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Zebrafish embryo-larval testing reveals differential toxicity of new psychoactive substances

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ABSTRACT

New psychoactive substances (NPS) have emerged as a significant public health concern, with synthetic cannabinoid receptor agonists (SCRAs) and ketamine derivatives being among the most frequently detected compounds in the forensic context worldwide. The Fish Embryo Acute Toxicity (FET) and Maximum Tolerated Concentration (MTC) tests are used to evaluate the acute toxicity of chemicals. In this study, we used these assays to evaluate the acute toxicity of three NPS in zebrafish embryos and larvae: the SCRA MDMB-4en-PINACA and the ketamine derivatives deschloroketamine (DCK) and 2-fluorodeschloroketamine (2F-DCK). Our findings demonstrated that MDMB-4en-PINACA induced severe developmental abnormalities, including pericardial edema and yolk edema, along with high embryo mortality (10 μM), characterized by endpoints such as coagulation, lack of heartbeat, and lack of somite formation. In contrast, DCK and 2F-DCK exhibited low embryo mortality even at higher concentrations. In larval stages, MDMB-4en-PINACA presented 8 % larvae mortality (10 μM) at eight days post-fertilization (dpf), whereas ketamine derivatives led to 100 % mortality at 2000 μM in the MTC test at eight dpf. The LC_{50} was calculated for the FET test with MDMB-4en-PINACA, and MTC test for both DCK and 2F-DCK. Additionally, our results support the absence of N-methyl-D-aspartate (NMDA) receptors in the early life stages of zebrafish described in previous studies and highlight the significance of ketamine derivatives intoxications when the NMDA receptor is expressed. Notably, MDMB-4en-PINACA exhibited significantly higher toxicity, with an LC_{50} of approximately 26 times lower than that of the ketamine derivatives. These results are particularly relevant given the increasing global prevalence of NPS-related intoxications and fatalities. Using zebrafish as an *in vivo* model for toxicological research provides an efficient approach for screening the acute effects of emerging compounds such as NPS.

1. Introduction

The New Psychoactive Substances (NPS) constitute a heterogeneous group of emergent compounds largely consumed within social contexts. The wide variety of substances is generally produced by structural modifications of drugs aiming not only to mimic their psychoactive effects but also to evade international drug control agencies, remaining as a legal alternative to illicit drugs [1,2]. As reported by the United Nations Office on Drugs and Crime (UNODC) in 2023, over 1200 NPS were notified by the Early Warning Advisory (EWA) considering 141 countries and territories worldwide. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) monitored around 930 NPS by the end of 2022, only in Europe [2,3]. Nowadays, the most common classes of NPS are synthetic cathinones, synthetic cannabinoid receptor agonists (SCRA), and synthetic opioids. However, other classes such as phenethylamines, tryptamines, arylcycloalkylamines, benzodiazepines, and piperazines also deserve attention. Since they are mostly composed of new molecules, the health risks are generally unknown and, in most

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cases, NPS are even more powerful than the classic precursor drug [2,4]. Some NPS can also be included into the broader category of chemicals that may pose adverse health effects while only scarce data is available. These substances are referred to as Chemicals of Emerging Concern (CECs) [5,6].

Synthetic cannabinoid receptor agonists (SCRA) are compounds that bind to cannabinoid receptors (CB_1 and CB_2) and mimic the effects of delta-9-tetrahydrocannabinol (Δ^9-THC). They are mostly commercialized by spraying on herbal plant material for smoking use [4,7]. Many SCRA are potent CB_1 agonists, leading to more intense THC-like symptoms such as increased heart rate, vomiting, agitation, confusion, and hallucinations. This class of NPS is described as a high potency group and can even lead to death [7]. MDMB-4en-PINACA stands out as one of the most potent SCRA. This substance was first reported in 2018 in Slovenia as a yellow powder material [8,9]. In 2020, the EMCDDA classified this substance under intensive monitoring due to the increase in the frequency of its identification in Europe and the related risks associated with its consumption. As described by The Center for Forensic Science Research & Education (CFSRE), this designer drug represents the most identified SCRA in the United States between 2020 and 2023 [10]. In Brazil, MDMB-4en-PINACA was the second most detected drug in infused paper samples seized between 2019 and 2020 [11].

