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Early preclinical screening using zebrafish (*Danio rerio*) reveals the safety of the candidate anti-inflammatory therapeutic agent *Tn*P

João Batista-Filho [a,b], Maria Alice Pimentel Falcão [a], Adolfo Luis Almeida Maleski [a,b], Amanda Beatriz Silva Soares [a], Leticia Balan-Lima [a], Geonildo Rodrigo Disner [a], Carla Lima [a,1], Monica Lopes-Ferreira [a,1,*]

[a] *Immunoregulation Unit of the Laboratory of Applied Toxinology (CeTICs/FAPESP), Butantan Institute, Vital Brazil Avenue, 1500, Butantan, 05503-009, São Paulo, Brazil*

[b] *Post-Graduation Program of Toxinology, Butantan Institute, São Paulo, SP, Brazil*

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Dr. A.M Tsatsaka

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ABSTRACT

The patented anti-inflammatory peptide *Tn*P had its effectiveness recently confirmed *in vivo* in a murine model of multiple sclerosis and asthma. In this work, the safety of the *Tn*P was evaluated in investigative toxicology tests using zebrafish (*Danio rerio*) as a model. We conducted the OECD #236 test to investigate effects of the *Tn*P on the survival, hatching performance, and morphological formation of zebrafish embryos. After determining these endpoints, morphometric analysis termination of locomotion eartbeat rate in zebrafish larvae were evaluated to identify adverse effects such as neurotoxicity and cardiotoxicity. The results highlight a wide therapeutic index for *Tn*P with non-lethal and safe doses rom 1 nM to 10 μM, without causing neurotoxicity or cardiotoxic effect. The low frequencyf abnormalities by *Tn*P was associated with high safety of the molecule and the developing embryo's ability to process and eliminate it. *Tn*P crossed the blood-brain barrier without disturbing the normal architecture of forebrain, midbrain and hindbrain. Our data reinforce the importance of zebrafish as an accurate investigative toxicology model to assess acute toxicity as well as cardiotoxicity and neurotoxicity of molecules in the preclinical phase of development.

1. Introduction

Due to the high specificity of their targets, marine organisms and their toxins have been increasingly used to identify molecules with therapeutic activity, which are tested in numerous experimental models of diseases [1,2]. Our group identified new molecules denominated *Tn*P family derived from the venom of the Brazilian fish *Thalassophryne nattereri*, which their synthetic products were subjected to a patent application in several countries and currently is patented in Europe (EP2046815B1)Mexico (MX300187), United States (US8304382B2) Canada (CA2657338C) China (CN101511861B) Hong Kong (HK1135406) India (IN256624) South Korea (KR1399175B1), and Japan (JP5635771B2).

The *Tn*P family invention [3] refers to synthetic peptides with anti-inflammatory and anti-allergic activities containing a sequence of 13 L-amino acids in their primary structure. The basic cyclic peptide *Tn*P ($C_{63}H_{114}N_{22}O_{13}S_4$, H-Ile-Pro-Arg-Cys-Arg-Lys-Met-Pro-Gly-Val-Lys-Met -Cys-NH2 with disulfide bond between Cys4 and Cys13 with 1514.8 Da) is in a preclinical development stage indicated for chronic inflammatory diseases such as asthma and multiple sclerosis (MS).

Peptides represent a small portion (2%) of the worldwide drug market, but Copaxone (Glatiramer acetate), Lupron, Zoladex, Sandostatin, and Velcade blockbuster peptide drugs on the market together account for about $20 billion [4]. There are currently ~70 approved peptide drugs on the market, ~200 in clinical development, and ~600 in the preclinical drug discovery stage. The peptide market is growing twice as quickly as the rest of the drug market, suggesting peptides might soon occupy a larger niche [5].

One of the challenges in MS research is understand the shortcomings of the remyelination process and developing strategies to restore myelination. Relapsing-remitting MS (RR-MS) is partially alleviated by current first-line disease-modifying therapies (DMT) composed

* Corresponding author at: Immunoregulation Unit, Laboratory of Applied Toxinology, Butantan Institute, Brazil.
E-mail address: monica.lopesferreira@butantan.gov.br (M. Lopes-Ferreira).
[1] The authors contributed equally to this work.

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Interferonbeta and Glatiramer acetate while non-responsive patients are treated with second-line DMT sphingosine 1-phosphate receptor modulator Fingolimod and the monoclonal antibody Natalizumab [6].

Our group developed a large study that demonstrated that *Tn*P has important characteristics for develoing an effective drug control neuroinflammation and prevention of demyelination in MS [7]. We found that subcutaneous *Tn*P treatment (prophylactic, therapeutic or continuous regime) successfully ameliorates the severity of the clinical signs of MOG-induced EAE (experimental autoimmune encephalomyelitis) a well characterized mouse model for MS, delaying the onset of maximal symptoms (4 days) and decreasing the severity of symptoms by 40% compared to control EAE-mice treated with vehicle alone. *Tn*P beneficially interferes with the immune circuit in several stages by mechanisms partially dependent on IL-10: 1) suppresses the activation state of the conventional dendritic cells (DC) and provides the emergence of plasmacytoid DC and regulatory cells during the EAE induction phase; 2) blocks leukocyte transit and infiltration into the central nervous system (CNS) by suppressing MMP-9 activity and CD18 expression; 3) blocks the reactivation and permanence of pathogenic Th1 or Th17 lymphocytes in the CNS; 4) prevents the expansion of microglia cells and the infiltration of macrophages in the CNS; 5) favors the localized increase of regulatory T cells; 6) and also leads to accelerated remyelination in a cuprizone model.

In pharmaceutical drug discovery, ninetyeight percent of drug compounds tested in animals are eventually abandoned before clinical trials [8–10]. In most cases, this is because either the compound did not show sufficient therapeutic activity (efficacy) *in vivo* or it had adverse effects during clinical development and was considered unsafe [11,12]. Although we have proven the efficiency of *Tn*P interfering in the complex immune circuit, it is urgent to evaluate the safety of *Tn*P in accurate preclinical toxicology studies.

During the early phase of preclinical development process when investigative toxicology demonstrates the safety and reveal toxic mechanisms of pre-drug candidates through simultaneous activity on multiple targets, many platforms are considered alternatives to animal models such as rodents or higher mammals in line with the 3Rs (reduction, refinement and replacement) philosophy [13–15].

