Evaluation of the Anti-biofilm Efficacy of Kyotorphin Derivatives and Biosafety Assessment: In Vitro and In Vivo Investigations Targeting Bacterial and Fungal Pathogens

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Abstract

Kyotorphin (KTP) dipeptide (L-Tyrosine-L-Arginine) and their derivatives possess a multitude of functions, qualifying them as "multifunctional peptides." Considering the escalating bacterial resistance to antibiotics, antimicrobial peptides offer a promising road, forming the central focus of this current investigation. The effectiveness of KTP derivatives, GABA-KTP-NH_2 and Indol-KTP-NH_2, were assessed for biofilm inhibition in bacterial and fungal strains. The viability of these derivatives was tested in fibroblasts and B16-F10-Nex2 cells. In vivo toxicity was evaluated using the model organisms *Galleria mellonella* and *Danio rerio*. Notably, both GABA-KTP-NH_2 and Indol-KTP-NH_2 derivatives effectively hindered biofilm formation in *E. coli*, *S. pneumoniae*, and *C. krusei*. In the *G. mellonella* model, the derivatives exhibited significant larval survival rates in toxicity tests, while in infection tests, they demonstrated efficient treatment against the evaluated microorganisms. Conversely, zebrafish assays revealed that Indol-KTP-NH_2 induced substantial mortality rates in embryos after 72 and 96 h of exposure. Similarly, the GABA-KTP-NH_2 derivative exhibited heightened lethality, noticeable at the 100 µM concentration after the same exposure periods. Importantly, toxicity assessments unveiled a relatively lower toxicity profile, coupled with a reduced potential for inducing abnormalities. These results highlight the necessity of employing a comprehensive approach that integrates diverse techniques to thoroughly assess toxicity implications.

Keywords Kyotorphin · Antimicrobial · Biofilm · *Galleria mellonella* · *Danio rerio* · Toxicity

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Introduction

In 1979, Japanese researchers (Takagi et al. 1979) discovered Kyotorphin (KTP), a dipeptide consisting of L-Tyrosine and L-Arginine, originally isolated from bovine brain (Takagi et al. 1979). Subsequently, this compound was identified in the brain synaptosomes of various mammals, including humans. When directly administered into the central nervous system (CNS) or used at high concentrations (200 mg/kg), KTP demonstrates opioid and analgesic effects (Fukui et al. 1983). Limited effects arise upon systemic administration due to KTP's limited ability to traverse the blood–brain barrier (BBB), as revealed in studies (Chen et al. 1998), thereby presenting a significant obstacle for its potential as a drug candidate.

Enzymatic degradation susceptibility poses another significant challenge for the potential use of KTP as a pharmaceutical agent. To address this concern, numerous research

groups have undertaken diverse approaches involving the incorporation and alteration of the original peptide structure (Chen et al. 1998; Rybal'chenko et al. 1999; Wang et al. 2001; Lopes et al. 2006a, b; Ribeiro et al. 2011a, b; Dzimbova et al. 2014; Serrano et al. 2015). Within this context, two KTP analogues that are the focal point of the present study were synthesized: GABA-KTP-NH$_2$, achieved by introducing aminobutyric acid (GABA) at the N-terminus, and Indol-KTP-NH$_2$, which involves substituting the tyrosyl residue with an indole moiety (Serrano et al. 2015). These alterations have exhibited noteworthy influences on permeability and analgesic efficacy, suggesting their potential to traverse the blood–brain barrier through passive diffusion across lipid bilayers (Serrano et al. 2015).

Certain bioactive peptides are capable of eliciting multiple physiological responses within living systems, earning them the designation of multifunctional peptides (MPs). An instance of such MPs is found in lactoferricin hydrolysates, a 25-sequence pepsin hydrolysate of lactoferrin, which has demonstrated attributes encompassing anticancer, antitumor, antiparasitic, and antimicrobial properties (Eliassen et al. 2002; Korhonen and Pihlanto 2006). Similarly, the KTP derivatives are classified as MPs due to their capacity to serve as analgesics, neuromodulators, antimicrobial peptides, biomarkers in Alzheimer's disease, and more (Perazzo et al. 2017; Ueda 2021).

In terms of its antimicrobial effects, KTP-NH$_2$ prompted membrane blebbing, disruption, and lysis of *Staphylococcus aureus*, as observed through atomic force microscopy experimentation (Ribeiro et al. 2011b). Additionally, the IbKTP-NH$_2$ variant demonstrated intriguing antifungal capabilities against *Candida species*, as highlighted in the research conducted by Martins de Andrade et al. (2020). The advancement of antimicrobial agents, particularly peptides known as AMPs, holds significant importance, given the substantial bacterial resistance to antibiotics. This avenue serves as an alternative approach to counteract such resistance. Antibiotics, commonly employed to combat bacterial infections, encounter challenges in penetrating the blood–brain barrier (BBB) and attaining therapeutic levels within the brain. The success of antibiotics in treating central nervous system (CNS) infections hinges on their capacity to access the infection site and unleash their antimicrobial effects. However, the BBB acts as a barrier that blocks the entry of numerous antibiotics into the brain, curtailing their effectiveness against CNS pathogens (Haddad et al. 2022). We hypothesized that derivatives such as GABA-KTP-NH$_2$ and Indol-KTP-NH$_2$, with their potential to translocate the BBB, might not only display analgesic properties but also present antimicrobial and antibiofilm activity.

The association of microorganisms within biofilms presents a protective mechanism for their growth, rendering them resilient to various environments, the host's immune system, and antimicrobial agents (Dunne 2018). Within the biofilm's matrix, the extracellular polymeric substances (EPS) are composed of polysaccharides, proteins, and nucleic acids (Stoodley et al. 2002). This matrix, characterized by porosity, contains water channels that facilitate the exchange of nutrients, oxygen, and excreted metabolites (Davey and O'toole 2000). EPS plays a pivotal role in diminishing antimicrobial penetration across all biofilm regions, acting as a physical hindrance to diffusion by sequestering most antimicrobial agents (Nichols et al. 1988). Microbial adhesion and the formation of biofilms are critical processes in pathogenesis, contributing to the development of persistent infections, as highlighted by the US agency "National Institutes of Health" (Costerton et al. 1999; Wilson et al. 2017).

The assessment of potential drug prototypes' toxicity commonly initiates with in vitro studies employing diverse cell lines across varying sample concentrations. Subsequently, these prototypes are subjected to testing in different animal models, historically including mice and rats, before proceeding to clinical trials. As elucidated across multiple references, the utility of alternative animal models in intermediate toxicity assessment is underscored (Rizzo et al. 2013; D'amora and Giordani 2018). Moreover, driven by ethical considerations, financial constraints, evolving safety requisites, and alignment with the 3Rs principle (Refinement, Reduction, Replacement), the utilization of mammalian experimental animals is increasingly curtailed to meet essential needs. Consequently, the establishment of standardized alternative models becomes imperative (Russell and Burch 1959; Hartung 2010). Alternative methods or models are designed to complement or substitute traditional mammalian tests in both biomedical research and education. Within this framework, the significance of employing experimental models like *Galleria mellonella* and *Danio rerio* (zebrafish) for studying host–pathogen interactions and conducting preclinical evaluations of new drugs is expanding (Martins de Andrade et al. 2020; Skalska et al. 2020), which coincidentally were the models adopted in the present study.

In the last years, methodologies have emerged for in vivo infection investigations using invertebrates, including the *G. mellonella* moth larva. Research has demonstrated that despite the taxonomic distinctions between invertebrates and mammals, they share analogous attributes within their innate immune systems. This invertebrate model confers numerous advantages compared to other counterparts, encompassing economical breeding, minimal technical constraints, uncomplicated management, and notably, swift outcomes (Mylonakis et al. 2005). The larvae can be readily and precisely subjected to inoculation via injection, forced feeding, or overlay with a spore layer. Notably, this approach allows for the examination of diverse parameters based on the rapid responses to infection, encompassing mortality rates,

melanization processes, immune modifications, microbial loads, gene expression alterations, and shifts in the proteome (Arvanitis et al. 2013; Kavanagh and Sheehan 2018). The evaluation of survival post-infection was carried out to determine suitable concentrations of the derivatives studied in zebrafish.

Zebrafish emerges as an optimal vertebrate model for toxicity screening due to its ability to emulate the intricate interactions within the human body during embryo development. Possessing a substantial genomic similarity (70%) and biological system resemblance to humans, zebrafish embryos allow the monitoring of phenotypic and genotypic abnormalities subsequent to exposure to various compounds. The assessment of alterations in organ dimensions and structures during development serves as a straightforward yet valuable gauge of toxicity. The cost-effective maintenance, swift developmental timeline, and ease of manipulation enable high-throughput and economically viable preclinical screenings (Yang et al. 2018; Batista-Filho et al. 2020).

Finally, in an attempt to find new activities associated with KTP derivatives, Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$ and evaluate their toxicity, the present study evaluated the inhibition of biofilm form of selected microorganisms strains, followed by in vitro analysis of cell viability. Additionally, the toxicity and anti-infective action of the KTP derivatives were investigated in vivo in *G. mellonella* and *D. rerio*.

Materials and Methods

Synthesis of KTP Derivatives

Details on peptide synthesis of Indol-KTP-NH$_2$ (331,2 g. mol-1) and GABA-KTP-NH$_2$ (422,2 g.mol-1) are provided in Supplementary Material. Briefly, both derivatives of KTP, were synthesized by solution phase synthesis and analyzed for purity on high performance liquid chromatography (HPLC). Moreover, the two kyotorphin derivatives synthesized, as well as the intermediate compounds of the synthetic sequence of GABA-KTP-NH$_2$, were completely characterized by nuclear magnetic resonance (NMR) and mass spectra under electrospray ionization (ESI–MS) (see Supplementary Material).

Determination of the Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of kyotorphins was determined using a modified broth microdilution method according to the Clinical and Laboratory Standards Institute (Komatsuzawa et al. 2000; Ouhara et al. 2008) the following microorganisms: *Escherichia coli* (ATCC 25922) and *Pseudomonas*

aeruginosa (ATCC 15442) as gram-negative bacteria and *Streptococcus pneumoniae* (ATCC 25922) as gram-positive bacteria. It also tested three Candida's species: *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 63) and *C. tropicalis* (140-S). Briefly, a cell suspension of each pathogens species was adjusted to 10^3 CFU/ml using brain heart infusion (BHI) broth (Sigma) or Mueller Hinton Broth media (MH) for bacteria. Aliquots (100 mL) of the suspension were dispensed in polystyrene, flat-bottomed, 96-well microtiter plates. Peptide stock solutions were diluted in BHI broth, and 100 mL of the dilution was added to the wells at a final concentration ranging from 25 μM to 1000 μM. The plates were incubated at 37 C for 24 h. Absorbance was measured at an optical density of 590 nm using a microplate reader (Synergy H1 Hybrid Reader, BIOTEK, USA). The assays were repeated independently three times using different cell suspensions. All the tests were performed in triplicates. MIC$_{50}$ was defined as the peptide concentration that inhibited 50% of the isolates from each group and MIC$_{90}$ was defined as the peptide concentration that inhibited 90% of the isolates from each group. MIC$_{50}$ and MIC$_{90}$ were calculated as follows: MIC$_{50}$ = n°. of isolates (n) × 0.5 and MIC$_{90}$ = n°. of isolates (n) × 0.9.