Ketamine derivatives, together with analogs of phencyclidine, are molecules classified as arylcyclohexylamines or phencyclidine-type substances, being mainly dissociative or stimulant drugs [7,12]. Stimulants act by altering the actions of dopamine, norepinephrine, and/or serotonin, mimicking the effects of traditional drugs such as cocaine, amphetamine, and ecstasy. Dissociative drugs, in turn, modulate the effects on the N-methyl-D-aspartate (NMDA) receptors in the brain, deceiving the perception of time, motions, colors, sound, and even self [12]. Among ketamine derivatives, two substances can be highlighted: deschloroketamine (DCK) and 2-fluorodeschloroketamine (2F-DCK). DCK was originally produced for clinical purposes intending to treat infections and immunological disfunctions [13]. In 2015, DCK was detected in seized powders in Italy, characterizing its use as recreational drug since then [14]. 2F-DCK was synthesized in 2014 [15] and it was first notified in Spain two years later [16]. Other reports described 2F-DCK detection in seized material, biological samples, and wastewater [17].

The use of zebrafish toxicological assays has increased in recent years due to several advantages over other *in vivo* models. Zebrafish offer small body size, minimal drug consumption, high reproductive rates, optical transparency (embryos and larvae), external and rapid embryo development, genetic similarity to humans, low cost, and the potential for high-throughput analyses [18–20]. Additionally, zebrafish have been proven valuable for studying complex responses to drugs of abuse, including sensitization, tolerance, withdrawal, drug seeking, extinction, and relapse [21–23]. Beyond these advantages, zebrafish provides significant understanding both for environmental and human health risks, aligning with the One Health perspective [24,25]. Human exposure to NPS, whether voluntary or involuntary, offers significant risks for human health, highlighting the need for improved monitoring and regulatory strategies [26]. Furthermore, many NPS and/or their metabolites can enter aquatic ecosystems primarily through wastewater contamination [27–29], representing risks not only to aquatic organisms but also to humans via water sources and the food chain.

The Fish Embryo Acute Toxicity (FET) test is described in the Test Guideline (TG) 236 from the Organization for Economic Co-operation and Development (OECD) to assess the acute toxicity of chemical substances using embryonic stages of zebrafish. The test consists of exposing newly fertilized eggs to the targeted substance for 96 h. At 24-hours, up to four key observations can be recorded, including indicators of lethality such as coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat. If a positive outcome is observed for at least one of the four endpoints, the mean lethal concentration (LC_{50}) can be determined [30,31]. The FET test has been successfully applied to evaluate different compounds, including NPS such as 25H-NBOMe and 25H-NBOH [32], meta-chlorophenylpiperazine (mCPP) [33], fentanyl and other synthetic opioids [34], etazene [35], and 5F-APINAC [18].

In contrast to the protocoled FET test, the Maximum Tolerated Concentration (MTC) test is not standardized but is well-described in the literature. In the MTC test, zebrafish larvae are exposed to the targeted substance and evaluated every 24 hours until the end of the experiment. The test aims to identify concentrations at which signs of acute toxicity, such as locomotor impairment (hypoactivity, absence/decrease of touch response, shaking and loss of posture), deformations, lack/decrease of heartbeat, or death are observed. The MTC is defined as the highest concentration tested in which no signs of toxicity are noticed [36,37]. Similar to the FET test, the MTC has been utilized to assess different substances, including NPS [37–40].

To evaluate and compare the acute toxicity of three NPS belonging to the SCRA (MDMB-4en-PINACA) and ketamine derivative (DCK and 2F-DCK) classes, both embryonic and larval stages of zebrafish were employed and subjected to the FET and the MTC tests. To our knowledge, this is the first study to systematically assess and directly compare the acute toxicity of these NPS across different zebrafish developmental stages, providing novel insights into their differential toxicological effects. Although the metabolism of MDMB-4en-PINACA has been previously investigated in adult zebrafish [41], no prior study has evaluated its acute toxicity in early life stages. Similarly, although ketamine derivatives such as esketamine, deschloro-N-ethyl-ketamine, fluoro-N-ethyl-ketamine, and bromoketamine have been studied in zebrafish to evaluate their effects on behavior, gene transcription pathways, and metabolism [42,43], no research to date has explored the toxicity of DCK and 2F-DCK using this model.

2. Materials and methods

2.1. Standards and reagents

Reference materials of MDMB-4en-PINACA, deschloroketamine (DCK), and 2-fluorodeschloroketamine (2F-DCK) were obtained from Cayman Chemical (Ann Arbor, MI, USA). Methanol (MeOH) and 3,4-dichloroaniline were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was provided by a Milli-Q RG system from the company Millipore (Burlington, MA, USA). Red sea-salt was purchased from Red Sea Fish (Houston, TX, USA) and prepared by diluting 1.7 g in 1 L of ultrapure water, producing the reconstituted water (RW).