Thus, the lower non-mammalian vertebrate zebrafish model offers the potential to reduce time and costs and is suitable for high-throughput drug screening in the early preclinical drug discoveryor evaluating candidate therapeutic agents [16–19] and satisf specific legislation requirements of the pharmaceutical sector.

It is important to mention that the validated alternative zebrafish OECD #236 test (embryo model to assess fish acute toxicity) [20] classically used to identify toxicity of toxins, particulate matter and nano particles [21,22] and monitoring of various environmental contaminants including pesticides, ethanol, and pharmaceuticals [23,24] is also being explored as a potential replacement for one of the regulatory *in vivo* mammalian embryoetal developmental toxicity studies for human pharmaceuticals [25].

Zebrafish toxicological preclinical screening provides, in addition to the advantages of morphological characteristics, the advantage of being performed on a vertebrate organism with conserved physiology and metabolism, in contrast to other alternative tests such as total embryo culture and embryonic stem cells. The advantages of using an intact animal as a focus for screening are particularly evident for the discovery of neurological drugs [26], in which the complexities of cell-cell interactions and endocrine signaling are challenging.

Although many findings exist about the biochemical and therapeutic characteristics of *Tn*P, we have not yet conducted studies evaluating toxicity in an *in vivo* model. Thus, the objective of this work is to take advantage of zebrafish as a model organism of preclinical studies of toxicology for evaluation of safety and biodistribution of the pre-drug *Tn*P which could reveal toxicity mechanisms and reduce the risk of failure in the later of preclinical and clinical phases.

2. Materials and methods

2.1. TnP

*Tn*P trifluoroacetate compound synthetized in solid phase was purchased from GenScript (#P13821401, 97.3 % of purity). *Tn*P solutions were prepared by diluting the powder in fish water E2 0.5x medium (7.5 mM KH_2PO_4, 2.5 mM Na_2HPO_4, 15 mM NaCl, 0.5 mM KCl, 1 mM $MgSO_4{+}7H_2O$, 1 mM $CaCl_2{+}2H_2O$, 0.7 mM $NaHCO_3$).

2.2. Zebrafish husbandry

Adult zebrafish (<18 months old) from AB strain (International Zebrafish Resource Center, Eugene, OR) were kept separated by sex and breed under standard conditions of temperature at 28 °C, pH 7 and lightdark cycle (14/10 h) in individual aquariums on an ALESCO (Campinas, Brazil) shelf using system water (60 μg mL^{-1} Instant Ocean sea salts). The experiments were carried out under the laws of the National Council for Animal Experiment Control (CONCEA) and approved by the Butantan Institute's Animal Use Ethics Commission (CEUAIB #9857030619). The fertilized embryos visualized on the Leica EZ4W stereomicroscope (Leica Microsystems, Cambridge, United Kingdom) were transferred to plastic dishes (100 × 20 mm) containing E2 0.5x medium and classified according to Kimmel et al. [27].

2.3. Zebrafish anesthesia and euthanasia

Anesthesia was performed by immersing larvae in 2 mL of E2 0.5x medium containing 0.4 % tricaine (ethyl-3-aminobenzoate, #MS-222, Sigma Chemical Co., St. Louis, MO) for 2 min before analysis. At the end of the experiments, euthanasia was obtained by immersion in 4 % tricaine diluted in E2 0.5x medium. After exposure, larvae were checked for visualization of heartbeat stop in a stereomicroscope Leica (M205C) before being placed in a 10 % bleach solution.

2.4. Evaluation of TnP interference in zebrafish survival and development

According to OECD #236: Fish Embryo Acute Toxicity (FET) Test, embryos up to 4 h post fertilization (hpf) were distributed in 24-well microplate (5 embryos/well, quadruplicate), exposed to 2 mL of E2 0.5x medium without or with different *Tn*P doses (0.001, 0.01, 0.1, 1, 10, 100, 1,000 and 10,000 μM) and analyzed after 24, 48, 72, and 96 h post exposition (hpe). Mortality (egg coagulation) was assessed under Leica M205C stereomicroscope, and dead individuals were counted daily without solution renewal.

2.5. Phenotype-based screening

In the surviving individuals, the occurrence of deformities such as absence of somites and non-detachment of the tail (lethality parameters); head/eye malformation, abnormal yolk sac absorption, edema in the pericardium, yolk sac edema, uninflated swimming bladder, and abnormal pigmentation (sub-lethality parameters); and curved tail, shortened tail, spinal deformity, and delayed growth (teratogenicity parameters) were annotated and photographed under Leica M205C stereomicroscope.

2.6. Larval length

The surviving 96 hpf larvae exposed to the acute toxicity regime of *Tn*P were aligned in a glass dish in lateral position for the total body length measurement. The images obtained using a Leica M205C stereomicroscope were used to measure the larvae from the top of the head to the tip of the tail using Leica Application Suite software (LAS v4.11).

