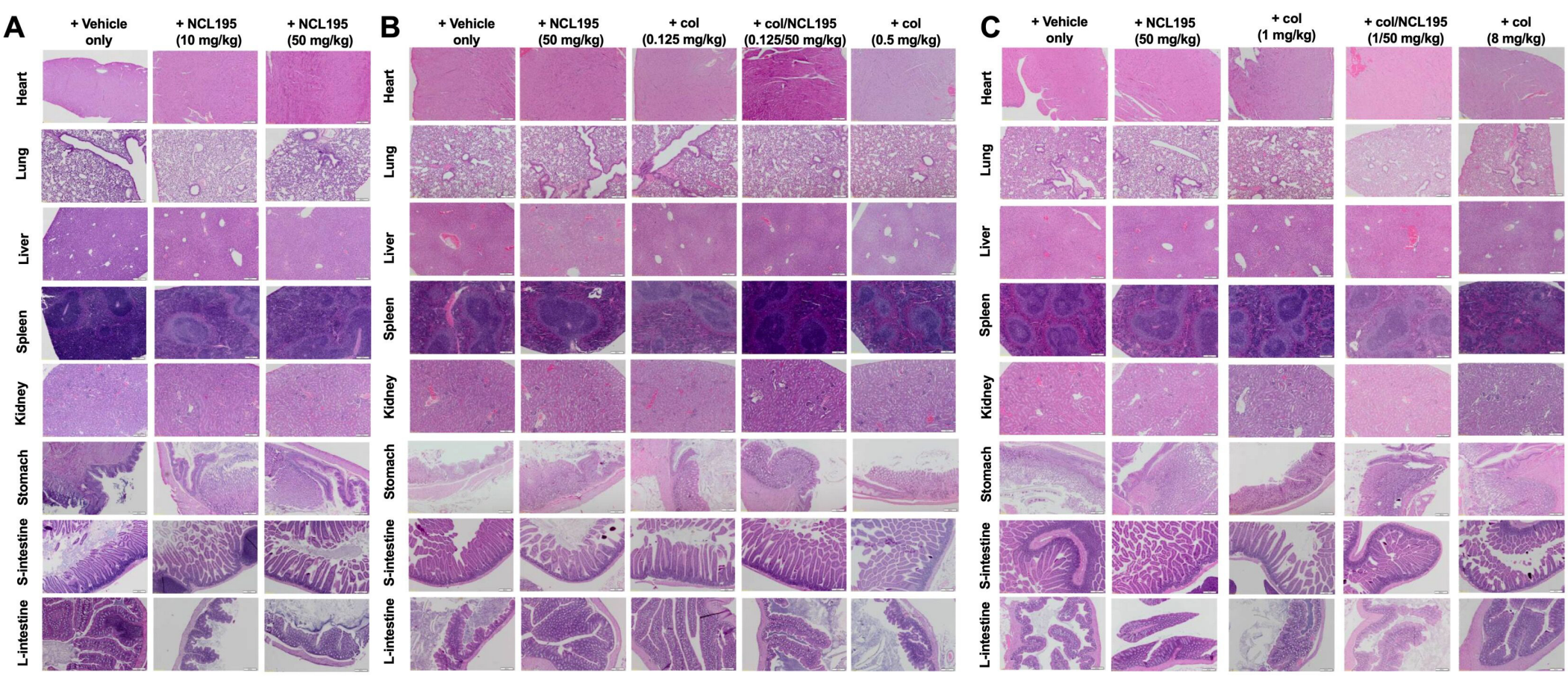
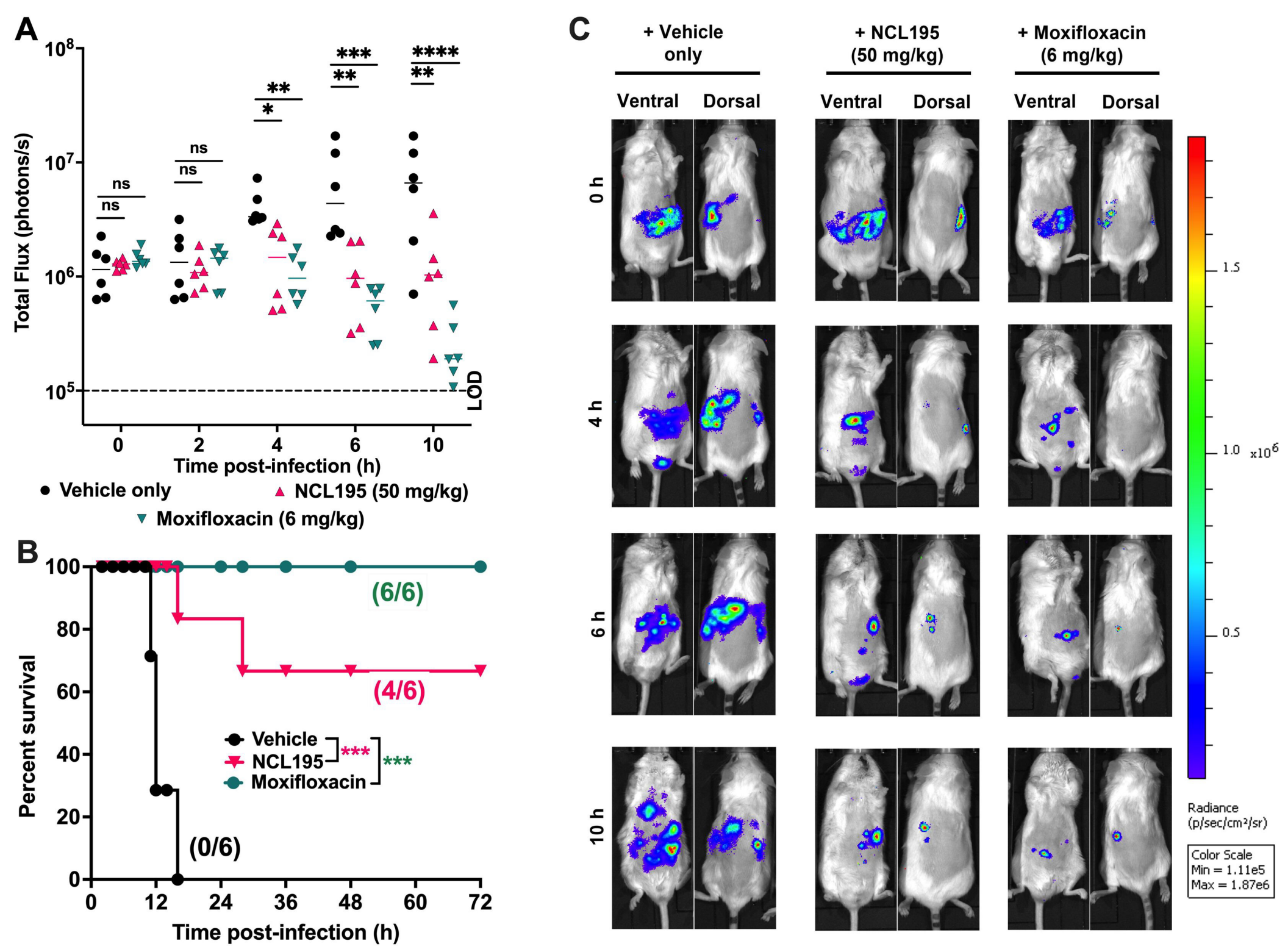
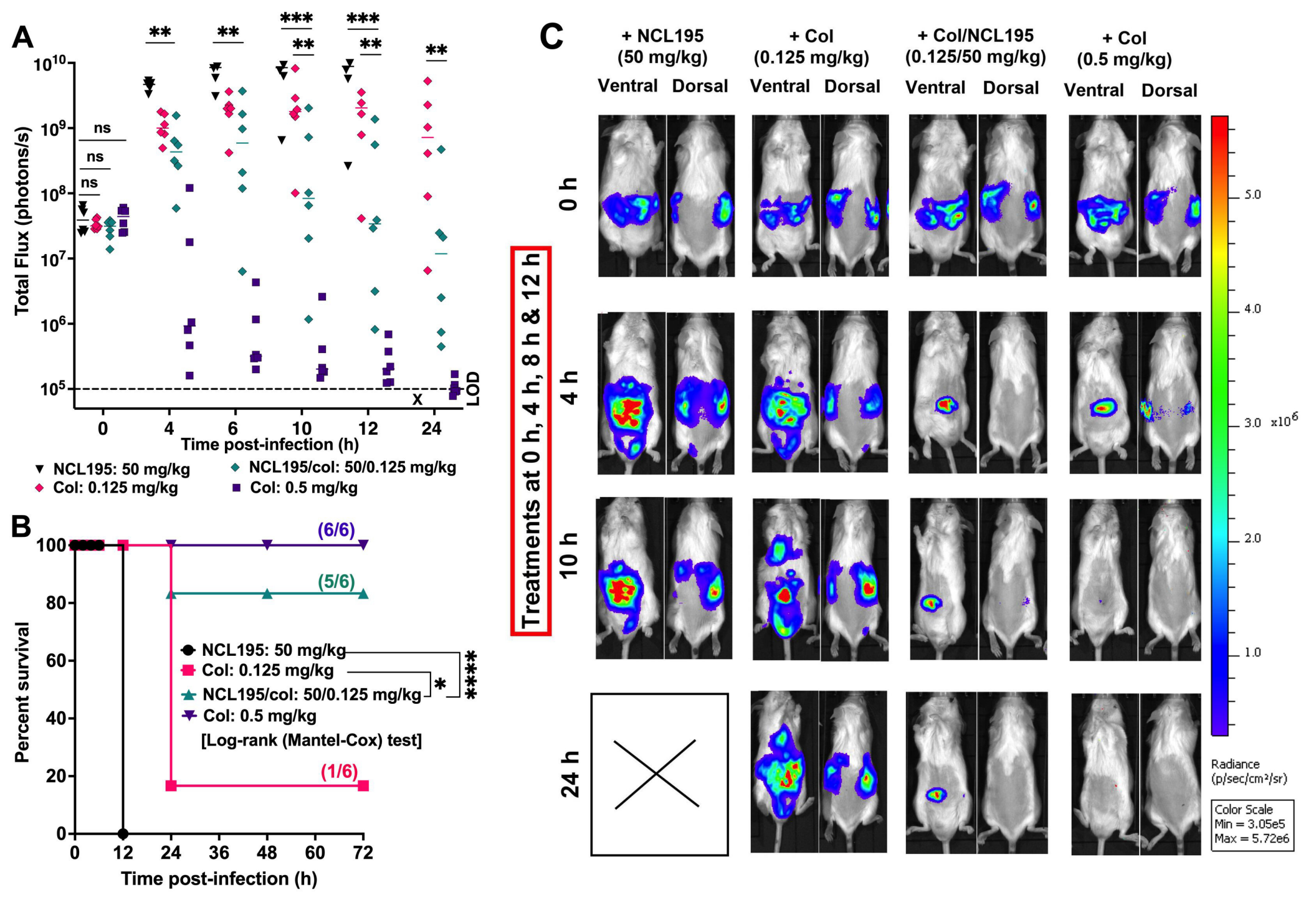
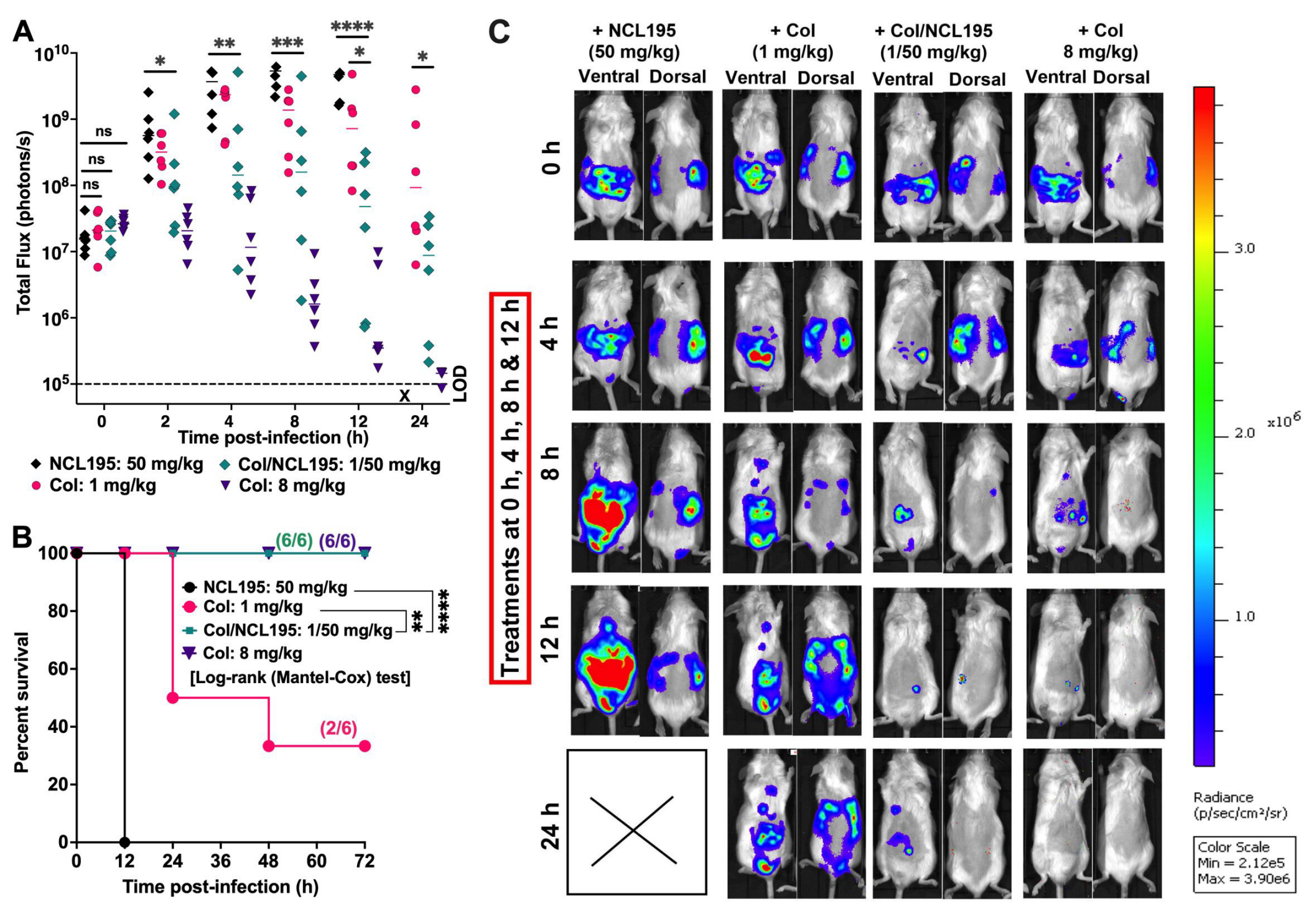
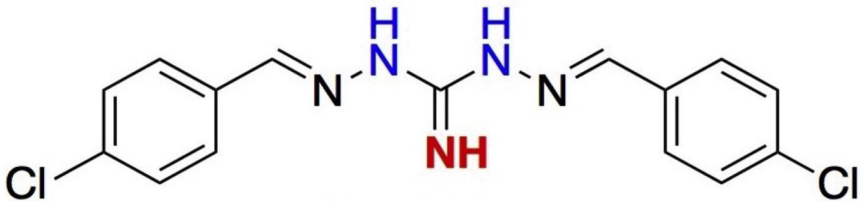

A
+ Vehicle only
+ NCL195 (10 mg/kg)
+ NCL195 (50 mg/kg)
Heart
Lung
Liver
Spleen
Kidney
Stomach
S-intestine
L-intestine
B
+ Vehicle only
+ NCL195 (50 mg/kg)
+ col (0.125 mg/kg)
+ col/NCL195 (0.125/50 mg/kg)