Determination of Biofilm Inhibition

The inhibition of the biofilm formation was evaluated using the protocol to static biofilm culture (Kwasny and Opperman 2010). For this, it was chosen only the microorganisms that were inhibited in the MIC$_{90}$ assay, *E. coli*, *S. pneumoniae*, and *C. krusei*. It was transferred 190 μL of the inoculum in the concentration of 10^6 and 10 μL of the peptide for the well of a sterile 96-well assay plate, also having a triplicate of each concentration. Amphotericin B and Chloramphenicol (Sigma, USA) were used as positive controls according to CLSI (Clinical and Laboratory Standards Institute, 2008; Clinical and Laboratory Standards Institute, 2023). After the 18 h incubation at 37 °C, it was measured the (OD) at λ = 600 nm to quantify overall growth. For evaluation of the biomass of biofilm, the medium was removed from each well and washed three times with NaCl 0.9% solution, ensuring the preservation of the biofilm structure at the bottom of each assay well. The washed plate was incubated at 60 °C for 1 h to fix the biofilm. For *S. pneumoniae* and *C. krusei*, it was transferred 200 μL of a 0.06% solution of crystal violet to each well to stain the biofilm for 5 min at 60 °C. For *E. coli* was used safranin (0.06%) to stain the biofilm. The dye was removed from each well and washed with NaCl 0.9%, followed by addition of 200 μL of acetic acid 30% in the wells and the samples were transferred to a new plate. For the evaluation of the viable cells on biofilms, colony forming unit (CFU) counting assay (Wilson et al. 2017) was conducted removing sample aliquots from the biofilm formation (without staining) to identify the number of viable cells.

After incubation for 24 h at 37 °C, microorganism colonies were counted, and viable microorganisms (CFU/mL) were reported as percentage of the control.

Cytotoxicity Test

Cell viability was monitored using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Riss et al. 2013; Formaggio et al. 2022) with mice embryonic fibroblasts (MEF) and murine melanoma tumor cells (B16-F10-Nex2). A total of 1×10^4 MEF and B16-F10-Nex2 per mL were sown in a 96-well microplate with Dulbecco's Modified Eagle Medium (DMEM) culture medium. The cells were maintained for 24 h in DMEM in the incubator with 5% CO_2 at 37 °C. After this period, the medium was removed, and the peptides were added in triplicate into microplate wells together with 240 μL of DMEM. Dimethyl sulfoxide (DMSO) was used as the positive control, and culture medium was used as the negative control. The plate was incubated for 24 h at 37 °C and 5% CO_2. The culture medium was removed, and the cells were washed in phosphate-buffered saline (PBS). MTT (5 mg/mL, prepared in PBS) was added to each well, and the plates were incubated at 37 °C and 5% CO_2 for 3 h. Then, MTT was removed, and DMSO was added to each well. Absorbance was measured in a spectrophotometer (Synergy H1 Hybrid Reader, BIOTEK, EUA) at 540 nm.

G. mellonella Toxicity and Infection Assay

G. mellonella larvae were kept in a metal container at 25 °C in the dark and fed a specific manipulated feed. For the assays, larvae without color changes and with adequate weight (150–200 mg) were selected and kept without food in petri dishes at 37 °C in the dark for 24 h prior to the tests. The toxicity activity of Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$ was conducted according to the methodology by Allegra et al. (2018). Ten *G. mellonela* larvae were used in each of eight treatment groups (Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$ in 200, 100 and 50 μM). A volume of 10 μL of each different peptide concentrations were inoculated in each group. One control group (inoculated with PBS) was included and served as a control for overall viability. The larvae were treated and counted for 7 consecutive days in three independent experiments and the toxicity was measured by the number of live larvae. For the infection assay, initially the lethal concentration of microorganisms for the larvae was standardized (Martins de Andrade et al. 2020). Ten *G. mellonella* larvae were infected with 10 μL of selected strains and kyotorphin derivatives by injection in the last pair of prolegs of each larva (Cotter et al. 2000). The negative control gets 10 μL of saline (NaCl 0.9%). For all experiments, larvae were incubated at 30 °C in standard petri dishes. The larvae were treated and counted for 7 consecutive days in three independent experiments and the peptides treatment against infection was measured by the number of live larvae.

Evaluation of KTP Derivatives Interference in Zebrafish Survival and Development

The in vivo toxicity activity of Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$, was tested with the zebrafish model. Wild-type strains of zebrafish were conditioned under standard conditions: 28 °C and pH 7.0 with a light:dark photo cycle of 12:12. Adult animals are kept in individual aquariums (Alesco) suitable for breeding zebrafish. The experiments were carried out under the laws of the National Council for Animal Experiment Control (CONCEA) and approved by the UNIFESP Animal Use Ethics Commission (CEUA nº 8861040323). The experiment begins with the separation of fertilized and unfertilized embryos. The fertilized embryos (n = 20) visualized on the Leica EZ4W stereomicroscope (Leica Microsystems, Cambridge, United Kingdom) were individually transferred to 24-well polystyrene plates containing different concentrations of the peptides to be tested. The E2 0.5 × medium was used as a control. In order to ensure the reproducibility of the tests, the embryos were kept in ovens at a constant temperature of 28 °C and the tests were carried out in triplicate. The tests were performed according to the Fish Embryo Acute Toxicity (FET) Test, according to OECD #236. Embryos were observed daily with Leica M205C stereomicroscope. Four parameters were evaluated as indicators of acute lethality in fish (according to FET test protocol): (1) coagulation of fertilized eggs; (2) absence of somite formation; (3) lack of separation between the tail and the yolk sac and, finally, the absence of a heartbeat. Parameters indicative of teratogenicity were also observed according to data obtained from Lantz-McPeak et al. (2015) and Brannen et al. (2010).

Statistical Analysis

The values were presented as the mean ± standard deviation. All experiments were conducted independently in triplicate. Statistical analyses were performed for each assay as follows: MIC statistical analysis employed a Two-way ANOVA followed by Dunnett's test; Biofilm inhibition and Quantification of viable cells underwent statistical analysis with ANOVA followed by Tukey's test; Quantification of cell viability in the MTT assay was subjected to a Two-way ANOVA followed by Dunnett's test; Toxicity test and Survival percentage in *G. mellonella* were analyzed using the Log-rank test and Gehan-Breslow-Wilcoxon test; Mortality percentage of zebrafish embryos was statistically assessed via Two-way ANOVA followed by Tukey's test. Data were

analyzed utilizing GraphPad Prism 7.00 (GraphPad Software, Inc., California, CA, USA), with a significance threshold set at $p < 0.005$.

Results

The antimicrobial activities of KTP derivatives against the microorganism were determined using microdilution method to obtain MIC_{50} and MIC_{90} of planktonic cells (Schwartz et al. 2010). As observed in Table 1, both KTP derivatives were found to be active against Gram-negative bacteria *E. coli* at concentration below 35 µM. This concentration against *E. coli* had a 90% inhibition, at least. The assay with *S. pneumoniae* showed a 50% inhibition with Indol-KTP-NH$_2$ at 101.8 µM concentration, this was the better value of inhibition with this derivative in the MIC test. As to *P. aeruginosa*, all derivatives were found inactive up to 250 µM. The Indol-KTP-NH$_2$ did not inhibit yeast growth, for this reason only the GABA-KTP-NH$_2$ activity was tested against the Candida species. The GABA-KTP-NH$_2$ derivative It showed an efficacy of 50% inhibition on Candida species in a concentration range of 5.86 a 23.97 uM, as seen in Table 1.

In the test of biofilm formation Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$ has almost 95% of inhibition in the biofilm formation for *E. coli* and *S. pneumoniae*, respectively, at the 62.5 µM and 250 µM, as presented in Fig. 1 A and 1 B. Moreover, biofilms of *C. krusei* were weakly inhibited by the two KTP-derivatives tested (below 30% inhibition) (Fig. 1C). The biofilm inhibition mean values and standard deviation are presented in Table 2 (see Supplementary Material).

The efficacy of Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$ against preformed (24 h) biofilms of microorganisms was evaluated by CFU count after 24 h of treatment. The number of CFU/mL of *E. coli* sample decreased significantly compared to the control group at the highest concentrations of the two KTP- derivatives (Fig. 2A). The result of the number of viable cells obtained by CFU corroborates with previous

results on biofilm inhibition. The results of the CFU test for the biofilm of *S. pneumoniae* didn't have the same satisfactory result (Fig. 2B). There was no significant difference between the number of cells in the negative control group and the groups with KTP- derivatives, although the percentage of inhibition was significant. The results for the *C. krusei* (Fig. 2C) demonstrated a significant decrease of CFU/mL compared to the control group in all concentrations of the derivatives tested, even though the percentage of biofilm inhibition was low. The biofilm viability mean values and standard deviation are presented in Table 3 (see Supplementary Material).

The MTT test showed that Indol-KTP-NH$_2$ are cytotoxic to the MEF cell since the viability is below 60% in the lowest concentration. However, GABA-KTP-NH$_2$ did not show cytotoxicity for the cells. Instead, the cell viability was 80% to 98% in the highest and lowest concentration, respectively, as seen in Fig. 3A. Also, in this test the derivatives showed no effect in tumor cells B16. The tumor cells had a high percentage of viability, as seen in Fig. 3B. After the analysis, the IC_{50} values were calculated for both peptides. In MEF cells, the concentrations for Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$ were 164 µM $\pm$ 41.87 and 1042 µM $\pm$ 210.8, respectively.

The *G. mellonella* model was used to carry out the toxicity tests of the peptides Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$. This test exhibited a high survival percentage on both derivatives, similar to the survival percentage of PBS (positive control), as presented in Fig. 4. The lethal concentration of *E. coli*, *S. pneumoniae* and *C.krusei* for the larvae of *G. mellonella* was evaluated using the microorganism strain standardization assay (supplementary material). For microorganisms *E. coli* and *C. krusei* the concentration of 10^8 cells/mL was chosen and for the *S. pneumoniae* the concentration of 10^5 cells/mL was chosen for subsequent trials.

