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Rapidly synthesized zinc oxide nanoparticles can increase the activity of antimicrobial drugs against clinical isolates of *Pseudomonas aeruginosa* and *Escherichia coli*

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ABSTRACT

This study aimed to investigate the effectiveness of combining zinc oxide nanoparticles (ZON) to antimicrobial drugs against clinical isolates of *Pseudomonas aeruginosa* and *Escherichia coli*. We explored two different methods to combine nitrofurantoin, cefepime, imipenem, azithromycin, gentamicin and sulfamethoxazole to ZON, using paper disks and 96 well plates. ZON was synthesized using the microwave-hydrothermal method and was characterized by UV-visible and Raman spectroscopy, X-ray diffraction, scanning electron microscopy and energy-dispersive X-ray spectroscopy. ZON cytotoxicity was tested against BGM cells, and its anti-inflammatory potential was also tested *in vitro*. The nanoparticles average size was of approximately 85 nm, and they decreased significantly the minimal inhibitory concentration of the tested antimicrobial drugs (ranging from 16 to more than 2000 times) when combined to them at the concentrations of 8 or 16 μg/mL - except for azithromycin against *E. coli* isolates. It also lacked cytotoxicity even at 1000 μg/mL. ZON were more effective than tenoxicam on the anti-inflammatory test. Further *in vivo* studies are necessary to set safe doses on living organisms.

Keywords: Antimicrobial, cytotoxicity, *Escherichia coli, Pseudomonas aeruginosa,* zinc oxide nanoparticles

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INTRODUCTION

Nanoparticles are fragments of materials such as metals, polymers and lipids, with sizes usually ranging from 1-500 nm, varied shapes, increased surface area and unique electromagnetic, optical and other physicochemical properties such as light absorption and emission[1, 2]. They are used as superconductors, semiconductors, for preparation of paints and coatings, and to increase the strength and durability of materials of varied interests, such as dentistry, orthopedics and textile industries[1, 2]. There is a growing interest in using nanoparticles for diagnostic and treatment of diseases, due to their possible interactions to molecular targets of interest. Such interactions may provide evidence of ongoing pathophysiological processes (in a more reliable and faster way compared to currently available methods) and/or change the course of diseases[2, 3]. Therapeutic benefits might be achieved using nanoparticles isolated or combined to clinically relevant drugs, increasing their pharmacological activity[2, 4].

Zinc oxide is used in several ways in its bulk form, such as in sunscreens and pharmaceutical anti-inflammatory formulations. Zinc oxide nanoparticles (ZON) are considered as safe for mammals as the bulk form, and have been explored in antimicrobial and antitumoral studies[3, 4]. Concerning their antimicrobial properties, ZON are mostly explored alone or functionalized with antimicrobial drugs[5], what usually requires complex reactions of organic synthesis. Combining nanoparticles to antimicrobial drugs is a simple strategy that can be useful to treat infectious diseases; however, this approach is poorly explored, mostly due to solubility and compatibility difficulties. In the current scenario of bacterial resistance, such combinations would be of interest if proven to be effective.

Bacterial resistance to antimicrobial drugs is a world health issue for which several economic and social problems have been predicted[6]. The global gross domestic product might fall around 4% by 2050 due to bacterial resistance impacts in people's work and academic productivity[7]. Mechanisms of bacterial resistance include molecular modification of drug targets, enzymatic inactivation or modification of drugs, efflux pumps, and biofilm formation[8-11]. A post-antibiotic scenario (i.e., a context in which infectious diseases of low clinical complexity - or even self-limiting - are not easily managed with the currently available antimicrobials) has been acknowledged, given the growing shortage of effective therapies[8]. Furthermore, new antimicrobials are not top interests of pharmaceutical industries[9]. Thus, strategies such as exploring nanoparticles to make the currently available drugs more effective are relevant.

This study aimed to describe the synergistic potential of ZON with relevant antimicrobial drugs against clinical isolates of *Pseudomonas aeruginosa* and *Escherichia coli,* which are relevant Gram-negative pathogens. Both are related to urinary and gastrointestinal infections, and reports on bacterial resistance to antimicrobials of these species are growing[10, 11]. ZON were synthesized using a rapid microwave-assisted method. We analyzed ZON using UV-visible and Raman spectroscopy, X-ray diffraction, scanning electron microscopy and energy-dispersive X-ray spectroscopy. We used an *in vitro* method to demonstrate ZON anti-inflammatory potential, and cytotoxicity assays suggested its safety. Our data open doors for further studies with the combinations using *in vivo* models of infectious diseases.