+ col (0.5 mg/kg)
Heart
Lung
Liver
Spleen
Kidney
Stomach
S-intestine
L-intestine
C
+ Vehicle only
+ NCL195 (50 mg/kg)
+ col (1 mg/kg)
+ col/NCL195 (1/50 mg/kg)
+ col (8 mg/kg)
Heart
Lung
Liver
Spleen
Kidney
Stomach
S-intestine
L-intestine

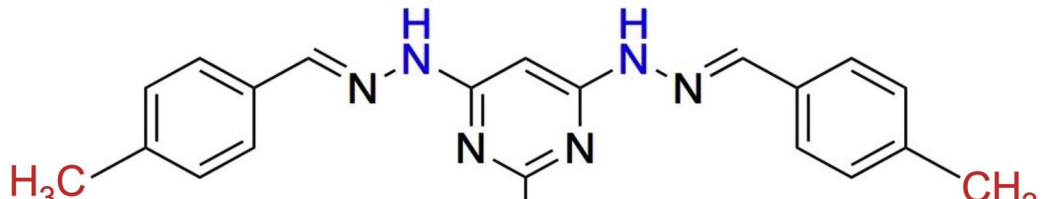

A
Total Flux (photons/s)
Time post-infection (h)
LOD
ns
*
**
***
****
Vehicle only
NCL195 (50 mg/kg)
Moxifloxacin (6 mg/kg)
B
Percent survival