The infection test showed that the peptides were efficient in the treatment against infection caused by all microorganisms evaluated and the survival remained above 80% (Fig. 5). For *E. coli* the two derivatives demonstrated an

Table 1 Quantification of the inhibition activity of KTP derivatives tested in MIC assay. Percentage of inhibition for Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$, as determined by absorbance at 530 nm

Microorganism	Indol-KTP-NH$_2$		GABA-KTP-NH$_2$	
	MIC_{50} (µM)	MIC_{90} (µM)	MIC_{50} (µM)	MIC_{90} (µM)
Gram negative bacteria				
Escherichia coli (ATCC 25922)	3.88	34.92	3.09	27.81
Pseudomonas aeruginosa (ATCC 15442)	> 250	> 250	> 250	> 250
Gram positive bacteria				
Streptococcus pneumoniae (ATCC 25922)	101.8	> 250	152.2	> 250
Yeast				
Candida krusei (ATCC 6258)	ND	ND	5.86	52.74
Candida parapsilosis (ATCC 63)	ND	ND	23.97	215.73
Candida tropicalis (140-S)	ND	ND	16.24	146.16

Fig. 1 Percentage of biofilm inhibition of KTP derivatives. **A** Bacteria Gram-negative *E. coli*; **B** Bacteria Gram-positive *S. pneumoniae*; **C** Fungi *C. krusei*. Percentage of inhibition for Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$, Chloramphenicol (positive control for bacteria) and Amphotericin (positive control for fungi). These values were determined by absorbance at 590 nm. $p < 0.05$ '*'
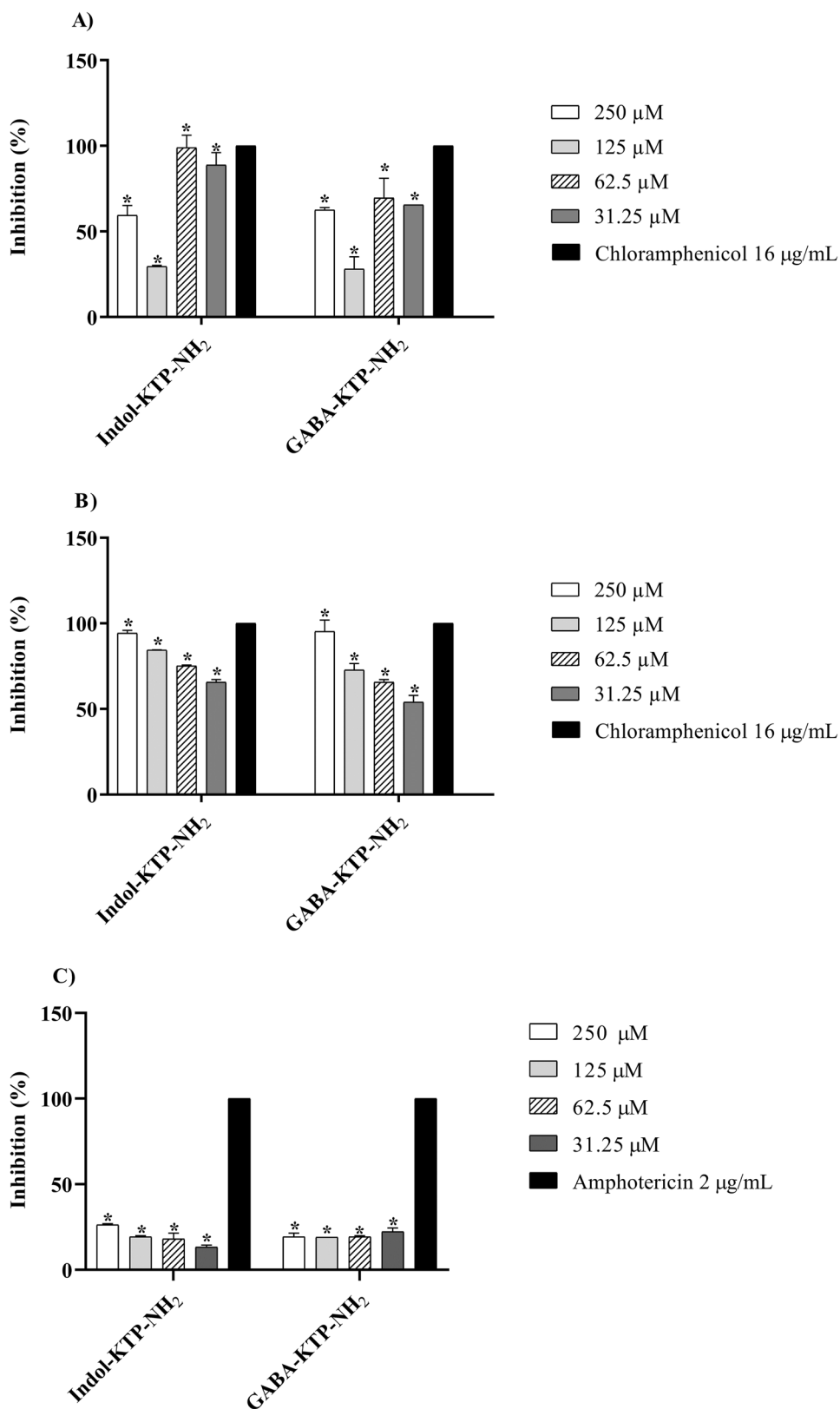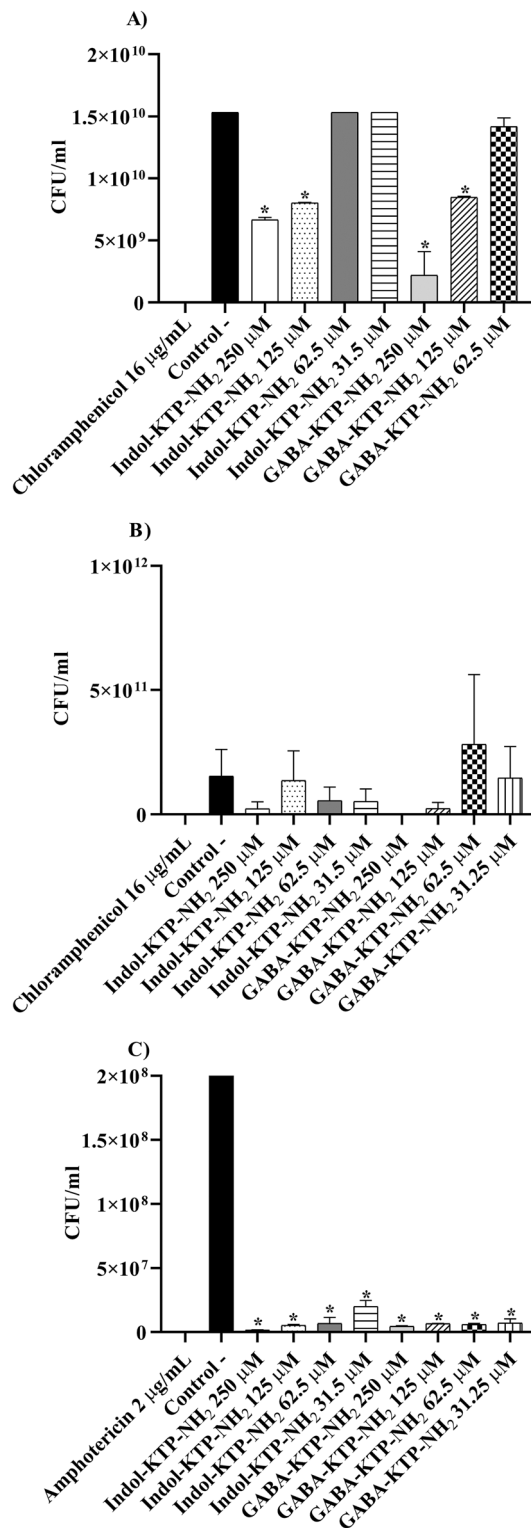
Fig. 2 Quantification of viable cells from **A** Gram-negative bacteria *E. coli*, **B** Gram-positive bacteria *S. pneumoniae* and **C** Fungi *C. krusei*. Biofilm inhibition test by Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$ derivatives. *p* value < 0.05 '*'

effective action of the three tested concentrations, GABA-KTP-NH$_2$ (200 μM) and Indol-KTP-NH$_2$ (200 μM and 100 μM) presented total effectiveness (Fig. 5A). Moreover, both derivatives in all tested concentrations showed total efficacy against the *S. pneumoniae* (Fig. 5B). For the *C. krusei* we evaluated only the peptide GABA-KTP-NH$_2$, once the Indol-KTP-NH$_2$ did not inhibit fungus growth in MIC assay. The results demonstrated an effective action of the three tested concentrations (200, 100 and 50 μM). The rate of survival of infected larvae was 100% in the 200 μM concentration, followed by 90% for the 100 μM concentration and 80% for the 50 μM concentration (Fig. 5C).

Acute toxicity of KTP derivatives, based on mortality of embryos, was evaluated each 24 h until 96 h using 0 hpf zebrafish embryos exposed to three doses (100, 50 and 25 μM) (Fig. 6). Four parameters were evaluated as indicators of acute lethality: (1) coagulation of fertilized eggs; (2) absence of somite formation; (3) lack of separation between the tail and the yolk sac and (4) the absence of a heartbeat. Our results in Fig. 6 demonstrated that KTP derivatives presented embryo mortality from 48 h for the Indol-KTP-NH$_2$ and 72 h for the GABA-KTP-NH$_2$. The GABA-KTP-NH$_2$ derivative showed mortality rate statistically significant at the concentration of 100 μM in the periods of 72 hpf ($p = 0.0119$) and 96 hpf ($p = 0.0119$). The Indol-KTP-NH$_2$ derivative showed mortality rate statistically significant at the three concentrations evaluated in the periods of 72 hpf (100 μM $p < 0.0001$; 50 μM $p = 0.0033$; 25 μM $p = 0.0005$) and 96 hpf (100 μM $p < 0.0001$; 50 μM $p = 0.0033$; 25 μM $p = 0.0005$). In the period of 48 hpf, only the concentrations of 100 μM ($p = 0.0005$) and 25 μM ($p = 0.0183$) showed a statistically significant mortality rate.

In the search for the phenotype-based malformations induced by non-lethal doses of KTP derivatives, embryos were analyzed for sub-lethality (yolk sac edema, absent eyes and pericardial edema) and teratogenicity (retarded growth, spinal deformity and curved tail) after 4 days of exposure (96 hpf) without renewal of the E2 0.5 × medium (Fig. 7). All tested concentrations of the two KTP derivatives caused some type of malformation after 96 h of exposure. The GABA-KTP-NH$_2$ showed more malformations at the highest tested concentration (100 μM), mainly in relation to teratogenicity, and the Indol-KTP-NH$_2$ showed more malformations at the lowest tested concentration (25 μM).

We documented zebrafish individuals exposed to KTP derivatives in all concentrations tested (100, 50 and 25 μM). Three defects were observed in the both KTP derivatives (GABA-KTP-NH$_2$ and Indol-KTP-NH$_2$) pericardial edema, yolk sac edema and curved tail (Fig. 8).