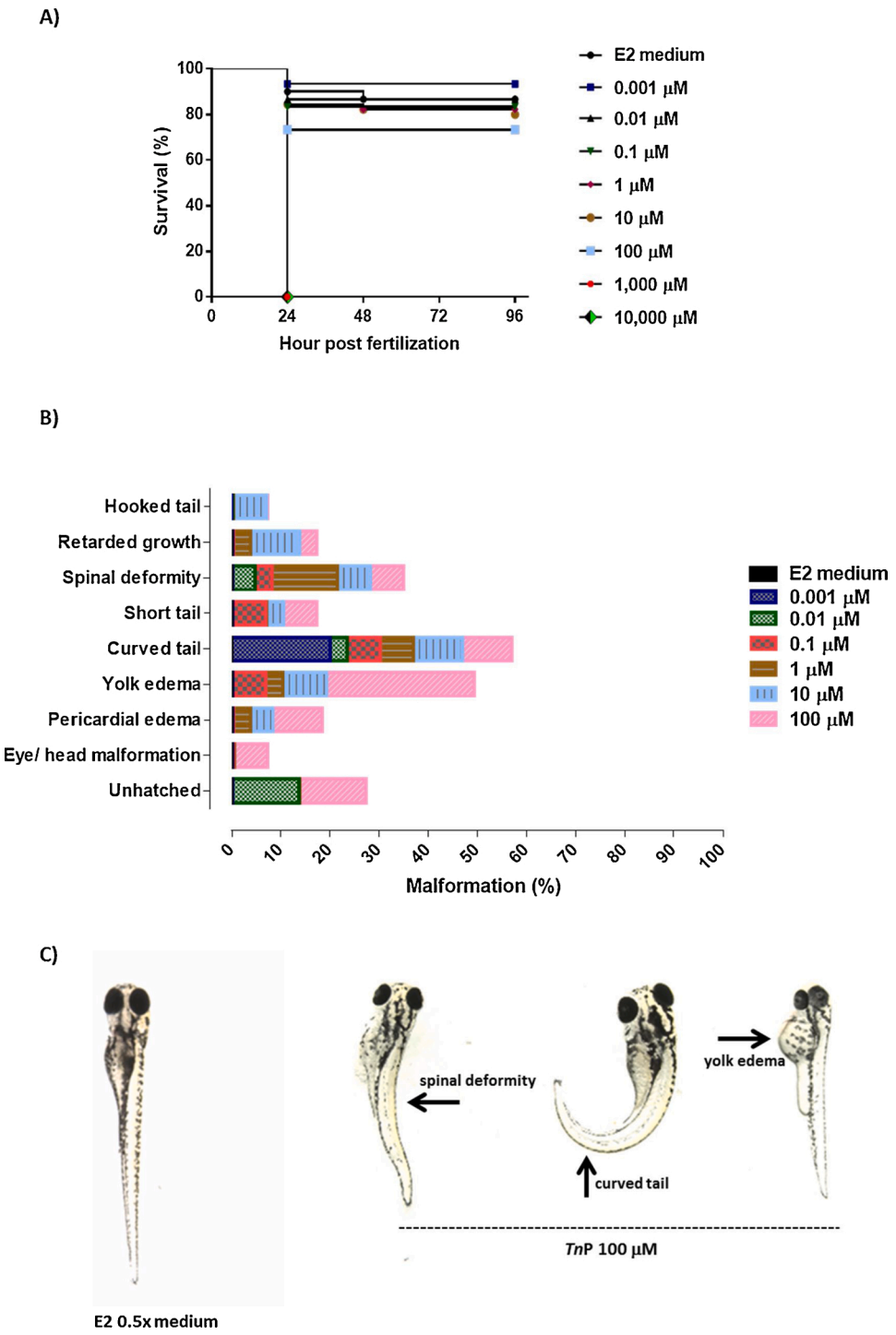


Fig. 1. *Tn*P exhibits a wide range of safe and non-lethal doses from 1 nM to 10 µM. Embryos at 0 hpf (n = 5 /well, quadruplicate) were treated by exposure to E2 0.5x medium without or with *Tn*P at doses of 0.001, 0.01, 0.1, 1, 10, 100, 1000, and 10,000 µM. Subjects were analyzed daily without medium renewal during 4 days to mortality rate count (***A***). *Tn*P treated embryos after 96 hpe were analyzed for the frequency of individuals affected with anomalies (***B***). The bars represent the mean percentage of each group. We documented zebrafish individuals exposed to *Tn*P at 100 µM (***C***) with the 3 defects (spinal deformity, curved tail, and yolk edema).

2.7. Cardiotoxicity and pericardial area measurement

The embryos up to 2 hpf were treated for 6 h with 0.00075 % N-phenylthiourea (PTU, #P7629, Sigma) to avoid differentiation of pigment-producing cells and with 1 dpf they were anesthetized and dechorionated by immersion for 5 min in pronase at 0.02 (#P5147, Sigma). After washing with E2 0.5x medium, the resulting larvae were distributed in 24-well plates (5 embryos/well, quadruplicate) and immersed in E2 0.5x medium without or with *Tn*P at doses of 0.001, 0.01, 0.1, 1, and 10 µM. The individuals were anesthetized and analyzed every 24 hpe for 3 consecutive days without renewing the medium. The heartbeats were counted and filmed under Leica M205C stereomicroscope at 50x (LAS V4.11 software) at 72 hpe larvae. Photographs were acquired from the 15-second videos of larvae exposed to the regime applied for cardiotoxicity analysis. The total pericardium area of larvae from different groups was measured using the ImageJ v.1.8.0_172 software.

2.8. TnP distribution

*Tn*P was conjugated to FITC (fluorescein isothiocyanate) using the EIT-Label FITC protein labeling kit (#53027, Pierce, Rockford, United States) according to the manufacturer's instructions. The coupling of *Tn*P to FITC was confirmed by LC—MS mass spectrometry positive

Table 1
The Low frequency of abnormalities induced by *Tn*P. Embryos up to 4 hpf distributed in 24-well microplate were exposed to 2 mL of E2 0.5x medium without or with different *Tn*P doses (0.001, 0.01, 0.1, 1, 10, and 100 μM) and analyzed after 24, 48, 72, and 96 h post exposition (hpe). In the surviving individuals, the occurrences of sub-lethality and teratogenicity phenotypes were counted.

		*Tn*P					
		100 μM	10 μM	1 μM	0.1 μM	0.01 μM	0.001 μM
Sub-Lethality parameters	**unhatched embryos**	**12 %**				**12 %**	
	head/eye malformation	6%					
	edema in the pericardium	**10 %**	3%	4%			
	yolk sac edema	**27 %**	9%	2%	6%		
Teratogenicity parameters	**curved tail**	9%	9%	6%	6%	3%	**19 %**
	shortened tail	6%	3%		6%		
	spinal deformity	6%	6%	**11 %**	3%	4%	
	delayed growth	3%	9%	4%			
	hooked tail		5%				

module (Thermo) and isolated in the Sep-Pak light TC18 column according to Komegae et al. [7]. Embryos up to 2 hpf were microinjected into the yolk with 2−3 nL of FITC at 2 or *Tn*P-FITC at 1.2 ng nL^{-1} (7.9 μM) using the Eppendorf InjectMan® 4 microinjector according to Li et al. [28]. The microinjection was carried out in a Leica M205C stereomicroscope and the images after 24, 48, and 72 h post injection (hpi) were obtained, under anesthesia, in a Lumar V12 stereomicroscope with Axiocam MRC REV 3 and the fluorescence was deconvolutioned in AxioVision® software (Carl Zeiss, Oberkochen, Germany).