2.2. Drug preparation

For each drug, the evaluated range consisted of five different concentrations. The range for MDMB-4en-PINACA was 0.001–10 μM. For DCK and 2F-DCK, two concentration ranges were tested. The initial range (1–500 μM) was selected based on literature data for ketamine-like compounds [44–46] but proved ineffective for DCK and 2F-DCK since no lethality was noticed. Therefore, a second phase was conducted with higher concentrations (100–2000 μM), including overlapping concentrations to enable comparison between experiments.

Stock solutions were prepared in methanol at concentrations of 27.98 μM (10 μg/mL) for MDMB-4en-PINACA, 4919.32 μM (1000 μg/mL) for DCK, and 4519.37 μM (1000 μg/mL) for 2F-DCK. Additionally, working solutions were also prepared in methanol for each substance. Each stock and working solution, as well as the final tests concentrations, are shown in Supplementary Table 1.

2.3. Animal husbandry

Adult wild-type zebrafish were obtained from the Zebrafish Laboratory and Husbandry at the School of Medical Sciences (Universidade Estadual de Campinas – UNICAMP) and were maintained in tanks

(30–50 L) filled with non-chlorinated water that was mechanically and chemically filtered. The fish density was two to three animals per liter. They were fed commercial fish food (Tetramin, Tetra, Blacksburg, VA, USA) three times per day, supplemented with brine shrimp once a day. Photoperiod cycles consisted of 14:10 hours (light:dark) and the temperature was set at 26 ± 2 °C. Fertilized eggs were collected after natural spawning, and embryos and larvae were housed in Petri dishes containing RW until the day of exposure. All experiments described in this study were reviewed and approved by the Ethical Committee for Animal Research for the Universidade Estadual de Campinas (6096–1/2022 and 6096–1(A)/2023).

2.4. Drug exposure

On each experiment day, before drug exposure, 100 μL of each working solution was aliquoted in 2 mL polypropylene tubes and dried under nitrogen flow at 30 °C until dryness in a TurboVap® Evaporator (Biotage, Uppsala, Sweden). The dried content was then resuspended with 100 μL of RW and these final solutions were used for drug exposure in zebrafish embryos and larvae, by adding it to 100 μL of RW.

2.5. Fish embryo acute toxicity (FET) test

To evaluate the acute toxicity of NPS during embryonic development, the FET test was performed following the OECD TG 236 recommendations, namely the early exposure of zebrafish embryos to the test substances. Zebrafish embryos were collected right after natural spawning and transferred to Petri dishes. Using a stereoscopic microscope (Nikon, Minato, Tokyo, Japan), fertilized eggs and up to 16-cell stage development were selected for the experiment. Exposures occurred on embryos (n = 20) aged between zero and four days post-fertilization (dpf). For ketamine derivatives, two different drug ranges were used. The first exposures were performed by adding 100 μL of DCK or 2F-DCK at 2, 20, 200, 500, and 1000 μM to wells containing 100 μL of RW, yielding final concentrations of 1, 10, 100, 250, and 500 μM. The second exposure employed higher concentrations, in which 100 μL of DCK or 2F-DCK was added in concentrations of 200, 500, 1000, 2000, and 4000 μM to wells containing 100 μL of RW, yielding final concentrations of 100, 250, 500, 1000, and 2000 μM. For MDMB-4en-PINACA, the exposure was performed by adding 100 μL of the drug at 0.002, 0.02, 0.2, 2, and 20 μM to wells containing 100 μL of RW, yielding final concentrations of 0.001, 0.01, 0.1, 1, and 10 μM. Each experiment included a negative control group (n = 20) where 200 μL of RW was added to the wells, and a positive control group (n = 20) where 100 μL of RW and 100 μL of 3,4-dichloroaniline (8 mg/L) were added to the wells. All the exposures were done in 96-well plates with one embryo per well, an adaptation from the OECD TG 236 guideline. Hence, at least one endpoint (egg coagulation, lack of heartbeat, lack of somite formation, and lack of detachment of the tail-bud from the yolk sac) was recorded as an indicator of lethality. Other changes were also observed as indicators of sublethal effects. The LC_{50} for each drug was determined based on the number of deceased embryos.