Fig. 3 Quantification of cell viability in MTT test with KTP derivatives. **A** MEF cells; **B** B16-F10-Nex2 cells. Percentage of cell viability with Indol-KTP-NH$_2$, GABA-KTP-NH$_2$ and DMSO 50% (positive control), as determined by absorbance at 540 nm. The different concentrations of peptides were compared with the positive control group. $*p < 0.0001$; $**p = 0.0251$; $***p = 0.0014$ and $****p = 0.0004$
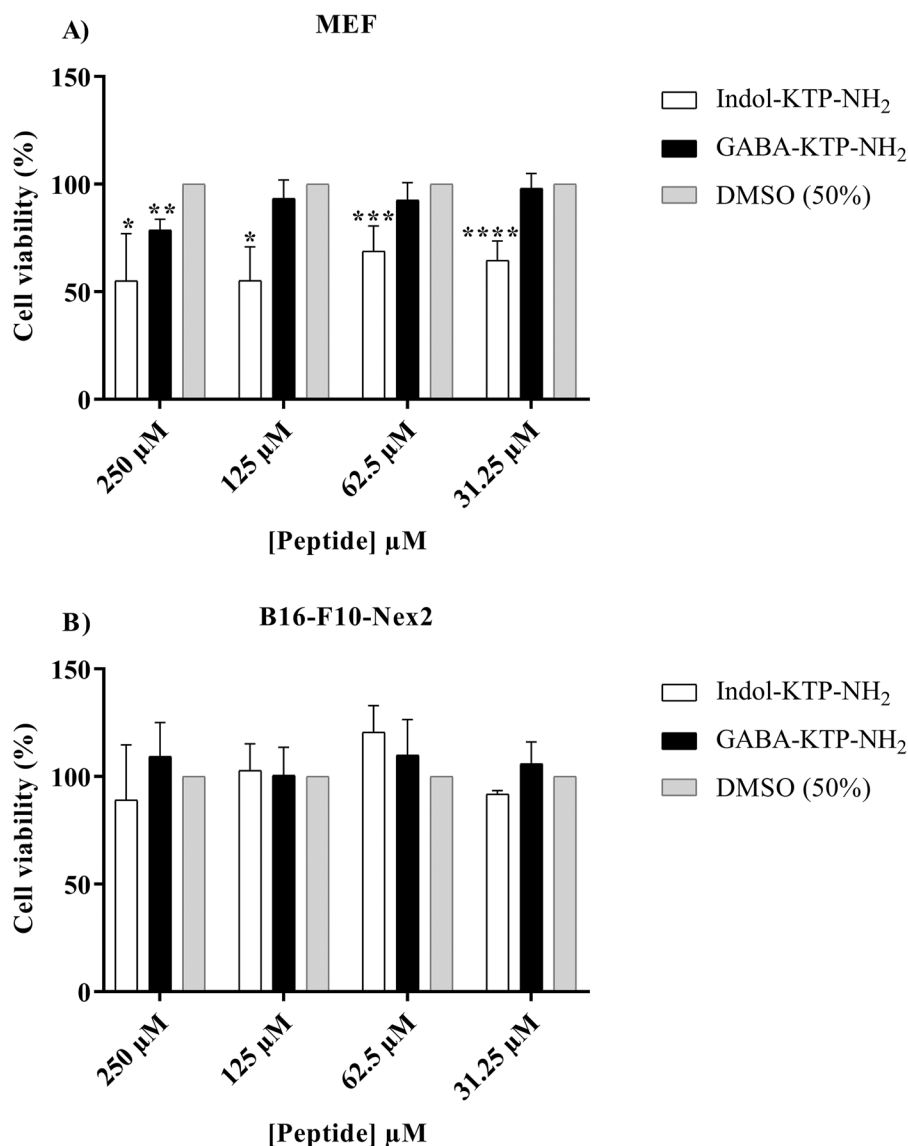

Discussion

The discovery of novel strategies to combat bacterial resistance to antibiotics highlights the renewed significance of bioactive peptides. Our hypothesis revolves around the antibiotic and antifungal potential of two KTP-derived compounds, namely GABA-KTP-NH$_2$ and Indol-KTP-NH$_2$, recognized for their multifunctional peptide properties. In the scope of this investigation, we assessed the efficacy of these two KTP derivatives against three microbial strains: *S. pneumoniae* (Gram-positive bacteria), *E. coli* (Gram-negative bacteria), and *Candida krusei* (fungi).

The results demonstrate the efficacy of KTP derivatives in inhibiting biofilm formation when tested against both *E. coli* and *S. pneumoniae*. Upon closer examination of the MIC values of these derivatives, it becomes evident that

all derivatives exhibited significant growth inhibition of *E. coli*. GABA-KTP-NH$_2$ displayed the highest potency against this Gram-negative bacteria. Intriguingly, this efficacy did not extend to *P. aeruginosa*, another gram-negative bacteria. Despite both bacteria sharing a Gram-negative classification and bacilli-shaped morphology, it is plausible that differences in zeta potential within their respective membranes could account for this variation in peptide interaction (Alves et al. 2010).

Multiple *Candida species* possess the capacity to infect critical organs, including the CNS (Li et al. 2017). This is attributed to their remarkable ability to cross the blood–brain barrier (Jong et al. 2001; Wu et al. 2019). Furthermore, infections stemming from yeast species within the Candida genus exhibit alarmingly high mortality rates (Gudlaugsson et al. 2003). Additionally, the limited repertoire of antifungal

Fig. 4 Results of toxicity test in *G. mellonella* with the KTP derivatives **A** Indol-KTP-NH$_2$ and **B** GABA-KTP-NH$_2$, and PBS as positive control, as determined by the count of live larvae. Values with *p* value < 0.05
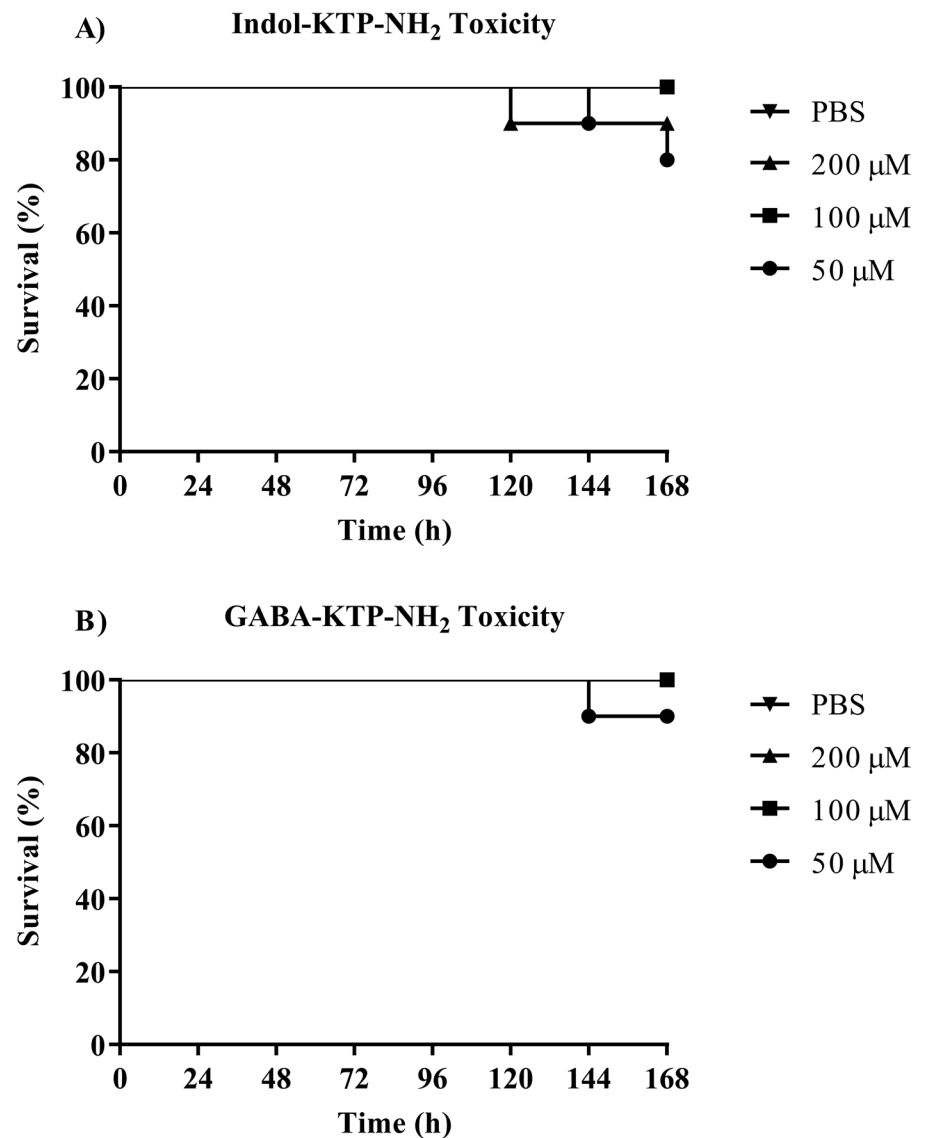

agents available for treating such infections underscores the pressing necessity for the development of novel antifungal compounds. Amphotericin B stands out as one of the foremost choices in treating these infections. However, it is not without its drawbacks, such as the potential for nephrotoxicity and the occurrence of fever accompanied by chills (Goodman and Gilman 1996). Consequently, our research embarked on evaluating alternative antifungal options for addressing the current shortcomings in medical treatment.

In a previous study conducted by our research group (Martins de Andrade et al. 2020), it was suggested that kyotorphins, due to their cationic characteristics, could potentially demonstrate significant antifungal properties. Indeed, Martins de Andrade et al. (2020) confirmed their hypothesis by demonstrating that IbKTP-NH$_2$, a kyotorphin derivative, displayed both antifungal and antibiofilm activities against *Candida species*, including *C. albicans* and non-albicans

strains. Remarkably, IbKTP-NH$_2$ demonstrated robust efficacy against *C. krusei*, with a 93% inhibition rate and complete eradication at a concentration of 1000 mM, and the CFU count after 24 h of treatment were weakly inhibited by 500 mM but strongly inhibited by 1000 mM. It's worth noting that *C. krusei* is recognized as one of the most pathogenic *Candida species* (Sullivan et al. 2004; Turner and Butler 2014). Therefore, IbKTP-NH$_2$ emerges as a promising candidate with substantial antifungal potential against *C. krusei* and other *Candida species*.

GABA-KTP-NH$_2$ and Ib-KTP-NH$_2$ are synthetized KTP-derivatives based in the amidation (-KTP-NH$_2$) and conjugation of residues, besides that they are membrane-active peptides (Ribeiro et al. 2011a, b; Serrano et al. 2015). Few synthetic membrane-active peptides targeting pathogenic fungi have been investigated. In our current investigation, GABA-KTP-NH$_2$ demonstrated a significantly decrease in