METHODOLOGY

Synthesis and characterization of zinc oxide nanoparticles (ZON)

Zinc acetate dihydrate was used to prepare ZON using the microwave-hydrothermal method described in detail by Marinho et al.[12], with reaction time of two minutes at 90 °C. ZON were collected by centrifugation, washed several times in distilled water and ethanol, and dried at 80 °C to obtain a fine powder. As they are poorly soluble in water, we tested different polar non-toxic solvents which would not hamper the biological assays.

ZON was characterized with different methods. Raman spectra was obtained at room temperature with an Ocean Optics portable spectrometer ($\lambda = 785$ nm, 499 mW), following the manufacturer instructions. X-ray diffraction (XRD) analyses were performed in a diffractometer (XRD-6000, Shimadzu, Japan), equipped with CuK radiation ($\lambda = 1.5406$ Å) in the 2θ range from 10° to 100° (0.02°/min scan increment, 2 s steps fixed time). Results were refined using the Rietveld's profile analysis method[13]. Scanning electron microscopy (SEM) was performed using the EVO MA 10 microscope (Zeiss, Germany), and energy-dispersive X-ray (EDX) spectra was obtained using an EDX analyzer operating at 200 kV (Oxford Instruments, UK).

Cytotoxicity assay

The cytotoxicity of ZON was assessed using BGM cells (American Type Cell Culture, USA), an immortalized fibroblast-like kidney cell line. ZON was tested at an initial concentration of 250 μg/mL. Culture and test protocols used in this study were conducted as described by our group [14]. Cells were cultured in RPMI 1640 media (Sigma, USA), supplemented with glutamine (0.3 mg/L), penicillin (200 IU/mL), streptomycin (100 μg/mL) and fetal bovine serum (10%). Plates were prepared with 180 μL/well, with an estimated counting of

$1x10^4$ cells each. The plates were incubated for 4 h, and the uptake of neutral red vital dye (50 μg/mL, 20 μL) was measured with a microplate reader (λ = 540 nm). Untreated cells prepared in ZON-free RPMI media were used as control. This test was performed in triplicate. The IC_{50} index was calculated using GraphPad Prism for Windows.

Bacterial strains

A total of 10 bacterial isolates of *E. coli* and 10 isolates of *P. aeruginosa* were used in this study. *E. coli* isolates are from urinary infections and *P. aeruginosa* isolates are from tracheal secretions. All strains are part of the microorganisms collection from Pitagoras College. Their identity was confirmed with VITEK 2 system version R04.02 (bioMérieux, France). Similarity indexes of 90% (or higher) were considered confirmative of the species of each isolate.

Minimal inhibitory concentration (MIC) assay

The MIC of ZON was determined in triplicate using untreated sterile 96-well polystyrene microtiter plates following CLSI standards and a protocol standardized by our group[14, 15]. A stock solution of ZON (5 mg/mL) was prepared using propylene glycol and water (4:1, previously sterilized in autoclave), which was diluted in sterile water for the tests (100 μL/well). The bacterial inoculum was primarily prepared at 0.5 MacFarland scale ($1.5x10^8$ CFU/mL) in sterile saline (0.9%), then diluted to $1x10^5$ CFU/mL in fresh sterile double concentration Mueller Hinton broth (Difco, Becton Dickinson, USA). The final concentration of ZON ranged from 1024 to 8 μg/mL, and the final concentration of the bacterial suspensions was of $5x10^4$ CFU/mL (final volume of the wells: 200 μL). MIC was established as the lowest concentration in which resazurine staining (0.1 g/L, 50 μL) resulted in no color modification from blue to pink in all strains. ZON at 1 mg/mL was used as a negative control.

This procedure was also performed to determine the MIC of azithromycin, gentamicin and sulfamethoxazole (all from Sigma, USA), for each species. Stock solutions of the drugs (4 mg/mL) were prepared in sterile water, and their final concentration of the drugs ranged from 1024 to 8 μg/mL. The final concentration of the bacterial suspensions was of $5x10^4$ CFU/mL (final volume of the wells: 200 μL). Resazurine staining (0.1 g/L, 50 μL) was used as described above.