2.6. Maximum tolerated concentration (MTC) test

For larval experiments, the MTC test was performed. Zebrafish embryos were collected right after natural spawning and maintained in Petri dishes until five dpf. Then, larvae were observed in the microscope and those with normal development were selected. Exposure occurred with larvae (n = 12) between five and nine dpf, at the same doses tested in FET. For ketamine derivatives, the MTC was performed only with the higher range used in FET. Also, negative (n = 12) and positive (n = 12) control groups were used, and the experiments were done in 96-well plates with one larva per well.

For its evaluation, signs of acute locomotor impairment (hypoactivity, absence/decrease of touch response, shaking, and loss of posture), deformations, lack/decrease of heartbeat, and death were registered. To minimize handling-related stress and potential confounding effects on behavioral responses, evaluations were conducted at six and eight dpf, avoiding excessive manipulation. Additionally, since some toxicological effects develop gradually rather than acutely within 24-hour intervals, this approach allowed the capture of cumulative effects while ensuring an efficient experimental design.

Parameters such as locomotor impairment, deformations, heartbeat alterations, and mortality were assessed through visual inspection using the stereoscopic microscope. Each larva was observed for at least 30 seconds under the same conditions to ensure consistency across experimental groups. Locomotor impairment was evaluated based on the presence of hypoactivity (reduced spontaneous movement), shaking (irregular body tremors), and loss of posture (inability to maintain a stable position). The touch response test was performed by applying a mechanical stimulus to the caudal region of each larva using a fine micropipette tip, achieving three types of response: (i) increase (exaggerated or erratic movement following the stimulus), decrease (delayed or weakened reaction), and absence (no movement). All observations were initially made by one individual and subsequently confirmed by another.

The number of dead larvae was used to calculate the LC_{50} for each drug. Both FET and MTC observations were performed at the stereoscopic microscope. At the end of each procedure, all the surviving larvae were euthanized by transferring them into a mixture containing ice: water (5:1, *m/m*) for 10 minutes.

2.7. Statistical analysis

To calculate LC_{50} for the studied drugs in each experiment, version 5.0 GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) software was used. LC_{50} values were calculated by probit regression analysis. A one-way ANOVA completed with Tukey's posthoc comparison determined differences to independently compare the exposure to the control group, using $p < 0.05$ as the parameter for statistical significance [32].

3. Results

3.1. Fish embryo acute toxicity (FET) test

The FET evaluation was independently performed for MDMB-4en-PINACA, DCK, and 2F-DCK. For each drug, twenty embryos per concentration were tested, along with negative and positive control groups. MDMB-4en-PINACA caused a high embryo mortality rate (30 %) at 10 μM, with observed endpoints including coagulation, lack of heartbeat, and lack of somite formation. Sublethal effects were observed across all concentrations, including varying degrees of tail detachment (one dpf), scoliosis (three dpf), lordosis (three and four dpf), pericardial edema (two and three dpf), and yolk edema (three dpf). Furthermore, MDMB-4en-PINACA demonstrated a positive correlation between exposure concentration and lethal effects. For DCK and 2F-DCK, two ranges were evaluated, since the lowest one did not produce substantial modifications in zebrafish embryos (data not shown). The two ketamine derivatives exhibited low embryo mortality at the higher concentration range compared to MDMB-4en-PINACA, not exceeding 20 % for DCK and 10 % for 2F-DCK, respectively. The endpoints observed included embryo coagulation, lack of heartbeat, and lack of somite formation. The sublethal effects observed included varying degrees of lack of tail detachment (one dpf), lordosis (three dpf), kyphosis (three dpf), scoliosis (three dpf), lack of pigmentation (two, three, and four dpf), egg-hatching delay (four dpf), blood clotting (two, three, and four dpf), and pericardial edema (two, three, and four dpf). Fig. 1 shows a graphical representation of the observed effects of the three NPS on zebrafish embryos at the end of the evaluation period - four dpf. Fig. 2 displays graphical representations of the embryo mortality progression