2.9. Zebrafish locomotor behavior assessment

Possible neurotoxic effects of *Tn*P were determined by the swimming behavior of 144 hpf zebrafish larvae, as described by Ulhaq et al. [29]. Obtained embryos were kept in a dish with 10 mL of fresh daily renewed E2 0.5× medium until 6 dpf. Each larva (24/group) was added to a single well of a 96-well plate containing 200 μL of E2 0.5x medium without or with *Tn*P at doses of 0.001, 0.01, and 0.1 μM. After 30 min of acclimatization, the 96-well plate was taken to a ZebraBox system (Viewpoint Life sciences, Lyon, France) applying tracking settings during alternated 10 min light/dark. Locomotor activity was quantified and analyzed by ZebraLab™ software by Viewpoint.

2.10. TnP interference in brain morphological development

In order to test the potential effects of *Tn*P on zebrafish brain development, embryos 0 hpf microinjected into the yolk with 2−3 nL of 0.01 μM *Tn*P using the Eppendorf InjectMan® 4 microinjector were analyzed for deformities through gross morphology imaged with bright-field microscopy at 28 and 48 hpf. The microinjection was carried out in a Leica M205C stereomicroscope, under anesthesia in semi-solid agarose plate he images were obtained for antero-caudal and horizontal length measurements across the forebrain, midbrain and hindbrain.

2.11. Statistical analysis

All values were expressed as mean ± SEM. Experiments were performed independently two times. Parametric data were evaluated using analysis of variance, followed by the Bonferroni test for multiple comparisons. Non-parametric data were assessed using the Mann-Whitney test. Differences were considered statistically significant at $p < 0.001$ using GraphPad Prism (Graph Pad Software, v6.02, 2013, La Jolla, CA, USA).

3. Results

3.1. Low incidence of abnormalities in zebrafish embryos by non-lethal doses of TnP

Acute toxicity of *Tn*P, based on egg coagulation and mortality of embryos and larvae, was evaluated each 24 h until 96 h using 0 hpf zebrafish embryos exposed over a range of doses (1 nM up to 10 mM). Our results in Fig. 1A demonstrated that the highest doses of 1,000 and 10,000 μM were sufficient to promote 100 % embryo mortality in the first 24 h of exposure ($p < 0.001$) compared to the 90 % viability of control embryos only exposed to E2 0.5× medium. Importantly, a wide range of *Tn*P doses from 1 nM to 100 μM did not induce changes in the survival rate compared to control embryos, and survivors persisted until 96 hpf.

In the search for the phenotype-based malformations induced by non-lethal doses of *Tn*P, embryos were analyzed for sub-lethality (yolk edema, pericardial edema, eye/head malformation, and hatching delay) and teratogenicity (retarded growth, spinal deformity, hooked, short and curved tail) after 4 days of exposure (96 hpf) without renewal of the E2 0.5x medium.

The results in Fig. 1B and Table 1 represented the percentage of the remaining individuals that exhibited developmental defects at the doses tested (0.001, 0.01, 0.1, 1, 10, and 100 μM). We observe that the increment in *Tn*P doses promoted an increase in the incidence of reported anomalies. The lowest dose of *Tn*P (0.001 μM) caused only the appearance of curved tail (1 out of 9 reported defects), and the highest dose of 100 μM induced both sub-lethality and teratogenicity defects (8 out of 9 reported defects), except for not causing hooked tail anomaly.

The dose of 0.001 μM induced a 19 % frequency of curved tail, the dose 0.01 μM induced a frequency of 12 % of unhatched embryos, the dose of 1 μM induced an 11 % frequency of spinal deformity, and the dose of 100 μM induced a frequency of 12 % unhatched embryos, 10 % pericardial edema and 27 % yolk edema (Fig. 1B and Table 1).

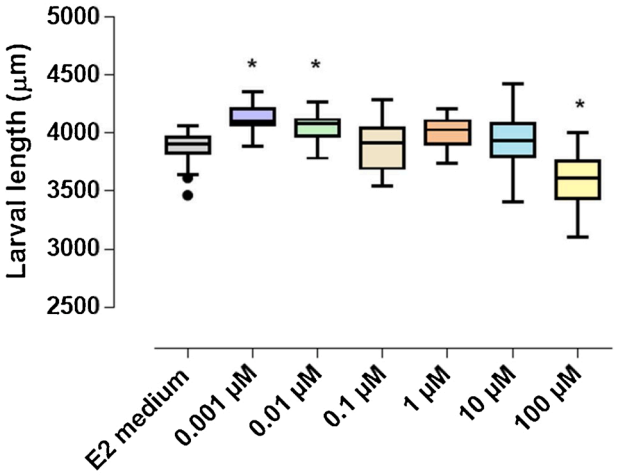
In addition, our result showed that curved tail, spinal deformity, and yolk edema were induced by virtually all doses of *Tn*P: 6 (0.001–100 μM), 5 (0.01–100 μM), and 4 (0.1–100 μM), respectively; and in Fig. 1C we showed representative images these defects in zebrafish individuals exposed to *Tn*P at the dose of 100 μM.

3.2. Phenotypic screening in zebrafish embryos exposed to TnP

To investigate the effect of *Tn*P on growth, we evaluated the larval body length at 96 hpf (Fig. 2A) We observed that the lowest doses (0.001 and 0.01 μM) were able to stimulate an increase of 6.5 % in the larval length when compared to the control larvae without *Tn*P treatment. On the other hand, at the highest dose (100 μM) the 96 hpf larvae showed a slight decrease of 7% in larval length compared to control. The doses of 0.1, 1 or 10 μM did not change the standard length.