Time post-infection (h)
(6/6)
(4/6)
(0/6)
Vehicle
NCL195
Moxifloxacin
***
C
+ Vehicle only
+ NCL195 (50 mg/kg)
+ Moxifloxacin (6 mg/kg)
Ventral
Dorsal
0 h
4 h
6 h
10 h
Radiance (p/sec/cm²/sr)
Color Scale
Min = 1.11e5
Max = 1.87e6


A
Total Flux (photons/s)
Time post-infection (h)
ns
**
***
LOD
NCL195: 50 mg/kg
NCL195/col: 50/0.125 mg/kg
Col: 0.125 mg/kg
Col: 0.5 mg/kg
B
Percent survival
Time post-infection (h)
(6/6)
(5/6)
(1/6)
NCL195: 50 mg/kg
Col: 0.125 mg/kg
NCL195/col: 50/0.125 mg/kg
Col: 0.5 mg/kg
[Log-rank (Mantel-Cox) test]
C
+ NCL195 (50 mg/kg)
+ Col (0.125 mg/kg)
+ Col/NCL195 (0.125/50 mg/kg)
+ Col (0.5 mg/kg)
Ventral
Dorsal
Treatments at 0 h, 4 h, 8 h & 12 h
0 h
4 h
10 h
24 h
Radiance (p/sec/cm²/sr)
Color Scale
Min = 3.05e5
Max = 5.72e6


A
Total Flux (photons/s)
Time post-infection (h)
ns
*
**
***
****
LOD
NCL195: 50 mg/kg
Col: 1 mg/kg
Col/NCL195: 1/50 mg/kg
Col: 8 mg/kg
B
Percent survival
Time post-infection (h)
(6/6) (6/6)
(2/6)
[Log-rank (Mantel-Cox) test]
C
+ NCL195 (50 mg/kg)
+ Col (1 mg/kg)
+ Col/NCL195 (1/50 mg/kg)
+ Col 8 mg/kg)
Ventral Dorsal
0 h
4 h
8 h
12 h
24 h
Treatments at 0 h, 4 h, 8 h & 12 h
x10^6
Radiance (p/sec/cm²/sr)
Color Scale
Min = 2.12e5
Max = 3.90e6



NCL812
(Robenidine)



NCL195