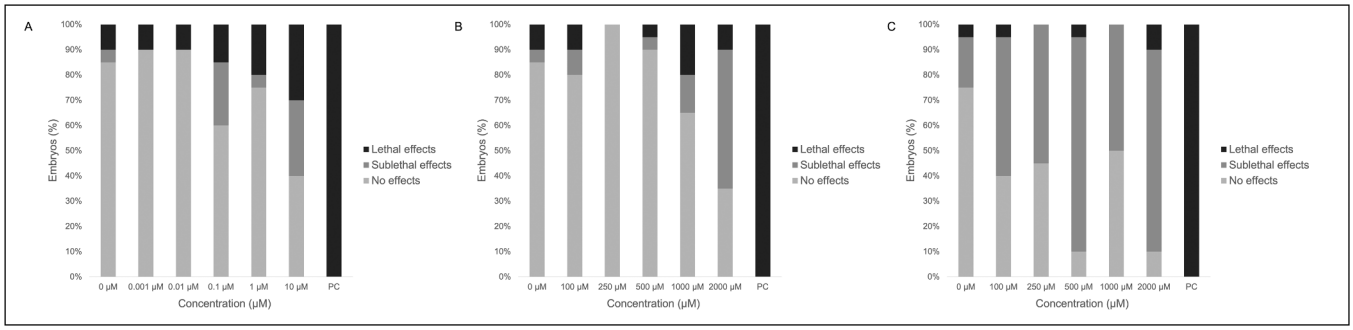

Fig. 1. Lethal and sublethal effects and absence of effects observed for the MDMB-4en-PINACA (A), deschloroketamine (DCK) (B), and 2-fluorodeschloroketamine (2F-DCK) (C) for zebrafish embryos at the end of the evaluated period - four dpf. PC positive control.

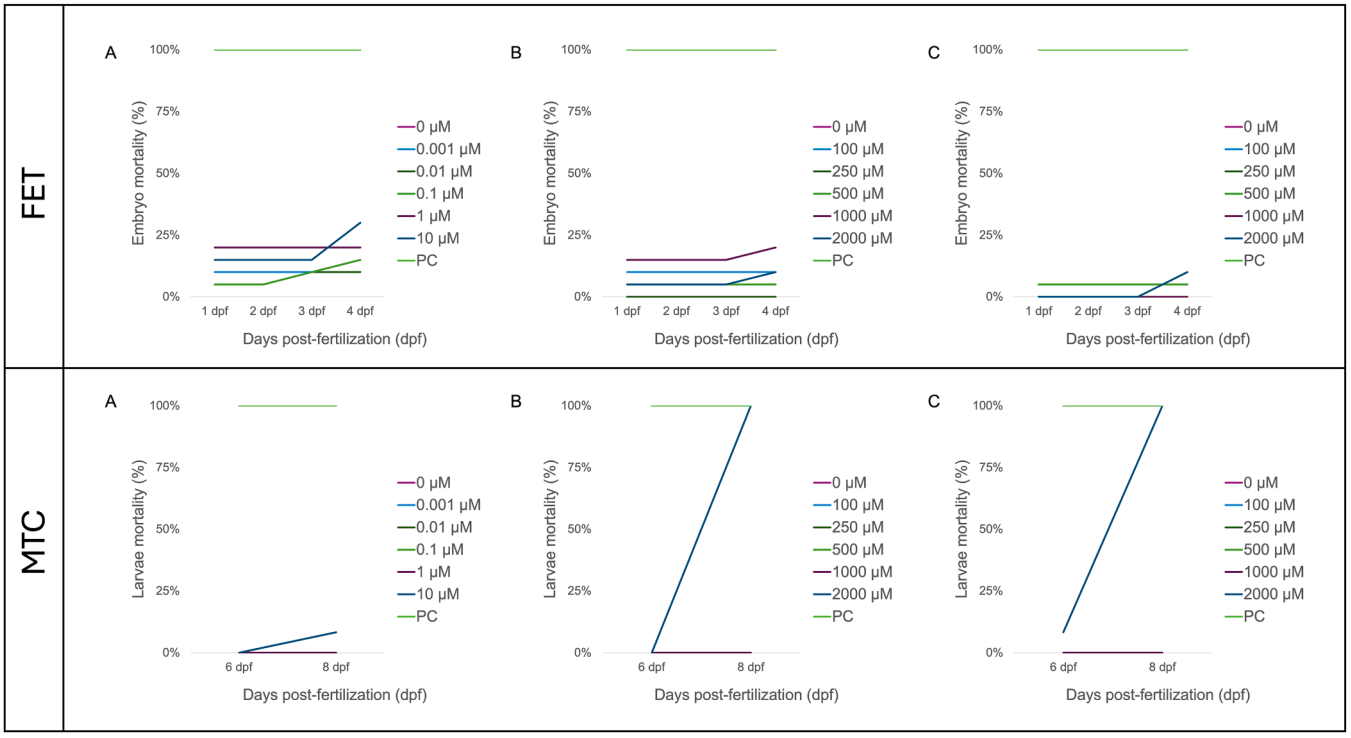

Fig. 2. Embryo and larvae mortality progression for the Fish Embryo Acute Toxicity (FET) (upper box) and Maximum Tolerated Concentration (MTC) (lower box) tests for the MDMB-4en-PINACA (A), deschloroketamine (DCK) (B), and 2-fluorodeschloroketamine (2F-DCK) (C). PC positive control.

for the FET test. Fig. 3 provides a visual representation of some of the endpoints and sublethal effects observed in zebrafish embryos during the four days of exposure.