Interference of ZON on antimicrobial drugs

The effects of combining ZON to antimicrobial drugs (synergism or antagonism) were assessed using two different methods, both performed in triplicate. First, we used an interference method standardized by our group[16] with three isolates of each species, as a preliminary assay. The selected disks were

nitrofurantoin 300 μg and cefepime 30 μg for *E. coli*, and imipenem 10 μg and azithromycin 15 μg for *P. aeruginosa* (all from Sensifar, Brazil), applied in petri dishes as for conventional susceptibility test. Following, briefly, 10 μL of the ZON solution at 1000 μg/mL was dispensed in each disk. Plates were incubated overnight at 37 °C, and the inhibition zone mean diameter was compared to control plates (untreated disks). Synergism and antagonism were inferred considering a 2 mm increase or decrease in the inhibition zone compared to the control, respectively.

We then used the checkerboard method[17] to test interactions of ZON and antimicrobials, with some modifications. Overnight-grown bacterial cultures were prepared in Mueller-Hinton broth as for the MIC assays. ZON was serially diluted vertically to reach final concentrations from 1024 to 8 μg/mL, and the antimicrobial drugs were serially diluted horizontally to reach final concentrations from 1024 to 0.5 μg/mL. Results were obtained using resazurine staining, as described at the MIC assay section.

In vitro anti-inflammatory potential of ZON

We used the bovine serum albumine (BSA - Thermo Fisher, USA) denaturation assay to investigate the anti-inflammatory potential of ZON (at 1000 μg/mL), as previously described[18], with slight modifications. BSA denaturation was conducted at 70 °C for 15 minutes, and Tenoxicam (Sigma, USA, 1000 μg/mL) was used as a positive control. BSA and Tenoxicam were prepared as aqueous solutions.

Statistics

Homocedacisty of data was checked by Bartlett's test, and normality was verified using Shapiro-Wilk test (square root transformation was performed when necessary). Differences on the activity of the antimicrobial drugs were analyzed using paired T-test. Calculated and observed XRD were analyzed using chi-square. The anti-inflammatory potential of ZON was analyzed using one-way ANOVA followed by Tukey test. All analyses were conducted using Bioestat 5.0 for Windows. Significant and highly significant levels were set as $p<0.05$ and $p<0.01$, respectively.

RESULTS and DISCUSSION

ZON characterization

XRD and Raman analyses indicated peaks that correspond to the hexagonal structure of wurtzite-like nanoparticles. For XRD, Rietveld's refinement of the results indicated that calculated and observed diffraction patterns are correlated (Fig 1), suggesting good long-range crystal ordering. The nanoparticles presented a single phase of wurtzite structure (JCPDS 36-1451), without the formation of secondary phases or impurities. Sharp and narrow diffraction peaks consistent with the zinc oxide wurtzite-type hexagonal structure were observed, indexed according to the JCPDS 36-1451 crystallographic record and $P6_3mc$ space group. The Rietveld refinement indexes, the lattice parameters and volume, obtained by Rietveld refinement for ZnO were a = b (Å) = 3.25014(5); c (Å) = 5.20715(9) and V (Å^3) = 47.636 (2); Rwp (%) = 4.86; Rp (%) = 3.68; Rbragg (%) = 1.59 and $\chi 2$ = 1.65. Raman spectra was more intense at E_{2H} at 438 cm^{-1} (Fig. 2). The characteristic bands are attributed to the active Raman modes of the zinc oxide wurtzite single crystal. These results are in good agreement with the work of Marinho et al.[12].

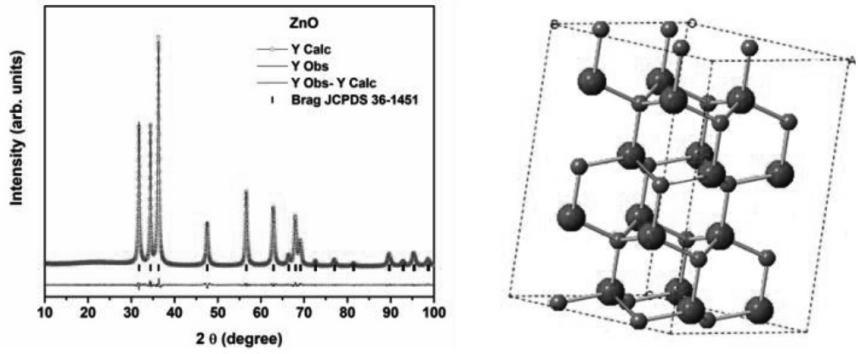

Figure 1. Results obtained by X-ray diffractogram of ZON after using Rietveld's refinement method.