Fig. 5 Survival of *G. mellonella* infected with *E. coli* (10^8 cells/mL), *S. pneumoniae* (10^5 cells/mL) and *C. krusei* (10^8 cells/mL) and treated with GABA-KTP-NH$_2$ (200, 100 and 50 μM), Indol-KTP-NH$_2$ (200,100 and 50 μM) and PBS (used as a negative control). Results are the average of three independent experiments. Statistically significant results when compared to the control group (PBS). Statistical significance was calculated using Gehan-Breslow-Wilcoxon test $p \le 0.05$. The null hypothesis assumes that the survival curves are identical in the overall populations
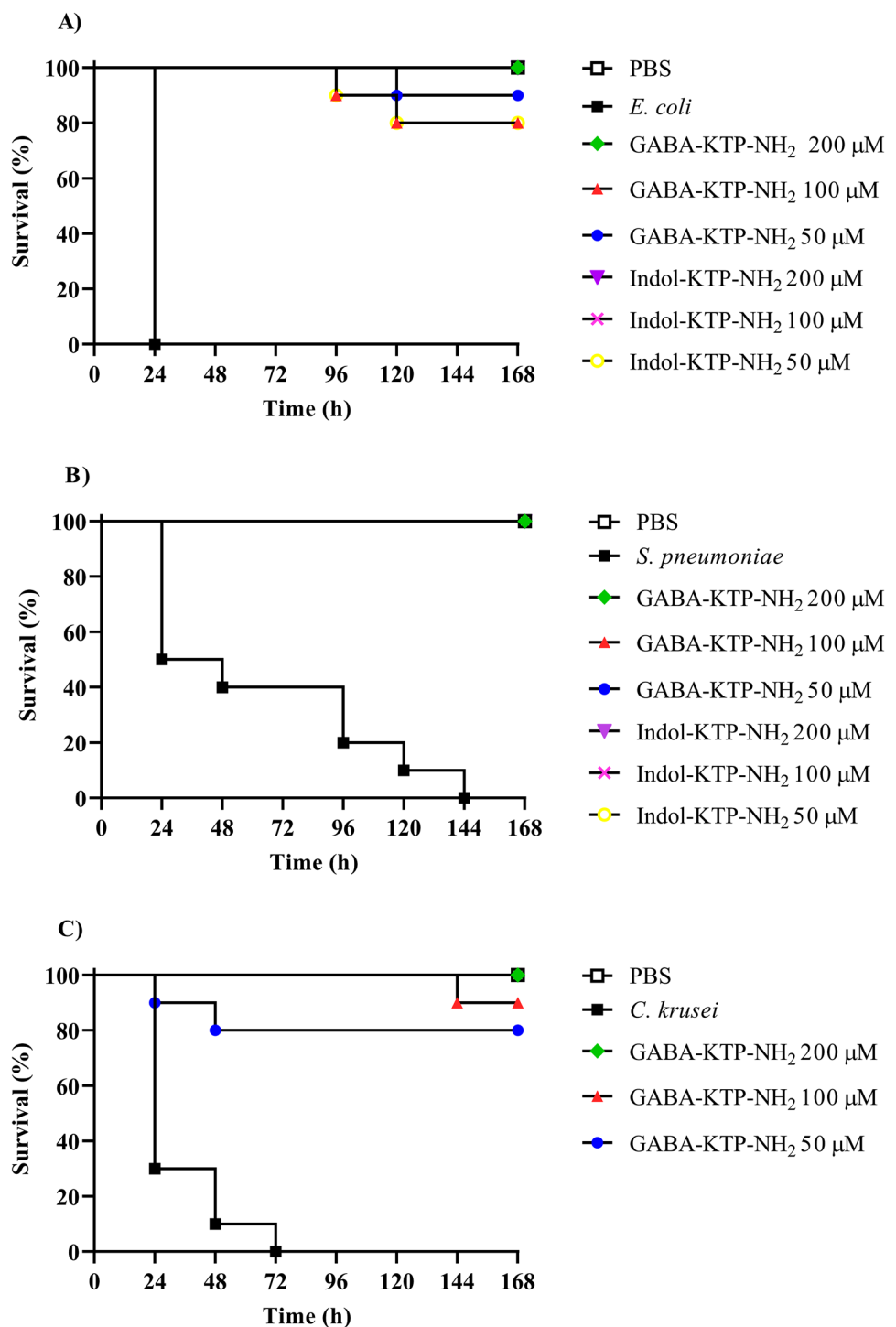
CFU/mL compared to the control group across all tested concentrations when challenged with *C. krusei*. Although the percentage of biofilm inhibition may have been relatively modest, these results indicate the potential of GABA-KTP-NH$_2$ as a promising antifungal candidate.

As previously observed, both Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$ exhibited a 50% inhibition of the MIC at concentrations exceeding 50 μM. In the context of the biofilm growth tests, it's important to note that the concentrations employed were higher. This difference can be attributed to the higher cell density within the biofilm tests compared to the MIC tests. It was observed that an intermediate concentration yielded a more favorable percentage of biofilm inhibition compared to the highest concentration, suggesting that a simple dose–response mechanism was not at play here. This observation aligns with a prior study

Fig. 6 Mortality percentage of zebrafish embryos exposed for 96 h to both KTP derivative. The embryos were immersed 0 hpf to different GABA-KTP-NH$_2$ or Indol-KTP-NH$_2$ concentrations diluted in E2 medium, and the effects were counted overtime every 24 h. The experiments were performed four times, using 5 embryos/concentration (final $n = 20$). The tested concentration was considered statistically significant compared with control (E2 medium) when $p > 0.05$
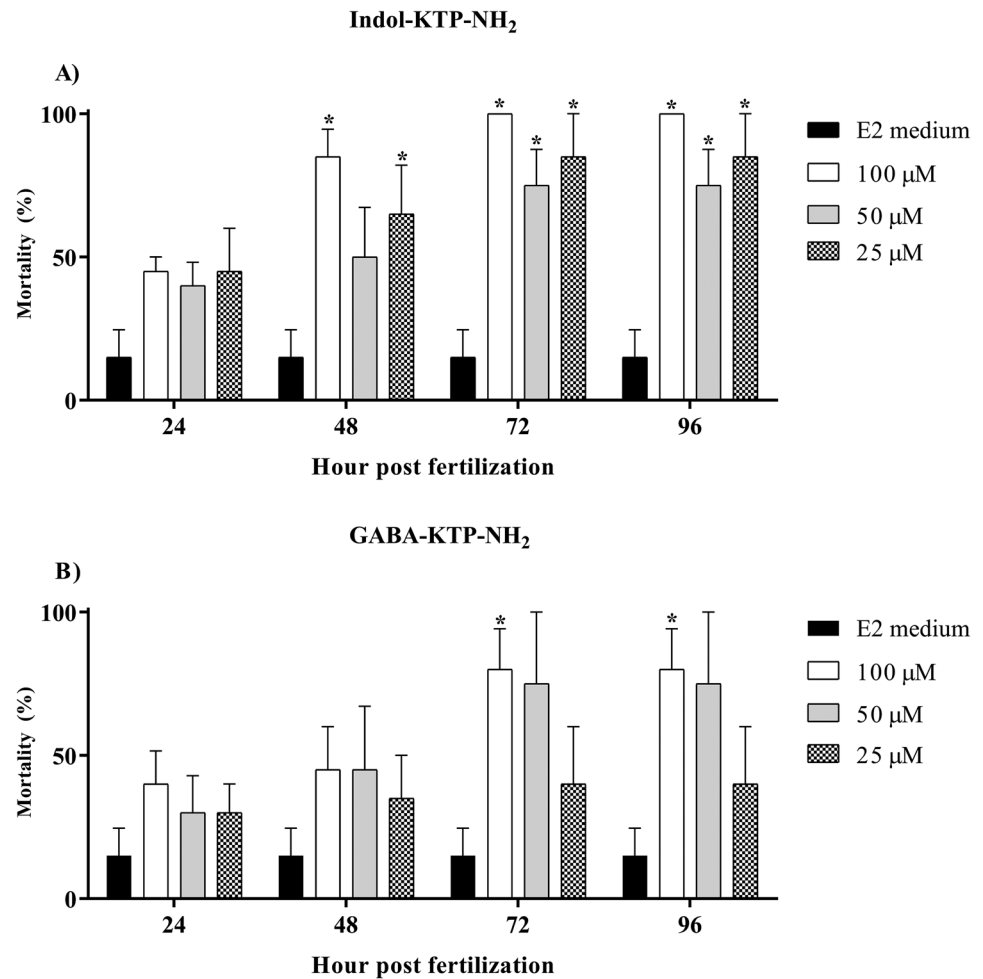

conducted by Overhage et al. (2008), which revealed that even lower concentrations of LL-37 peptide could exhibit enhanced efficacy against bacterial biofilm formation. This phenomenon is attributed to the fact that antimicrobial peptides often target various elements within bacterial cells, including cell membranes, cellular proteins, DNA, and RNA (Powers and Hancock 2003). Consequently, it is plausible that KTP derivatives interact with some of these targets to inhibit biofilm formation, and this interaction may reach a saturation point at which smaller doses prove to be more effective. This trend is further corroborated by our findings in the case of S. pneumoniae, observed in both the MIC and biofilm inhibition assays.

The CFU analysis for *E. coli* corroborates the findings from previous experiments. The data suggests that the concentration of the most prominent GABA-KTP-NH$_2$ derivative falls between the concentrations observed in MIC and biofilm tests, specifically at 35 μM and 62.5 μM. This assay assesses the count of viable cells in both planktonic and biofilm forms (Huang et al. 2017). In contrast, the results of the *S. pneumoniae* test indicate no reduction in the count of viable cells, despite a noticeable inhibition in biofilm

growth. The biofilm is a set of microorganisms entangled in an organic polymer matrix, which provides greater resistance to antimicrobial molecules. In a study conducted by Dias et al. (2022), the effectiveness of a spider-derived cyclic AMP gomesin analogue, [G1K,K8R]cGm, was investigated against bacterial cells embedded within S. aureus biofilms. The three-dimensional structure of [G1K, K8R]cGm was found to be crucial for its antibacterial and antibiofilm properties. Mechanistic examinations indicate that the peptide possesses the capability to permeate the biofilm and eradicate bacteria by selectively targeting and disrupting their cell membranes. This approach, involving antimicrobial peptides (AMPs) that can penetrate complex polymeric matrices and eliminate bacteria without relying on their metabolic activity, offers a promising alternative strategy for combating biofilm infections.

G. mellonella, commonly known as the greater wax moth larva, has emerged as a prominent model organism for investigating toxicity and infection dynamics. Over recent years, it has gained increasing popularity as a valuable tool in studies related to antibiotics and molecular antimicrobial potential (Tsai et al. 2016; Serrano et al. 2023). Recently this animal

Fig. 7 Malformations induced by non-lethal doses of KTP derivatives, embryos were analyzed for sub-lethality (yolk sac edema, absent eyes and pericardial edema) and teratogenicity (retarded growth, spinal deformity and curved tail) after 4 days of exposure (96 hpf) without renewal of the E2 $0.5 \times$ medium
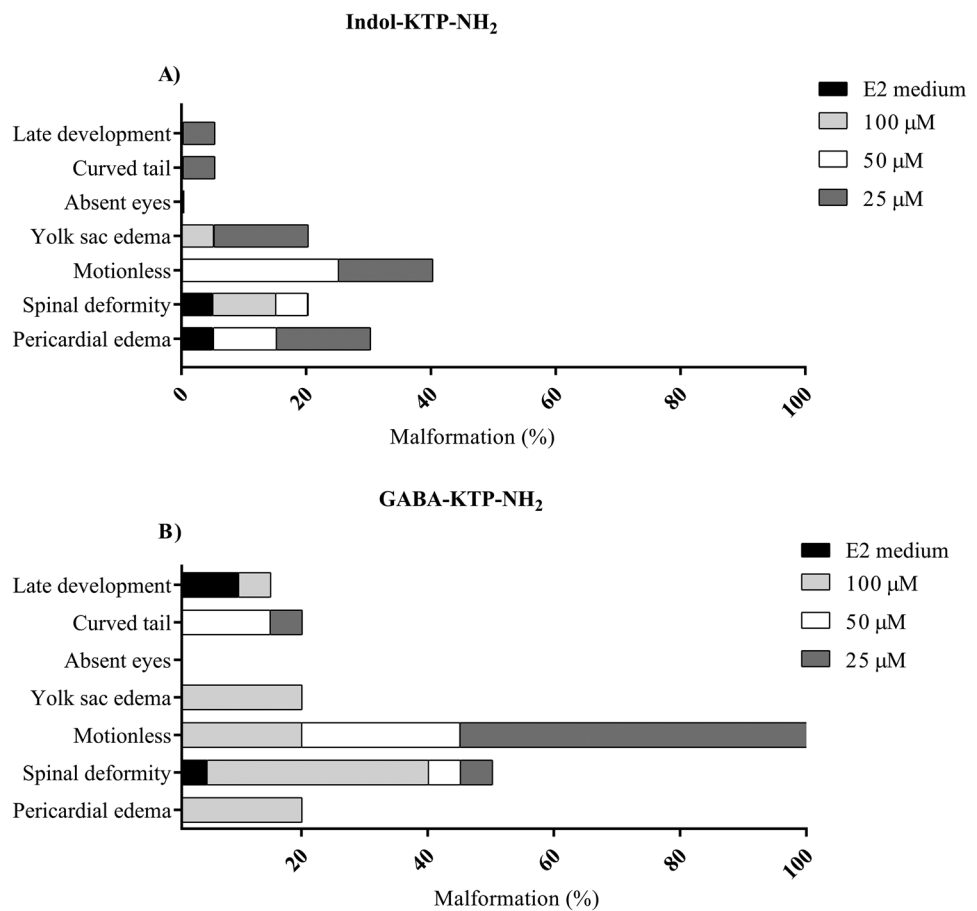
model has found a novel role in immunological research. Its significance lies in its capacity to facilitate the exploration of various facets of host–pathogen interactions, the elucidation of innate immune mechanisms, the assessment of virulence factors employed by human pathogens, the in vivo evaluation of the antimicrobial efficacy of emerging drugs, and the discovery of bioactive molecules with diverse applications, ranging from antimicrobial, antiviral, and anticancer properties to their potential use as biopesticides (Wojda et al. 2020; Serrano et al. 2023).