Since the gross morphology imaged with bright-field microscopy analysis showed that *Tn*P at doses of 100, 10 and 1 μM induced pericardial edema (Fig. 1) and that together to yolk sac edema represent classically “blue sac syndrome” as described in zebrafish by Hill et al. [30], the next step was to measure the extension of pericardial area as a way of understanding the toxic mechanism of *Tn*P (Fig. 2B). *Tn*P doses of 0.001–1 μM did not increase the pericardial area. However, only the

A)



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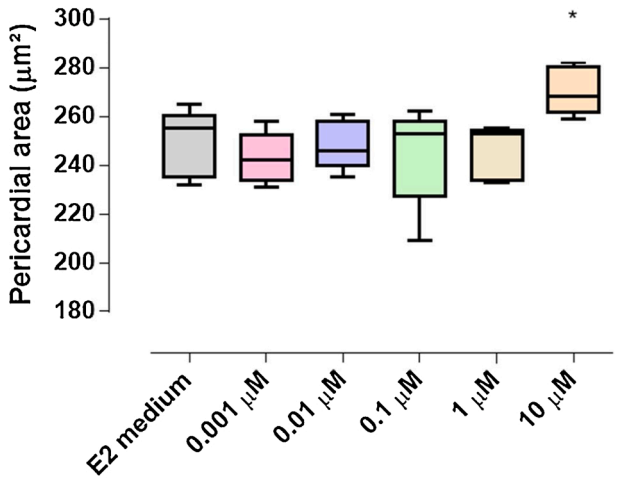

C)

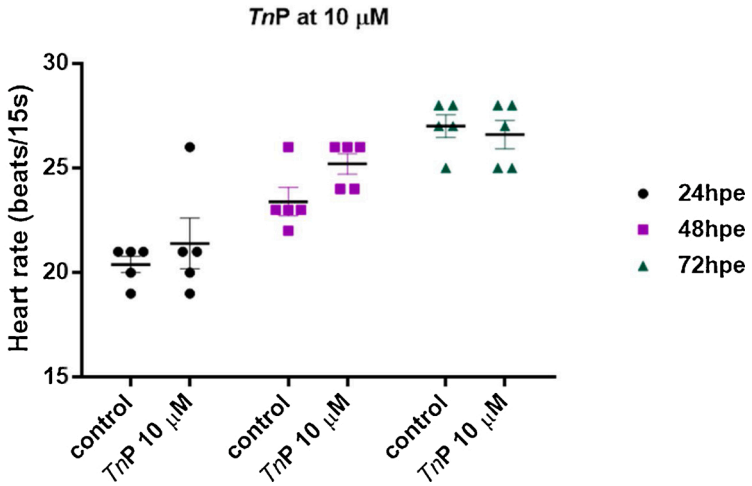

Fig. 2. Larval length of 96 hpf zebrafish exposed to *Tn*P. Embryos at 0 hpf (n = 5/well, quadruplicate) were treated by exposure to E2 0.5x medium without or with *Tn*P at doses of 0.001, 0.01, 0.1, 1, 10, and 100 μM. The 96 hpe individuals were anesthetized by immersion in 0.4 % tricaine, aligned, photographed in a Leica M205C stereomicroscope and the larval length was measured (***A***) from the top of the head to the tip of the tail in the images obtained using the Leica Application Suite software (LAS V4.11). Previously PTUtreated dechorionated larvae were treated with *Tn*P in doses of 0.001 to 10 μM for total pericardium area measurement at 72 hpe (***B***). The boxplot diagram results represent the median ± SEM and the black circle symbol indicates outliers. The heartbeat (***C***) was recorded on a Leica M205C stereomicroscope at 30x magnification (LAS V4.11 software) for 15 s. The results represent the mean ± SEM. *$p < 0.001$ compared with negative-control group (E2 0.5x medium).