3.2. Maximum tolerated concentration (MTC) test

Like the FET, the MTC test was performed for each drug separately. MTC was conducted with twelve larvae per drug concentration, as well as for negative and positive control groups. Surprisingly, MDMB-4en-PINACA presented only 8 % of larvae mortality at the highest concentration (10 µM) after eight dpf. Regarding this concentration, at six dpf, hypoactivity (83 %) was the only important effect noticed. On the other hand, for ketamine derivatives MTC, 100 % of the larvae died in 2000 µM after eight dpf. For DCK at six dpf, a positive correlation between drug concentration and hyperactivity was evident, as well as a decrease in touch response. At eight dpf, touch response showed an inverse correlation with drug concentration until the highest one, where all the larvae died. For 2F-DCK, at six dpf, we noticed that the loss of posture became more pronounced as the concentration increased, with effects detected at 500, 1000, and 2000 µM. Shaking behavior was noticed at 1000 µM for 58 % of the larvae. Also, 100 % of the larvae showed a reduced touch response at 1000 µM and 100 % showed an absence of touch response in 2000 µM. At eight dpf, 75 % of the larvae exposed to 1000 µM showed decrease in heartbeat rate and 100 % showed absence of touch response in this concentration. The larval mortality progression over both days of evaluation is graphically displayed in Fig. 2. Fig. 4 shows all the effects observed for the three NPS tested with zebrafish larvae at evaluated periods (six and eight dpf) and Fig. 5 contains the information related to touch response.

3.3. Mean lethal concentration calculation (LC_{50})

For the experiments in which the mortality was higher than 30 % in at least one of the concentrations tested, the LC_{50} was calculated. Consequently, data are available for the FET test with MDMB-4en-PINACA, MTC test with DCK, and MTC test with 2F-DCK. Other experiments did not achieve substantial mortality. Fig. 6 contains the LC_{50} plots for each of the tests cited.