Firstly an in vivo assay with *G. mellonella* was conducted to evaluate the KTP derivatives toxicity. Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$ exhibited good pharmacological potential by demonstrating non-toxic properties and yielding results akin to the positive control (PBS). This outcome holds significant importance due to the structural and functional resemblance of this model organism's innate immune system to that of mammals. Consequently, this larval model is ethically accepted as a viable alternative for toxicity research, offering valuable insights into predicting toxicity outcomes in mammalian systems (Wojda et al. 2020; Serrano et al. 2023). Subsequently, we conducted another in vivo assay involving the infection of *G. mellonella* larvae with *E. coli*, *S. pneumoniae*, and *C. krusei* to assess the therapeutic

potential of Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$. The results demonstrated the efficacy of both KTP derivatives against these infections. GABA-KTP-NH$_2$ exhibited a significant antifungal effect against *C. krusei*, underscoring its potential as an antifungal candidate. In contrast, Indol-KTP-NH$_2$ did not exhibit inhibition of yeast growth in the MIC assay; therefore, this derivative was not further evaluated against the fungus *C. krusei*.

Previous studies of our group (Martins de Andrade et al. 2020) demonstrated that IbKTP-NH$_2$, a derivative of kyotorphin, reduced the yeast cell count in the larval hemolymph. The survival curves not only revealed that IbKTP-NH$_2$ prevented the mortality of 80% of the sample but also extended the lifespan of infected larvae compared to those treated with PBS, and 20% of the treated larvae remained alive at the conclusion of the experimental period. Furthermore, the evaluation of IbKTP-NH$_2$'s toxicity using *G. mellonella* larvae demonstrated that the drug's toxicity remained low up to 2 days post-treatment, aligning with the treatment results. These discoveries underscore the substantial therapeutic potential of IbKTP-NH$_2$ as an antifungal agent. On the other hand, the study by Ridyard et al. (2023) presents data indicating high toxicity for the LL-32 peptide at a concentration of 32 μg/mL (½ MIC), resulting in the death of 100% of the
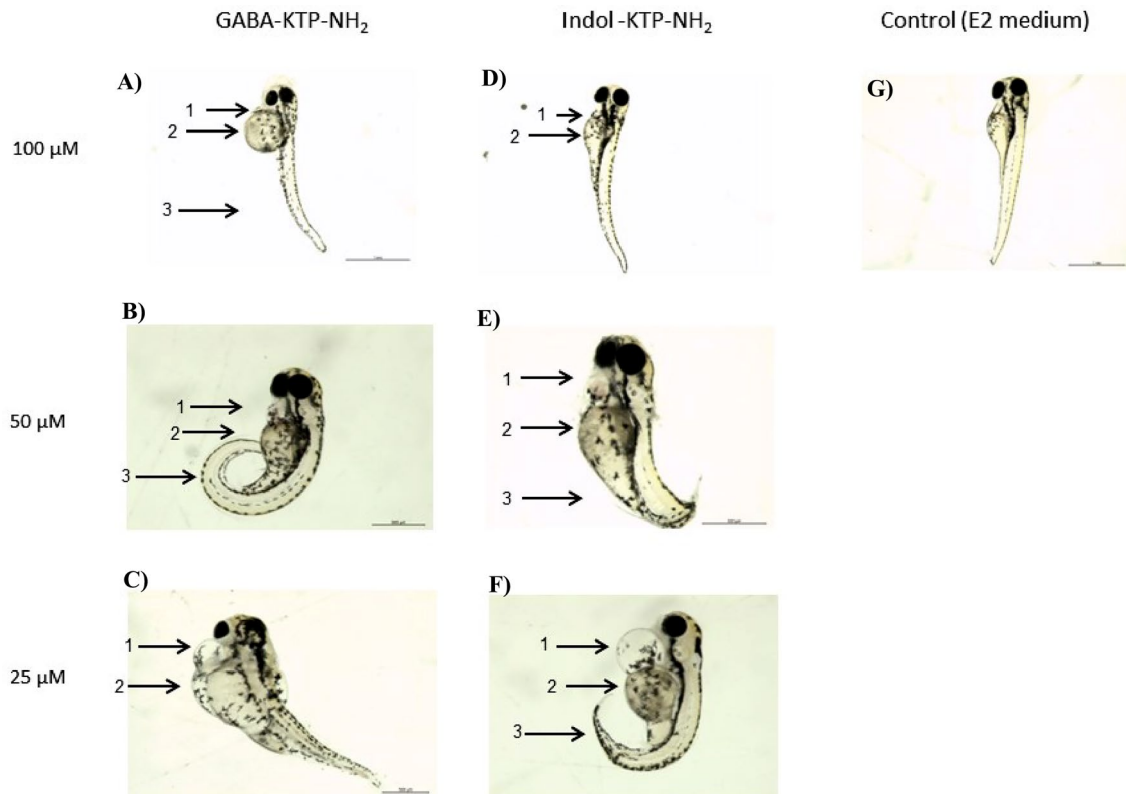
Fig. 8 Representative embryo morphological changes observed after 96 h of GABA-KTP-NH$_2$ (**A**, **B** and **C**) or Indol-KTP-NH$_2$ (**D**, **E** and **F**) exposition. Embryos presenting malformation in the tail (curved tail—arrow 3), edema in the cardiac cavity (arrow 1), and edema in the yolk sac (arrow 2). The control showed no morphological abnormalities (G)

larvae after 24 h of the experiment. Our findings regarding the GABA-KTP-NH$_2$ peptide align with those of Martins de Andrade et al. (2020) and suggest that this peptide represents a compelling candidate, particularly as an antifungal agent, within the realm of antimicrobial drug development.

The data obtained from treating *E. coli* infections with KTP derivatives can be correlated with the results obtained from in vitro tests. Both Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$ exhibited significant efficacy against this infection. However, it's worth noting that these derivatives did not yield the same level of effectiveness against *S. pneumoniae*. In the *G. mellonella* infection test, both derivatives, across all tested concentrations, demonstrated complete efficacy against *S. pneumoniae*. Nonetheless, it's noteworthy that in the CFU test for *S. pneumoniae* biofilm, there was no significant difference observed between the number of cells in the negative control group and the groups treated with KTP derivatives, despite a notable percentage of inhibition. In general, antimicrobial peptides have demonstrated notably potent antimicrobial activity against biofilms formed by certain bacteria and fungi, as evidenced in the study conducted by Ribeiro and collaborators (Ribeiro et al. 2012). Our results have shown significant activity of Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$. Given that *E. coli*, *S. pneumoniae*,

and *C. krusei* are responsible for a range of common infections, these derivatives hold promise for further development as therapeutic antimicrobials.

The zebrafish (*Danio rerio*) is widely used in environmental toxicity assessment of chemicals. More recently, however, this animal model, particularly zebrafish embryos, has gained substantial traction in the realm of new drug development (Barros et al. 2008; Kalueff et al. 2014; Kanungo et al. 2014; Raldúa and Piña 2014; Augustine-Rauch et al. 2016; Jia et al. 2019; Batista-Filho et al. 2020). The validated alternative zebrafish OECD #236 test embryo model to assess fish acute toxicity (OECD, 2013) originally designed for identifying the toxicity of toxins, particulate matter, and nanoparticles (Ahkin Chin Tai and Freeman 2020; Rahman et al. 2020), and monitoring of various environmental contaminants including pesticides, ethanol, and pharmaceuticals (Vargas and Ponce-Canchihuaman 2017; Disner et al. 2021), has evolved into a versatile tool. It is now being explored as a potential substitute for one of the regulatory in vivo mammalian embryo fetal developmental toxicity studies for human pharmaceuticals. Recent studies have used this animal model, particularly zebrafish embryos, for drug discovery, early drug development, and toxicological screening (Barros et al. 2008; Kalueff et al. 2014; Kanungo

et al. 2014; Raldúa and Piña 2014; Augustine-Rauch et al. 2016; Jia et al. 2019; Batista-Filho et al. 2020).

In our current investigation, we conducted the FET test to investigate the impact of Indol-KTP-NH$_2$ and GABA-KTP-NH$_2$ on the survival and morphological development of zebrafish embryos. The results showed that Indol-KTP-NH$_2$ displayed a pronounced association with lethality. All tested doses of Indol-KTP-NH$_2$ induced high mortality rates in the embryos after both 72 h and 96 h of exposure. Furthermore, after the 48-h mark, lethality was evident at concentrations of 100 μM and 25 μM. Conversely, malformations were predominantly observed in embryos exposed to the 25 μM concentration. These malformations, indicative of sub-lethality and teratogenic effects, occurred at relatively low rates, underscoring the heightened toxicity of Indol-KTP-NH$_2$ at higher concentrations. This finding aligns with the outcomes of the MTT assay, which demonstrated cytotoxicity of Indol-KTP-NH$_2$ to MEF cells across all tested concentrations. In contrast, when subjected to toxicity testing using the model organism *G. mellonella*, Indol-KTP-NH2 exhibited no discernible toxicity.