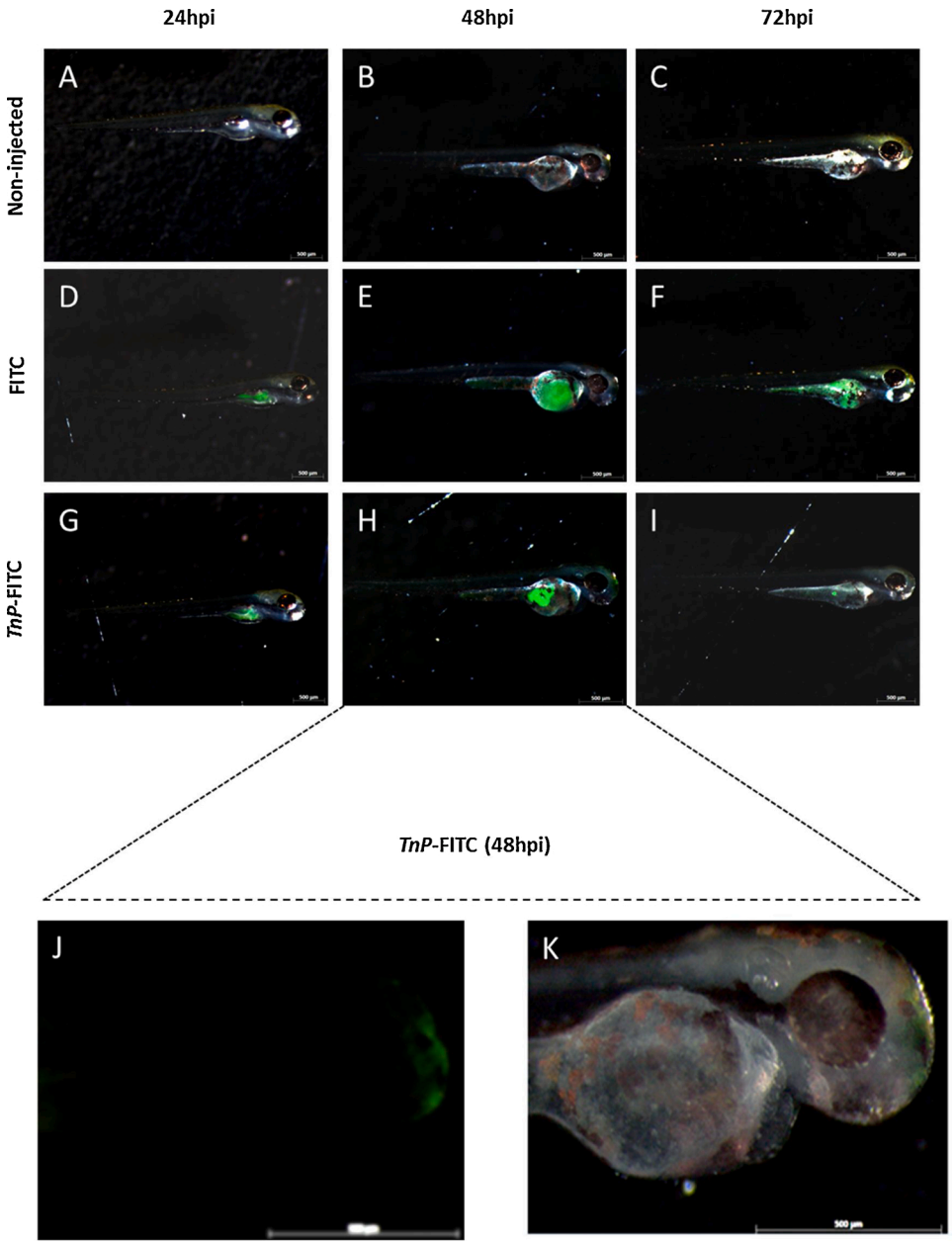

Fig. 3. Kinetic determination of *Tn*P distribution in zebrafish by fluorescence. Embryos at 0 hpf were microinjected into the yolk with 2-3 nL of FITC 2 or *Tn*P-FITC 1.2 mg.mL^{-1} using Eppendorf InjectMan® 4 microinjector. The microinjection was conducted in a Leica M205C stereomicroscopehe images of 24 (***A***, ***D***, and ***G***), 48 (***B***, ***E***, ***H***, ***J***, and ***K***) or 72 (***C***, ***F***, and ***I***) hpi of non-injected or *Tn*P-FITC injected larvae were obtained, under 0.4 % tricaine anesthesia, in Lumar V12 stereomicroscope with Axiocam MRC REV 3 and fluorescence deconvolved in AxioVision® software (Carl Zeiss, Germany) by applying the calibration parameters: 0.8x lenses, 52x magnification, -0.10 brightness, 5.78 contrast and gamma 2.20.

highest dose of *Tn*P (10 μM) increased the pericardial area at 8.4 % (270 μm^2 ± 2.8) when compared to the control larvae (249 μm^2 ± 4.3).

Impairment of cardiac development can be followed by reduced blood circulation, heart cavity congestion, and reduced heartbeat rate without contraction [31]. Then, we assessed whether the dose of 10 μM could affect the cardiac function of 1 dpf embryos by measuring the heartbeat count according to MacRae & Fishman [32]. In Fig. 2C, we observe a normal increase in the heartbeats of the control larvae from 2 to 4 dpf reaching at last day 27 beats/15 s as described by Yalcin et al. [33]. We found the same heart rate pattern after treatment with *Tn*P at 10 μM, and no differences were observed in the heartbeats of *Tn*P-treated larvae compared with control.

These results demonstrate that milder toxic effect induced by *Tn*P at 10 μM increasing the pericardial area (8.4 %) did not reflect in cardiac dysfunction.

3.3. TnP crosses the blood-brain barrier but does not induce a neurotoxic effect

We analyzed whether *Tn*P could be transported through the blood-brain barrier (BBB). For this, we visualized the tissue distribution of peptide through the fluorescence signal (FITC) after 24, 48, and 72 hpi of *Tn*P-FITC microinjected into the yolk of the embryos at 2 hpf.

Non-injected larvae did not present green fluorescence at 24 (Fig. 3A), 48 (Fig. 3B), or 72 hpi (Fig. 3C). Twenty-four hours after injection into the yolk, the *Tn*P-FITC was observed only at the microinjection site (Fig. 3G) similar to FITC-injected larvae (Fig. 3D). However, at 48 hpi the presence of the *Tn*P-FITC was verified in the zebrafish brain (Fig. 3H, J, and K) in contrast to FITC concentration in the yolk of FITC-injected larvae (Fig. 3E). At 72 hpi, a slight fluorescence signal was observed in the embryos injected with *Tn*P-FITC (Fig. 3I) in contrast to fluorescence signal in the yolk of FITC-injected larvae (Fig. 3F).

The photomotor response assay, consisting of the automatic tracking of larval movement in response to alternate illumination conditions, is

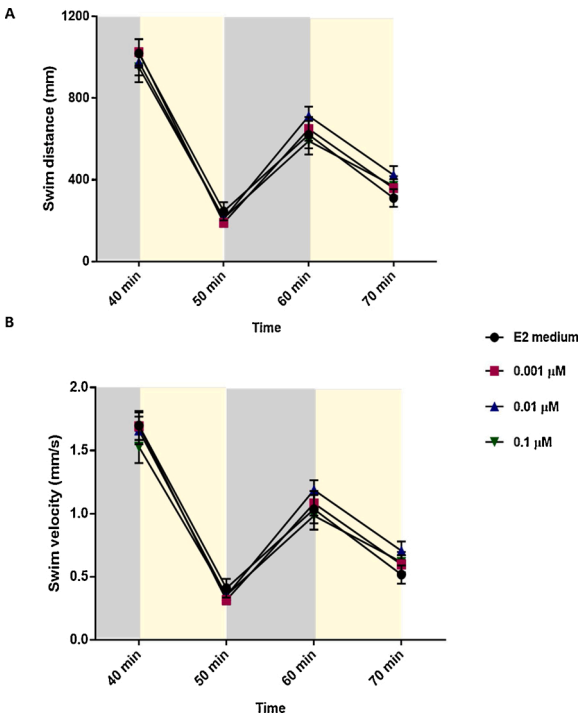

Fig. 4. Locomotor activity determination in *Tn*P-treated zebrafish. 144 hpf zebrafish larvae (n = 24/group) treated by exposure to 200 µL of E2 0.5x medium without or with *Tn*P at doses of 0.001, 0.01, and 0.1 µM were analyzed after alternate light or dark periods to detect changes in the swimming activity as the distance (***A***) and velocity (***B***). The results represent the mean ± SEM.

extensively used screen neurotoxic effects. The lightdark transition increases locomotor activity, while the darklight transition decreases locomotor activity [34].

The locomotor activity represented by the distance swam by the larva and velocity of 144 hpf larvae treated with *Tn*P submitted to alternate light or dark periods is demonstrated in Fig. 4A and B, respectively. We observed less activity recorded during light followed by immediate and robust hyperactivity in locomotor behavior during darkness period for control larvae o deviations from the behavior were recorded after exposure to all doses of *Tn*P.