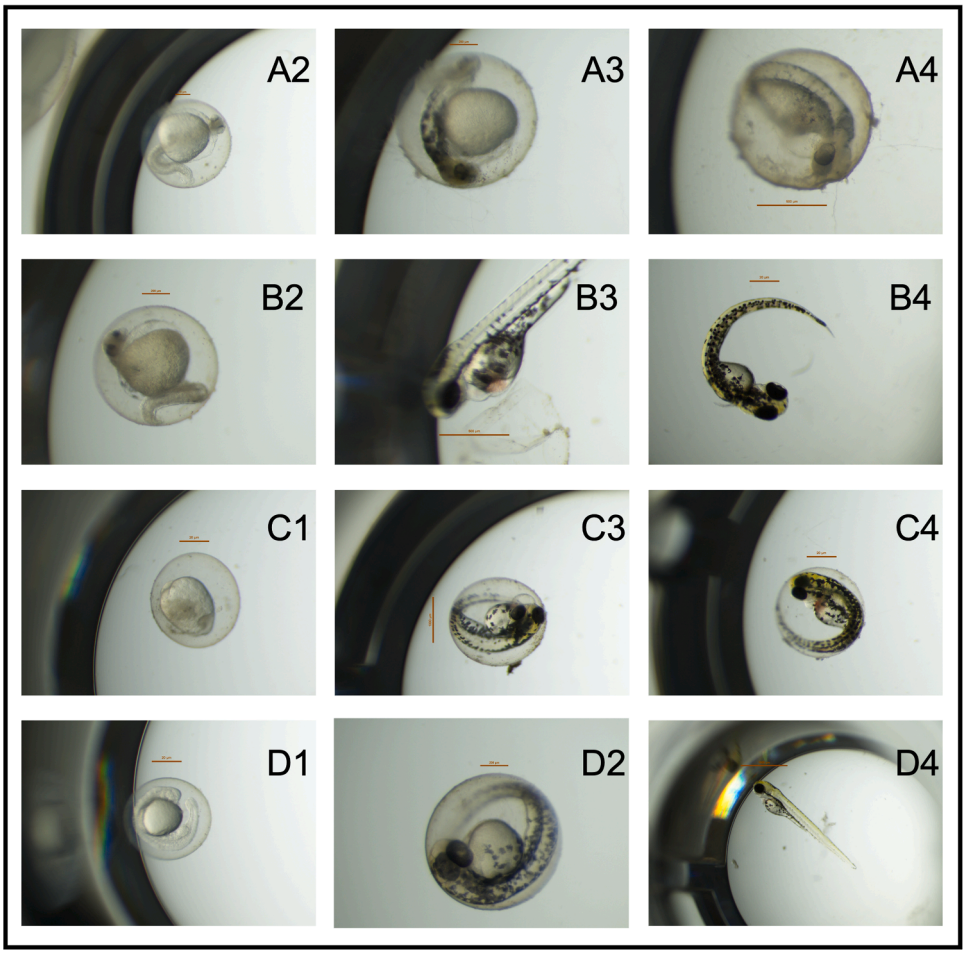

Fig. 3. Illustrative images of the endpoints and sublethal effects observed in zebrafish embryos during four days of exposure with MDMB-4en-PINACA (A), deschloroketamine (DCK) (B), and 2-fluorodeschloroketamine (2F-DCK) (C) at one (1), two (2), three (3), and four (4) days post-fertilization (dpf). The effects were: A2 – lack of heartbeat and degrees of lack of tail detachment; A3 – lack of heartbeat and lack of somite formation; A4 – lack of heartbeat and lack of somite formation; B2 – pericardial edema and lack of pigmentation; B3 – pericardial edema and blood clotting; B4 – scoliosis; C1 – coagulation; C3 – lack of heartbeat and pericardial edema; C4 – pericardial edema and blood clotting; D1, D2, and D4 corresponds to the negative control, with normal development at 24 h, 48 h, and 96 h, respectively.

4. Discussion

The cannabinoid mechanism of action is intimately related to the endocannabinoid system, a complex endogenous arrangement of structures comprising endocannabinoid substances, enzymes of synthesis and degradation, and cannabinoid receptors. The two principal cannabinoid receptors are CB_1 and CB_2. In humans, CB_1 is mostly expressed in the Central Nervous System (CNS) and the CB_2 is primarily expressed in the immune and hematopoietic systems [47,48]. The CB_1 receptor is the main site of action for the SCRA, including the MDMB-4en-PINACA [9]. Baldwin (1993) demonstrated that the CB_1 receptor belongs to the G-protein coupled receptor (GPCR) family, specifically the rhodopsin family. These structures are well known for their conformational changes after agonists binding, triggering plenty of cellular responses [49]. Several amino acid residues also play a critical role in the maintenance, structure, and functions of these receptors [50]. Lam *et al.* (2006) demonstrated that many amino acid residues in CB_1 receptors are conserved across zebrafish developmental stages, supporting the homology of this receptor between humans and zebrafish [50]. Furthermore, they showed that CB_1 expression is consistently detected in specific regions of the zebrafish embryonic and adult CNS, namely the dorsal telencephalon, pretectum, torus longitudinalis, and periventricular hypothalamus [50].

Among the SCRA, MDMB-4en-PINACA stands out for its high potency in activating CB_1 receptor, sharing some similarities with the psychoactive constituents of *Cannabis* [51,52]. Gu *et al.* (2022) studied the effects of the MDMB-4en-PINACA in the metabolism of adult zebrafish, but the study did not consider the toxic effects of this substance [53]. Our study, on the other hand, was able to elucidate some morphological and behavioral effects of this SCRA in embryos and larvae of zebrafish. For the embryo evaluation, several sublethal effects such as lack of tail detachment, lordosis, pericardial edema, yolk edema, and egg hatching delay were observed between zero and four dpf, as well as lethal endpoints that led to a 30 % mortality rate at the highest concentration. The presence of these effects could emphasize not only the presence of CB_1 receptors in zebrafish embryos but also the severity of MDMB-4en-PINACA toxicity in early life stages. However, the toxicity profile differed when considering zebrafish larvae. Sublethal effects and lethal endpoints were also observed but to a lesser extent. Hypoactivity was the most frequent sublethal effect, observed at 10 μM but only at six dpf.