On the other hand, the GABA-KTP-NH$_2$ derivative exhibited a lower degree of lethality, with lethality being observed only at the 100 μM concentration after 72 h and 96 h of exposure. Nevertheless, it displayed elevated rates of malformation at this same concentration, particularly concerning teratogenic effects. Moreover, these KTP derivatives also demonstrated substantial malformation rates at lower concentrations. Additionally, in the MTT assay, GABA-KTP-NH$_2$ did not exhibit cytotoxicity towards the two tested cell types and in the toxicity assessment, involving the model organism *G. mellonella*, GABA-KTP-NH$_2$ displayed no discernible toxicity. Batista-Filho et al. 2020 conducted investigative toxicology assessments on the patented anti-inflammatory peptide TnP using zebrafish as the model organism. The results indicated that elevated concentrations of the peptide were associated with increased lethality and the induction of dose-dependent abnormalities in the developing embryos, in addition, lower concentrations of the peptide exhibited a reduced propensity to induce such abnormalities. Based on this premise, it is prudent to contemplate future tests at concentrations equal to or less than 25 μM for the GABA-KTP-NH$_2$ peptide, particularly in the context of its effectiveness against the fungus *C. krusei*.

Our results emphasize the notable antifungal effectiveness of the GABA-KTP-NH$_2$ peptide in the infection test, and the toxicity evaluations indicate a comparatively lower toxicity profile with a reduced likelihood of inducing abnormalities. Nevertheless, it's noteworthy that our results caution against the use of these derivatives at higher tested concentrations, as they led to both mortality and abnormalities in the developing embryos. Furthermore, this study's outcomes emphasize the pivotal role of the zebrafish as a precise model for investigative toxicology, particularly in evaluating the acute toxicity of molecules during the preclinical development phase. They also underscore the importance of employing a multifaceted approach involving various techniques for a comprehensive assessment of toxicity.

Research Involving Human and Animal Participants

The authors declare that all animals was kept according to animal welfare standards. The experiments were carried out under the laws of the National Council for Animal Experiment Control (CONCEA) and approved by the UNIFESP Animal Use Ethics Commission (CEUA nº 8861040323).

Author contributions   Isabel Chaves Silva Carvalho: Data Curation, Formal analysis, Investigation, Validation, Writing—original draft. Fernanda da Silva Seiffert Simões: Investigation, Writing—original draft. Vitor Martins de Andrade: Formal analysis, Investigation, Validation. Dayane Batista Tada, Montserrat Heras, Eduard Bardaji and Monica Lopes Ferreira: Data Curation, Formal analysis, Investigation, Validation, Writing—original draft, Writing—review & editing. Miguel Augusto Rico Botas Castanho: Investigation, Writing—original draft, Writing—review & editing. Katia Conceição: Funding acquisition, Investigation, Project administration, Writing—original draft, Writing—review and editing. All authors read and approved the manuscript.

Declarations

Competing interests   The authors declare no competing interests.

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References

Ahkin Chin Tai JK, Freeman JL (2020) Zebrafish as an integrative vertebrate model to identify miRNA mechanisms regulating toxicity. Toxicol Rep 7:559–570. https://doi.org/10.1016/j.toxrep.2020.03.010

Allegra E, Titball RW, Carter J, Champion OL (2018) Galleria mellonella larvae allow the discrimination of toxic and non-toxic chemicals. Chemosphere 198:469–472. https://doi.org/10.1016/j.chemosphere.2018.01.175

Alves CS, Melo MN, Franquelim HG, Ferre R, Planas M, Feliu L, Bardají E, Kowalczyk W, Andreu D, Santos NC, Fernandes MX, Castanho MA (2010) Escherichia coli cell surface perturbation and disruption induced by antimicrobial peptides BP100 and

pepR. J Biol Chem 285:27536–27544. https://doi.org/10.1074/jbc.M110.130955

Arvanitis M, Glavis-Bloom J, Mylonakis E (2013) Invertebrate models of fungal infection. Biochim Biophys Acta 1832:1378–1383. https://doi.org/10.1016/j.bbadis.2013.03.008

Augustine-Rauch K, Zhang CX, Panzica-Kelly JM (2016) A developmental toxicology assay platform for screening teratogenic liability of pharmaceutical compounds. Birth Defects Res B Dev Reprod Toxicol 107:4–20. https://doi.org/10.1002/bdrb.21168

Barros TP, Alderton WK, Reynolds HM, Roach AG, Berghmans S (2008) Zebrafish: an emerging technology for in vivo pharmacological assessment to identify potential safety liabilities in early drug discovery. Br J Pharmacol 154:1400–1413. https://doi.org/10.1038/bjp.2008.249

Batista-Filho J, Falcão MAP, Maleski ALA, Soares ABS, Balan-Lima L, Disner GR, Lima C, Lopes-Ferreira M (2020) Early preclinical screening using zebrafish (Danio rerio) reveals the safety of the candidate anti-inflammatory therapeutic agent TnP. Toxicol Rep 8:13–22. https://doi.org/10.1016/j.toxrep.2020.12.004

Brannen KC, Panzica-Kelly JM, Danberry TL, Augustine-Rauch KA (2010) Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. Birth Defects Res B Dev Reprod Toxicol 89:66–77. https://doi.org/10.1002/bdrb.20223

Chen P, Bodor N, Wu WM, Prokai L (1998) Strategies to target kyotorphin analogues to the brain. J Med Chem 41:3773–3781. https://doi.org/10.1021/jm970715l

Clinical and Laboratory Standards Institute (2008) Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard, 2nd ed CLSI document M38-A2.

Clinical and Laboratory Standards Institute (2023) Performance standards for antimicrobial susceptibility testing; 33rd ed. CLSI Supplement M100.

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1222. https://doi.org/10.1126/science.284.5418.1318

Cotter G, Doyle S, Kavanagh K (2000) Development of an insect model for the in vivo pathogenicity testing of yeasts. FEMS Immunol Med Microbiol 27:163–169. https://doi.org/10.1111/j.1574-695X.2000.tb01427.x

D'Amora M, Giordani S (2018) The utility of zebrafish as a model for screening developmental neurotoxicity. Front Neurosci 12:976. https://doi.org/10.3389/fnins.2018.00976

Davey ME, O'toole GA (2000) Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 64:847–867. https://doi.org/10.1128/mmbr.64.4.847-867.2000

Dias SA, Pinto SN, Silva-Herdade AS, Cheneval O, Craik DJ, Coutinho A, Castanho MARB, Henriques ST, Veiga AS (2022) A designed cyclic analogue of gomesin has potent activity against Staphylococcus aureus biofilms. J Antimicrob Chemother 77:3256–3264. https://doi.org/10.1093/jac/dkac309

Disner GR, Falcão MAP, Andrade-Barros AI, Leite Dos Santos NV, Soares ABS, Marcolino-Souza M, Gomes KS, Lima C, Lopes-Ferreira M (2021) The Toxic effects of glyphosate, chlorpyrifos, abamectin, and 2,4-D on animal models: a systematic review of Brazilian studies. Integr Environ Assess Manag 17:507–520. https://doi.org/10.1002/ieam.4353Dunne

Dunne WM Jr (2002) Bacterial adhesion: seen any good biofilms lately? Clin Microbiol Rev 15:155–166. https://doi.org/10.1128/cmr.15.2.155-166.2002

Dzimbova T, Bocheva A, Pajpanova T (2014) Kyotorphin analogues containing unnatural amino acids: synthesis, analgesic activity and computer modeling of their interactions with m-receptor. Med Chem Res 23:3694–3704. https://doi.org/10.1007/s00044-014-0953-9

Eliassen LT, Berge G, Sveinbjørnsson B, Svendsen JS, Vorland LH, Rekdal Ø (2002) Evidence for a direct antitumor mechanism of action of bovine lactoferricin. Anticancer Res 22:2703–2710

Formaggio DMD, Magalhães JA, Andrade VM, Conceição K, Anastácio JM, Santiago GS, Arruda DC, Tada DB (2022) Co-Functionalization of Gold Nanoparticles with C7H2 and HuAL1 peptides: enhanced antimicrobial and antitumoral activities. Pharmaceutics 14:1324. https://doi.org/10.3390/pharmaceutics14071324

Fukui K, Shiomi H, Takagi H, Hayashi K, Kiso Y, Kitagawa K (1983) Isolation from bovine brain of a novel analgesic pentapeptide, neo-kyotorphin, containing the Tyr-Arg (kyotorphin) unit. Neuropharmacology 22:191–196. https://doi.org/10.1016/0028-3908(83)90008-4

Goodman & Gilman (1996) As Bases Farmacológicas da Terapêutica. Blackwell, London

Gudlaugsson O, Gillespie S, Lee K, Vande Berg J, Hu J, Messer S, Herwaldt L, Pfaller M, Diekema D (2003) Attributable mortality of nosocomial candidemia, revisited. Clin Infect Dis 37:1172–1177. https://doi.org/10.1086/378745

Haddad N, Carr M, Balian S, Lannin J, Kim Y, Toth C, Jarvis J (2022) The blood-brain barrier and pharmacokinetic/pharmacodynamic optimization of antibiotics for the treatment of central nervous system infections in adults. Antibiotics (basel) 11:1843. https://doi.org/10.3390/antibiotics11121843

Hartung T (2010) Lessons learned from alternative methods and their validation for a new toxicology in the 21st century. J Toxicol Environ Health B Crit Rev 13:277–290. https://doi.org/10.1080/10937404.2010.483945

Huang X, Zhang K, Deng M, Exterkate RAM, Liu C, Zhou X, Cheng L, Ten Cate JM (2017) Effect of arginine on the growth and biofilm formation of oral bacteria. Arch Oral Biol 82:256–262. https://doi.org/10.1016/j.archoralbio.2017.06.026

Jia HR, Zhu YX, Duan QY, Chen Z, Wu FG (2019) Nanomaterials meet zebrafish: toxicity evaluation and drug delivery applications. J Control Release 311–312:301–318. https://doi.org/10.1016/j.jconrel.2019.08.022

Jong AY, Stins MF, Huang SH, Chen SH, Kim KS (2001) Traversal of Candida albicans across human blood-brain barrier in vitro. Infect Immun 69:4536–4544. https://doi.org/10.1128/iai.69.7.4536-4544.2001

Kalueff AV, Stewart AM, Gerlai R (2014) Zebrafish as an emerging model for studying complex brain disorders. Trends Pharmacol Sci 35:63–75. https://doi.org/10.1016/j.tips.2013.12.002

Kanungo J, Cuevas E, Ali SF, Paule MG (2014) Zebrafish model in drug safety assessment. Curr Pharm Des 20:5416–5429. https://doi.org/10.2174/1381612820666140205145658

Kavanagh K, Sheehan G (2018) The use of galleria mellonella larvae to identify novel antimicrobial agents against fungal species of medical interest. J Fungi (basel) 4:113. https://doi.org/10.3390/jof4030113

Komatsuzawa H, Ohta K, Sugai M, Fujiwara T, Glanzmann P, Berger-BächiB SH (2000) Tn551-mediated insertional inactivation of the fmtB gene encoding a cell wall-associated protein abolishes methicillin resistance in Staphylococcus aureus. J Antimicrob Chemother 45:421–431. https://doi.org/10.1093/jac/45.4.421

Korhonen H, Pihlanto A (2006) Bioactive peptides: production and functionality. Int Dairy J 16:945–960. https://doi.org/10.1016/j.idairyj.2005.10.012

Kwasny SM, Opperman TJ (2010) Static biofilm cultures of Gram-positive pathogens grown in a microtiter format used for anti-biofilm drug discovery. Curr Protoc Pharmacol. https://doi.org/10.1002/0471141755.ph13a08s50