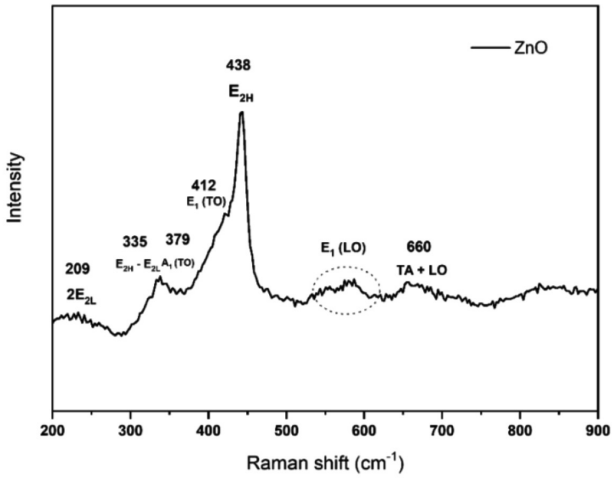

Figure 2. Raman spectra of ZON.

Scanning electron microscopy and EDX results are shown in figure 3. Approximately 100 particles were considered from the visual field images to determine average particle size. ZON presented regular and agglomerated shapes, with an average size of approximately 85 nm. Small, aggregated particles of approximately 30 nm were also observed. The peaks observed in EDX spectrum confirmed the pure composition of ZON.

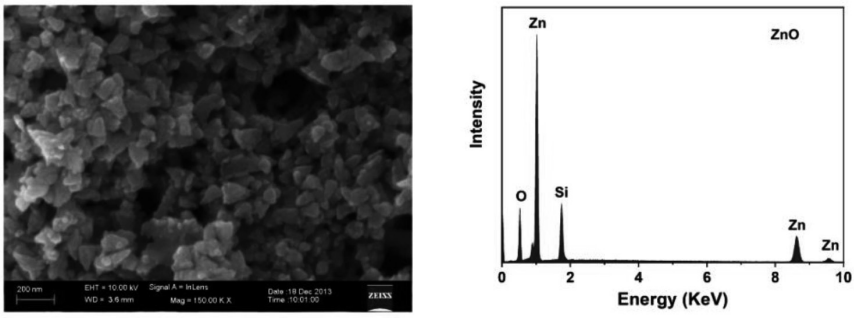

Figure 3. Scanning electron microscopy (left) and energy-dispersive X-ray spectra of ZON (right).

ZON solubility was tested with several possible solvents that would not interfere on the biological tests. A 4:1 blend of propylene glycol and water provided us the best solubility results.

MIC assays

The MIC values of the antimicrobial drugs are presented on table 1. Gentamicin was the most effective drug against *P. aeruginosa* isolates, as azithromycin was for *E. coli* isolates. ZON isolated presented no antimicrobial activity against any of the isolates of the tested species.

Table 1. MIC values of antimicrobial drugs

Species	Azithromycin	Gentamicin	Sulfamethoxazole
E. coli	16	128	>1024♦
P. aeruginosa	256	8	512

Data are expressed in μg/mL. Results are referent to all tested isolates.
♦ MIC was superior to the highest tested value.

Drug interaction assays

We used two different methods to check the possible effects of the combination of ZON at 1 mg/mL and antimicrobial drugs. Using the interference method[16], we detected significant increase of the inhibition zones of the antimicrobial drugs in disks (tables 2 and 3) upon the addition of ZON ($p<0.05$). The synergic effect was more evident for cefepime (against *E. coli*) and for azithromycin (against *P. aeruginosa*).

Table 2. Interference of ZON on the antimicrobial activity of drugs in disks for *E. coli*

Strain	Nitro	Nitro + ZON	Cef	Cef + ZON
E2	10	12*	27	34*
E5	17	19*	0	10*
E9	18	20*	0	12*

Nitro: Nitrofurantoin; Cef: cefepime; +ZON: addition of zinc oxide nanoparticles. Data are expressed as inhibition zone dimensions in millimeters. *All data with +ZON are significantly different of their ZON-free counterparts ($p<0.05$).