The main structures as forebrain, midbrain, and hindbrain are found in the zebrafish [35]. Finally, the length of the forebrain, midbrain or hindbrain was measured after 28 or 48 hpf, periods before and after the start of circulation, involved in the ventricular expansion process, according to Lowery & Sive [36]. As demonstrated in Fig. 5, no changes were observed in the length of forebrain (Fig. 5A), midbrain (Fig. 5B), and hindbrain (Fig. 5C) at 28 or 48 hpf of embryos treated with *Tn*P compared to non-injected control embryos, indicative of no effect of *Tn*P on brain growth.

4. Discussion

The zebrafish, especially the zebrafish embryos, increasingly used in drug discovery early drug development, and toxicological screens [37–42]. Here in this study, we conducted the OECD #236 test to investigate effects of *Tn*P on the survival, hatching performance, and morphological formation of zebrafish embryos. After determining these endpoints, morphometric analysis and determination of locomotion or swimming in zebrafish larvae were evaluated to identify adverse effects such as cardiotoxicity and neurotoxicity.

First, these data show that *Tn*P presented a low capacity to induce abnormalities and a wide range of safe doses from 1 nM to 10 µM he decrease in the *Tn*P doses promoted a shift from sub-lethal to teratogenic effects. Micromolar (10,000 and 1,000 µM) exposure of *Tn*P was associated with lethality, and the induction of abnormalities in embryonic development of *Tn*P-treated embryos was dose-dependent. As opposed to drugs with narrow therapeutic index that cause potentially fatal complications and can result in appearance of severe adverse toxic effects, the high therapeutic index of *Tn*P indicates the possibility to fit into less stringent regulatory standards for approval [43].

Second, we can associate the low frequency of abnormalities by *Tn*P with high safety of the molecule and the ability of the developing embryo to process and eliminate it. As opposed, we could expect possible cumulative toxicity of embryos for 96 hpf to *Tn*P without renewal of medium, even at the lowest doses. However, our data rule out this hypothesis as demonstrated by larvae'sability to eliminate *Tn*P-FITC. 72 hpf larvae demonstrate developed eliminating organs like the liver and kidneys as well as maturation of enzymatic processes that lead to increased elimination [44].

With accelerated excretion and low toxicity, peptides become attractive in develop new drugs [45]. The rapid metabolism of cyclic *Tn*P observed here in zebrafish, although favorable avoiding cumulative toxicity, is limiting from a pharmacokinetic point of view. Thus, our findings point to requirement to increase bioavailability of this type of molecule [46].

Here we demonstrated the opposite effect of *Tn*P to modulate larval length, at lowest doses (0.001 and 0.01 µM) stimulated an increase of 6.5 % but at a high dose (100 µM) decrease it at 7%. The study of Wincent et al. [47] reported that aryl hydrocarbon receptor (AhR)-dependent deregulation of Wnt/β-catenin signaling is a potential mechanism involved in reduc body growth in zebrafish. highlights a possible involvement of *Tn*P in the Wnt/β-catenin-AhR signaling deregulation, claiming further investigations.

Drug-induced cardiotoxicity is the major reason for drug withdrawal from the market. For instance, between 1994 and 2006, 45 % of discontinued medications had adverse effects such as cardiac ischemia and arrhythmogenesis [48]. In this line zebrafish has emerged as a model organism for cardiovascular research, investigating gene function and modeling a variety of human disease side effects of chemotherapeutic drugs or particularly to screen drug candidates. The toxicity effects reported from zebrafish-based experiments are considered representative for higher vertebrates including humans and can serve in drug development as a preclinical requirement decipher drug safety.

The cardiotoxicity evaluation of drugs is frequently performed in zebrafish. Zakaria and collaborators have done an extensive review and found a variety of drugs used in the therapeutic clinic with cardiotoxic activity [18]. These authors reviewed data on known human cardiotoxic drugs including aspirin, clomipramine hydrochloride, cyclophosphamide, nimodipine, quinidine, terfenadine, and verapamil hydrochloride tested in the zebrafish model. Also, they described that drugs includ 5-fluorouracil and mitoxantrone, as well as addictive drugs, anticancer drugs belong to the Anthracyclines class or kinase inhibitors, antiarrhythmic, anticonvulsant, and beta-blockers; and cyclophosphamide terfenadine affect the heart function of zebrafish administered *via* soaking or yolk sac microinjection with drug concentrations ranging from 0.01 to 1000 µM.

Furthermore, our data showed *Tn*P at 10 µM induced pericardial edema in 3% of the larvae with increased pericardium length (8.4 %) compared to control larvae. It is important to note that this slight morphological change induced by *Tn*P, without compromising the cardiac function corroborates our findings of the effect of *Tn*P as a possible inducer of muscle growth through modulation of the Wnt/β-catenin-AhR signaling.

Furthermore, our findings of distribution that demonstrate *Tn*P reached the CNS zebrafish through the BBB are of substantial importance for understanding its role as an immunomodulator in neuroinflammatory diseases such as multiple sclerosis that have the brain as the therapeutic target. Interestingly, zebrafish is an excellent model for the preclinical studies of neurological drugs [49–51] since its BBB has similar characteristics to higher vertebrates as rather low permeability,