Lantz-McPeak S, Guo X, Cuevas E, Dumas M, Newport GD, Ali SF, Paule MG, Kanungo J (2015) Developmental toxicity assay using high content screening of zebrafish embryos. J Appl Toxicol 35:261–272. https://doi.org/10.1002/jat.3029

Li H, Gong H, Qi Y, Li J, Ji X, Sun J, Tian R, Bao H, Song X, Chen Q, Liu G (2017) In vitro and in vivo antifungal activities and mechanism of heteropolytungstates against Candida species. Sci Rep 7:1–9. https://doi.org/10.1038/s41598-017-17239-8

Lopes SC, Fedorov A, Castanho MA (2006a) Chiral recognition of D-kyotorphin by lipidic membranes: relevance toward improved analgesic efficiency. Chem Med Chem 1:723–728. https://doi.org/10.1002/cmdc.200600096

Lopes SC, Soares CM, Baptista AM, Goormaghtigh E, Cabral BJ, Castanho MA (2006b) Conformational and orientational guidance of the analgesic dipeptide kyotorphin induced by lipidic membranes: putative correlation toward receptor docking. J Phys Chem B 110:3385–3394. https://doi.org/10.1021/jp053651w

Martins de Andrade V, Bardají E, Heras M, Ramu VG, Junqueira JC, Diane Dos Santos J, Castanho MARB, Conceição K (2020) Antifungal and anti-biofilm activity of designed derivatives from kyotorphin. Fungal Biol 124:316–326. https://doi.org/10.1016/j.funbio.2019.12.002

Mylonakis E, Moreno R, El Khoury JB, Idnurm A, Heitman J, Calderwood SB, Ausubel FM, Diener A (2005) Galleria mellonella as a model system to study Cryptococcus neoformans pathogenesis. Infect Immun 73:3842–3850. https://doi.org/10.1128/iai.73.7.3842-3850.2005

Nichols WW, Dorrington SM, Slack MP, Walmsley HL (1988) Inhibition of tobramycin diffusion by binding to alginate. Antimicrob Agents Chemother 32:518–523. https://doi.org/10.1128/aac.32.4.518

OECD (2013) Test No. 236: Fish Embryo Acute Toxicity (FET) Test, OECD, https://doi.org/10.1787/9789264203709-en

Ouhara K, Komatsuzawa H, Kawai T, Nishi H, Fujiwara T, Fujiue Y, Kuwabara M, Sayama K, Hashimoto K, Sugai M (2008) Increased resistance to cationic antimicrobial peptide LL-37 in methicillin-resistant strains of Staphylococcus aureus. J Antimicrob Chemother 61:1266–1269. https://doi.org/10.1093/jac/dkn106

Overhage J, Campisano A, Bains M, Torfs EC, Rehm BH, Hancock RE (2008) Human host defense peptide LL-37 prevents bacterial biofilm formation. Infect Immun 76:4176–4182. https://doi.org/10.1128/iai.00318-08

Perazzo J, Castanho MA, Sá Santos S (2017) Pharmacological potential of the endogenous dipeptide kyotorphin and selected derivatives. Front Pharmacol 7:530. https://doi.org/10.3389/fphar.2016.00530

Powers JP, Hancock RE (2003) The relationship between peptide structure and antibacterial activity. Peptides 24:1681–1691. https://doi.org/10.1016/j.peptides.2003.08.023

Rahman MS, Islam SMM, Haque A, Shahjahan M (2020) Toxicity of the organophosphate insecticide sumithion to embryo and larvae of zebrafish. Toxicol Rep 7:317–323. https://doi.org/10.1016/j.toxrep.2020.02.004

Raldúa D, Piña B (2014) In vivo zebrafish assays for analyzing drug toxicity. Expert Opin Drug Metab Toxicol 10:685–697. https://doi.org/10.1517/17425255.2014.896339

Ribeiro MM, Pinto A, Pinto M, Heras M, Martins I, Correia A, Bardaji E, Tavares I, Castanho M (2011a) Inhibition of nociceptive responses after systemic administration of amidated kyotorphin. Br J Pharmacol 163:964–973. https://doi.org/10.1111/j.1476-5381.2011.01290.x

Ribeiro MM, Pinto AR, Domingues MM, Serrano I, Heras M, Bardaji ER, Tavares I, Castanho MA (2011b) Chemical conjugation of the neuropeptide kyotorphin and ibuprofen enhances brain targeting and analgesia. Mol Pharm 8:1929–1940. https://doi.org/10.1021/mp2003016

Ribeiro MM, Franquelim HG, Torcato IM, Ramu VG, Heras M, Bardaji ER, Castanho MA (2012) Antimicrobial properties of analgesic kyotorphin peptides unraveled through atomic force microscopy. Biochem Biophys Res Commun 420:676–679. https://doi.org/10.1016/j.bbrc.2012.03.065

Ridyard KE, Elsawy M, Mattrasingh D, Klein D, Strehmel J, Beaulieu C, Wong A, Overhage J (2023) Synergy between human peptide LL-37 and polymyxin B against planktonic and biofilm cells of escherichia coli and pseudomonas aeruginosa. Antibiotics 12:389. https://doi.org/10.3390/antibiotics12020389

Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, Minor L. Cell Viability Assays. 2013 May 1 [updated 2016 Jul 1]. In: Markossian S, Grossman A, Brimacombe K, Arkin M, Auld D, Austin C, Baell J, Chung TDY, Coussens NP, Dahlin JL, Devanarayan V, Foley TL, Glicksman M, Gorshkov K, Haas JV, Hall MD, Hoare S, Inglese J, Iversen PW, Kales SC, Lal-Nag M, Li Z, McGee J, McManus O, Riss T, Saradjian P, Sittampalam GS, Tarselli M, Trask OJ Jr, Wang Y, Weidner JR, Wildey MJ, Wilson K, Xia M, Xu X, editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004–.

Rizzo LY, Golombek SK, Mertens ME, Pan Y, Laaf D, Broda J, Jayapaul J, Möckel D, Subr V, Hennink WE, Storm G, Simon U, Jahnen-Dechent W, Kiessling F, Lammers T (2013) In vivo nanotoxicity testing using the zebrafish embryo assay. J Mater Chem B. https://doi.org/10.1039/C3TB20528B

Russell WMS, Burch RL (1959) The principles of humane experimental. [S. l.: s. n.].

Rybal'chenko VK, Ostrovskaya GV, Poralo IV, Rybal'chenko TV, Melnik YM (1999) Membranotropic activity of optical isomers of the neuropeptide kyotorphin and a cardiotonic agent, suphan. Neurophysiology 31:223–225. https://doi.org/10.1007/BF02515077

Schwarz S, Silley P, Simjee S, Woodford N, Duijkeren E, Johnson AP, Gaastra W (2010) Editorial: assessing the antimicrobial susceptibility of bacteria obtained from animals. J Antimicrob Chemother 4:601–604. https://doi.org/10.1093/jac/dkq037

Serrano ID, Ramu VG, Pinto AR, Freire JM, Tavares I, Heras M, Bardaji ER, Castanho MA (2015) Correlation between membrane translocation and analgesic efficacy in kyotorphin derivatives. Biopolymers 104:1–10. https://doi.org/10.1002/bip.22580

Serrano I, Verdial C, Tavares L, Oliveira M (2023) The Virtuous galleria mellonella model for scientific experimentation. Antibiotics (basel) 12:505. https://doi.org/10.3390/antibiotics12030505

Skalska J, Andrade VM, Cena GL, Harvey PJ, Gaspar D, Mello ÉO, Henriques ST, Valle J, Gomes VM, Conceição K, Castanho MARB, Andreu D (2020) Synthesis, structure, and activity of the antifungal plant defensin PvD1. J Med Chem 63:9391–9402. https://doi.org/10.1021/acs.jmedchem.0c00543

Stoodley P, Sauer K, Davies DG, Costerton JW (2002) Biofilms as complex differentiated communities. Annu Rev Microbiol 56:187–209. https://doi.org/10.1146/annurev.micro.56.012302.160705

Sullivan DJ, Moran GP, Pinjon E, Al-Mosaid A, Stokes C, Vaughan C, Coleman DC (2004) Comparison of the epidemiology, drug resistance mechanisms, and virulence of Candida dubliniensis and Candida albicans. FEMS Yeast Res 4:369–376. https://doi.org/10.1016/S1567-1356(03)00240-X

Takagi H, Shiomi H, Ueda H, Amano H (1979) A novel analgesic dipeptide from bovine brain is a possible Met-enkephalin releaser. Nature 282:410–412. https://doi.org/10.1038/282410a0

Tsai CJ, Loh JM, Proft T (2016) Galleria mellonella infection models for the study of bacterial diseases and for antimicrobial drug testing. Virulence 7:214–229. https://doi.org/10.1080/21505594.2015.1135289

Turner SA, Butler G (2014) The Candida pathogenic species complex. Cold Spring Harb Perspect Med 4:a019778. https://doi.org/10.1101/cshperspect.a019778

Ueda H (2021) Review of kyotorphin research: a mysterious opioid analgesic dipeptide and its molecular, physiological, and pharmacological characteristics. Front Med Technol 3:662697. https://doi.org/10.3389/fmedt.2021.662697

Vargas R, Ponce-Canchihuamán J (2017) Emerging various environmental threats to brain and overview of surveillance system with zebrafish model. Toxicol Rep 4:467–473. https://doi.org/10.1016/j.toxrep.2017.08.002

Wang C, Zhao M, Yang J, Peng S (2001) Synthesis and analgesic effects of kyotorphin-steroid linkers. Steroids 66:811–815. https://doi.org/10.1016/S0039-128X(01)00112-X

Wiegand I, Hilpert K, Hancock RE (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3:163–175. https://doi.org/10.1038/nprot.2007.521

Wilson C, Lukowicz R, Merchant S, Valquier-Flynn H, Caballero J, Sandoval J, Okuom M, Huber C, Brooks TD, Wilson E, Clement B, Wentworth CD, Holmes AE (2017) Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini-review. Res Rev J Eng Technol 6(4). http://www.rroij.com/open-access/quantitative-and-qualitative-assessment-methods-for-biofilm-growth-a-minireview-.pdf.

Wojda I, Staniec B, Sułek M, Kordaczuk J (2020) The greater wax moth Galleria mellonella: biology and use in immune studies. Pathog Dis 78:ftaa057. https://doi.org/10.1093/femspd/ftaa057

Wu Y, Du S, Johnson JL, Tung HY, Landers CT, Liu Y, Seman BG, Wheeler RT, Costa-Mattioli M, Kheradmand F, Zheng H, Corry DB (2019) Microglia and amyloid precursor protein coordinate control of transient Candida cerebritis with memory deficits. Nat Commun 10:58. https://doi.org/10.1038/s41467-018-07991-4

Yang X, Jounaidi Y, Dai JB, Marte-Oquendo F, Halpin ES, Brown LE, Trilles R, Xu W, Daigle R, Yu B, Schaus SE, Porco JA Jr, Forman SA (2018) High-throughput Screening in larval zebrafish identifies novel potent sedative-hypnotics. Anesthesiology 129:459–476. https://doi.org/10.1097/ALN.0000000000002281