Table 3. Interference of ZON on the antimicrobial activity of drugs in disks for *P. aeruginosa*

Strain	Imip	Imip + ZON	Azit	Azit + ZON
P3	23	25*	17	19*
P6	21	23*	10	17*
P7	15	24*	0	20*

Imip: Imipenem; Azit: azithromycin; +ZON: addition of zinc oxide nanoparticles. Data are expressed as inhibition zone dimensions in millimeters. *All data with +ZON are significantly different of their ZON-free counterparts ($p<0.05$).

Given these results, we proceeded to the checkerboard method[17] to conduct a more detailed study on the effects of combining ZON to antimicrobial drugs. An adaptation on the interpretation of this method was necessary, as ZON presented no antimicrobial effect. Thus, we determined the new MIC of the drugs (table 4), instead of their fractional inhibitory concentration. For *P. aeruginosa* isolates, all new MIC values with addition of ZON were significantly lower than the MIC values obtained without ZON ($p<0.05$). For *E. coli,* the new MIC values for gentamicin and sulfamethoxazole with addition of ZON were significantly lower than the MIC values obtained without ZON ($p<0.05$). Surprisingly, the new MIC of azithromycin against *E. coli* increased significantly by the addition of ZON ($p<0.05$).

Table 4. New MIC values of antimicrobial drugs with the addition of ZON on checkerboard assay

Species	Azithromycin/ZON	Gentamicin/ZON	Sulfamethoxazole/ZON
E. coli	256 + 8	1 + 32	0.5 + 8
P. aeruginosa	0.5 + 8	0.5 + 8	8 + 16

Data are expressed as concentrations of drugs + ZON in μg/mL. Results are referent to all tested isolates.

Anti-inflammatory potential and cytotoxicity

ZON presented anti-inflammatory effect (Figure 4) and was more effective than tenoxicam at the highest concentration tested ($p<0.05$). ZON were not toxic to BGM cells even in the highest tested concentration (toxicity superior to 1000 μg/mL), suggesting their safety. IC_{50} could not be, therefore, calculated.

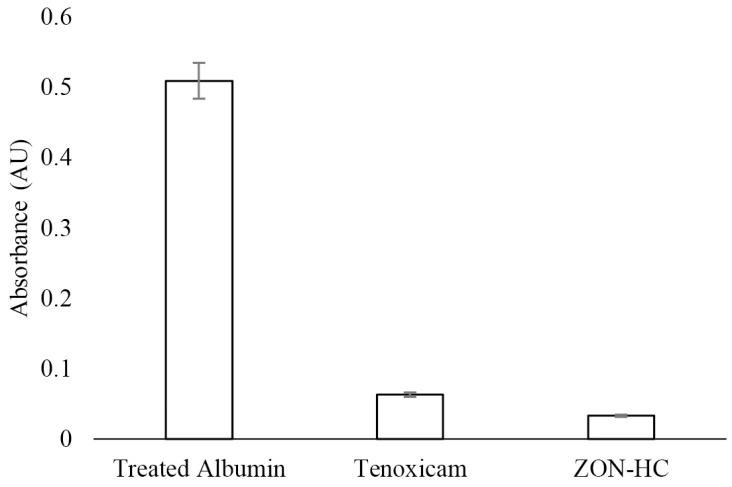

Figure 4. Comparative anti-inflammatory activities of ZON, tenoxicam and treated albumin (i.e. exposed to high temperature and free of ZON and tenoxicam). AU: arbitrary units, ZON-HC: ZON at its highest concentration (1024 µg/mL).

This study described that ZON synthesized by the microwave-hydrothermal method described by Marinho et al.[12] can increase the activity of antimicrobial drugs against Gram-negative pathogenic strains. ZON was synthesized without significant formation of impurities, and their nanoparticles features were confirmed by XRD and EDX spectrum peaks, Raman signals and scanning electron microscopy (Figs. 1-3). The antimicrobial mechanisms of action of ZON include cell disruption, induction of oxidative stress, and downregulation of several bacterial genes, without affecting human cells[19,20]. Generally, the cytotoxicity and poor water solubility of different nanoparticles hamper investigations on their possible biological properties. ZON lacked cytotoxicity at the tested concentrations (up to 1000 µg/mL), and we could overcome solubility issues using a blend of propylene glycol and water.