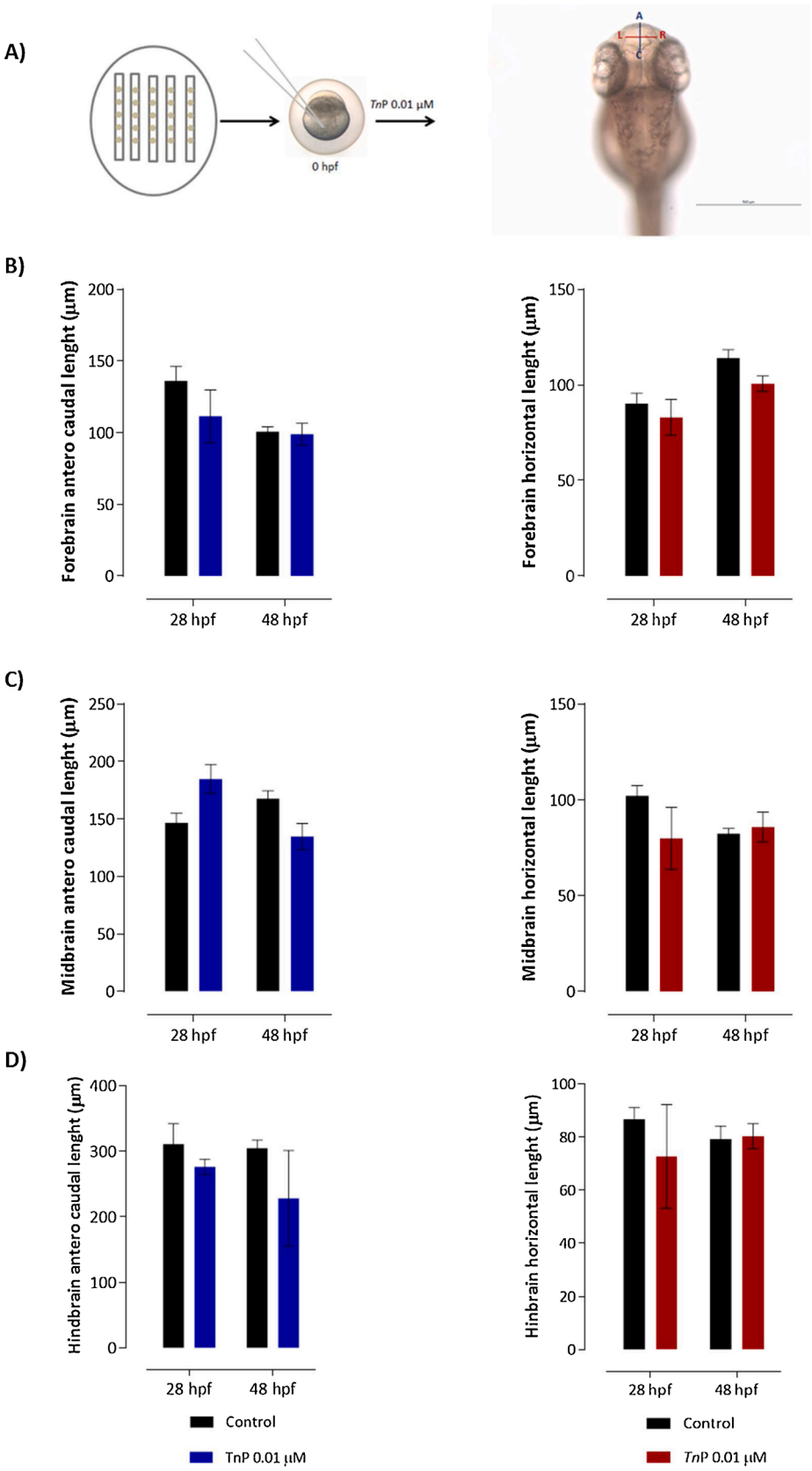

Fig. 5. Morphometric analysis of the brain of zebrafish embryos after *Tn*P treatment. 0 hpf zebrafish embryos (n = 30/group) were microinjected with 0.01 µM *Tn*P using microneedles coupled to the Injectman® 4 microinjector in a volume of 2-3 nL in the yolk and immersed in E2 0.5x medium up to 48 hpf without medium renewal (***A***). Embryos not microinjected and only treated with E2 0.5x medium were considered negative control. The embryos were analyzed at 28 and 48 hpf for brain development by measuring the length of the forebrain (***B***), midbrain (***C***) and hindbrain (***D***) in the antero-caudal and horizontal position, represented in the schematic Figure (**A**) on the right. The blue and red lines represent the measurement directions. The bars represent the mean ± SEM of the ventricle length.

strong tight junctions, and drug transporters [52].

However, BBB permeability determines whether a drug candidate has both neurological therapeutic or toxic impact. Once peptides can cross the BBB [53] and our results confirm that *Tn*P-FITC reaches the brain, we questioned whether *Tn*P could disturb the neurotransmitter circuit altering neurological functions [54]. To elucidate this issue we use a functional approach to assess the toxic impact of *Tn*P on the brain analyzing the larval zebrafish locomotor behavior, emerged as a powerful indicator of perturbations in the nervous system [55–57].

We found similar locomotor behavior in 144 hpf larvae treated with all doses of *Tn*P compared to control-larvae, suggesting that the passage of *Tn*P through BBB did not disturb the integrated response of the brain function, nervous system, and visual pathway [58,59].

Behavioral studies as locomotor activity have identified strong associations between the functions of zebrafish and human brain regions [38]. Finally, we confirmed that 0 hpf embryos microinjected with *Tn*P presented at 28 and 48 hpf a developed brain with forebrain, midbrain, and hindbrain, suggesting that the formation of structures or neurogenesis [60,61] were not affected by *Tn*P.

The results of this study confirm a broad therapeutic index for *Tn*P, a drug candidate for neuro-inflammatory diseases and highlight the importance of zebrafish as an accurate investigative toxicology model to assess acute toxicity as well as cardiotoxicity and neurotoxicity of molecules in the preclinical phase of development.

Competing financial and non-financial interests

The funders had no role in study design, data collection and analysis, decision to publish, or preparation.

Author statement

João Batista-Filho and Geonildo Rodrigo Disner: contributed to the study design, data collection, analysis, interpretation, and writing.

Maria Alice Pimentel Falcão, Adolfo Luis Almeida Maleski, Amanda Beatriz Silva Soares, Leticia Balan-Lima: contributed to the study design, data collection, analysis, and interpretation.

Carla Lima and Mônica Lopes-Ferreira: conceived and supervised the project, contributed to reagents/materials/analysis tools, helped with the data interpretation, and writing.

Conflict of Interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

João Batista-Filho: Investigation, Methodology, Formal analysis. **Maria Alice Pimentel Falcão:** Investigation, Methodology, Formal analysis. **Adolfo Luis Almeida Maleski:** Investigation, Methodology, Formal analysis. **Amanda Beatriz Silva Soares:** Investigation, Methodology, Formal analysis. **Leticia Balan-Lima:** Investigation, Methodology, Formal analysis. **Geonildo Rodrigo Disner:** Investigation, Methodology, Formal analysis. **Carla Lima:** Investigation, Methodology, Formal analysis. **Monica Lopes-Ferreira:** Investigation, Methodology, Formal analysis.

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