One may wonder why we tested the interactions of ZON with the antimicrobial drugs, as they were not active against bacteria. As previously described by our group, the activity of antimicrobial drugs might be increased or impaired by molecules with no antimicrobial properties[21-23]. Eventual synergism or antagonism resulting from such combinations are poorly predictable and require experimental evidence to be determined. In this context, our results are consistent with the observation of others that ZnO nanoparticles can present a synergistic behavior with antimicrobial drugs. A study described the combination of ZnO nanoparticles (average diameter of 20 nm) doped or not with Fe, Mn,

Cu and Co. The combination of doped ZnO nanoparticles to ciprofloxacin and ampicillin was synergic against *Bacillus subtilis, E. coli* and *S. aureus*, whilst combinations with pure ZnO nanoparticles resulted in additive effect[24]. ZnO nanoparticles (average diameter of 78 nm) in sub-inhibitory concentrations presented synergism when combined to ciprofloxacin and ceftazidime against *Acinetobacter baumannii*[25]. More recently, ZnO nanoparticles combined to fennel essential oil in a potato starch packing film were active against *S. aureus, E. coli* and *Aspergillus flavus*[26].

Among the drugs tested in the present study is azithromycin. Reports on the resistance of pathogenic bacterial species to this drug are increasing[27], especially due to its large use in patients with SARS-CoV-2 infections[28]. Azithromycin interaction with ZON resulted in decrease of MIC value for *P. aeruginosa* isolates from 256 to 0.5 μg/mL, whereas for *E. coli* isolates the MIC value increased. Similarly, a study reported an antagonistic behavior of ZnO nanoparticles (average diameter ranging from 10-30 nm) combined to β-lactams against *Yersinia intermedia*, whereas cephalosporins of second and third generations, tetracycline and nalidixic acid were not affected by the combination to the nanoparticle[29]. Recently, ZnO nanoparticles (average diameter of 50 nm) were combined to meropenem, ciprofloxacin and colistin against *P. aeruginosa*, and synergism was detected only for colistin[30].

The lack of antimicrobial activity of ZON alone might be at least partially explained by the method of synthesis, given that it influences morphological characteristics such as uniformity of size and contact surface. These properties may also interfere with their interactions to microbial targets[31]. Nevertheless, ZnO nanoparticles synthesized with other strategies might present antimicrobial activity. An investigation on the antimicrobial potential of ZnO nanoparticles (66 to 112 nm in diameter) prepared with *Albizia lebbeck* stem bark extract found that they were as effective as ciprofloxacin against *Bacillus cereus* and *Salmonella typhi,* using the agar diffusion method[32]. A study on ZnO nanoparticles prepared using the precipitation technique (232 to 692 nm in diameter) were shown to be active against *Aspergillus niger*[33].

The anti-inflammatory activity of ZON was expected, given that zinc oxide, in its bulk form, present this biological potential[34, 35]. We used an *in vitro* method based on the ability of a substance to inhibit BSA denaturation, exposure of its chromophore groups and protein aggregation, as observed in different inflammatory diseases[18]. ZON was more effective than tenoxicam in inhibiting BSA denaturation (Figure 4). The main known mechanisms that help to explain the anti-inflammatory potential of ZnO nanoparticles include suppression

of mRNA expression and protein levels of pro-inflammatory enzymes such as COX-2, and of cytokines such as TNF-α and IL-1β[36, 37].

Combinations of ZON and antimicrobial drugs were more effective than the drugs alone against clinical isolates of *E. coli* and *P. aeruginosa*. Furthermore, ZON lacked cytotoxicity and presented anti-inflammatory potential. The synthesis method is rapid, reproducible, and relatively simple, important aspects considering an eventual large-scale manufacturing. Although this study is not without limitations concerning the variety and number of bacterial strains, our data open doors for more studies exploring the combinations of antimicrobial drugs and ZON using *in vivo* models of infectious diseases.

STATEMENT OF ETHICS

All the necessary ethical rules were followed while performing research.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: MVDS

Methodology: MVDS, RCL

Validation: MVDS, RCL

Formal Analysis: LFCM, JZM, IPC, RCL, MVDS

Investigation: LFCM, JZM, IPC

Resources: MVDS, RCL, JZM

Writing - Original draft: LFCM, JZM

Writing - Review and editing: MVDS

Supervision and Project administration: MVDS

[†]Authors contributed equally to this work.

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