These results lead us to hypothesize that, for example, the process of developmental alteration caused by MDMB-4en-PINACA ends at five dpf as do many teratogens that cause morphological defects when added to the embryo, but not to the larvae, once initial development has been completed [54–56]. Another plausible hypothesis is that, as the larva develops, it is likely that the biotransformation capacity also improves, which may explain the absence of pronounced effects observed with this

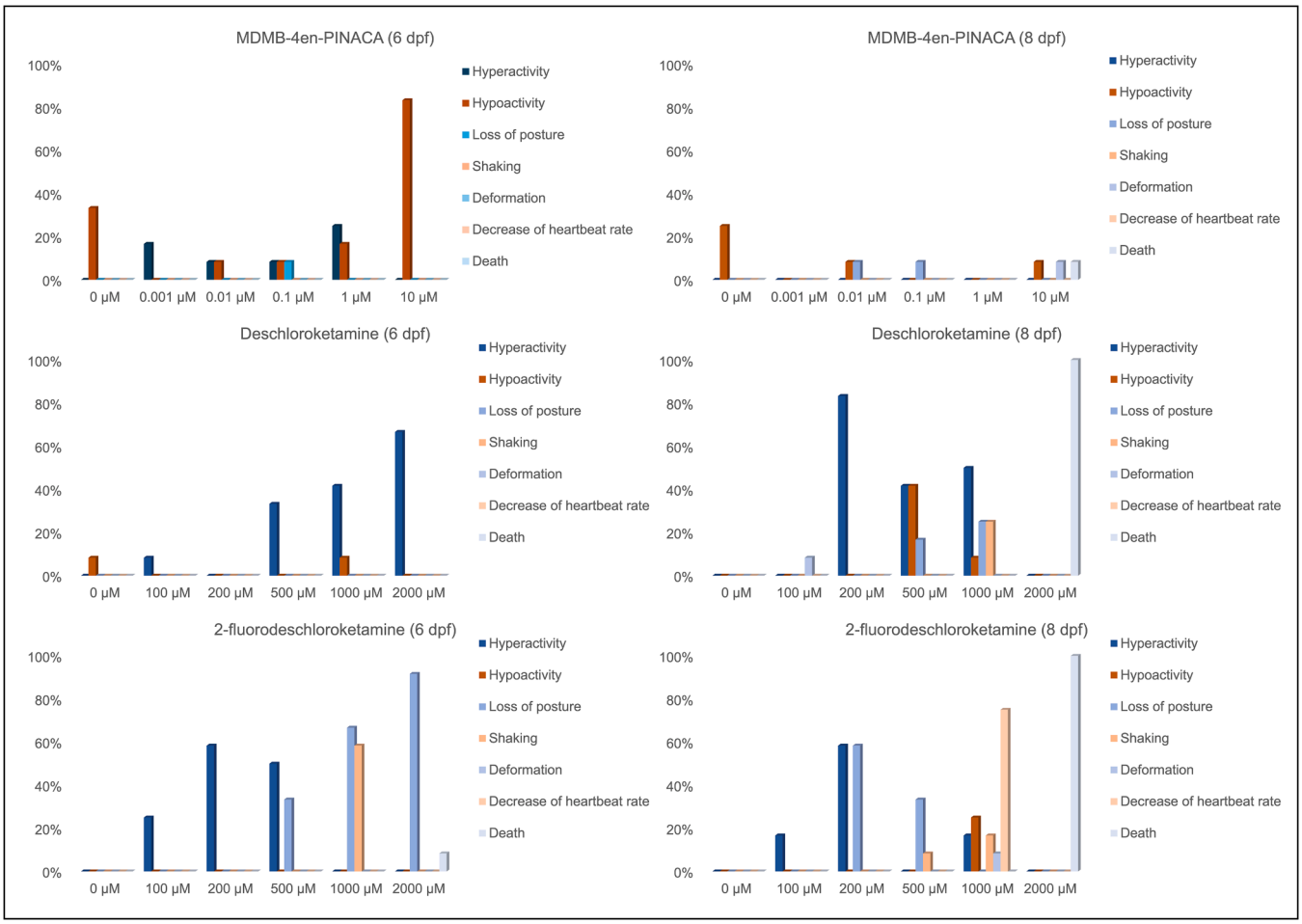

Fig. 4. Lethal and sublethal effects and absence of effects observed for the MDMB-4en-PINACA, deschloroketamine (DCK), and 2-fluorodeschloroketamine (2F-DCK) for zebrafish larvae at six and eight days post-fertilization (dpf).

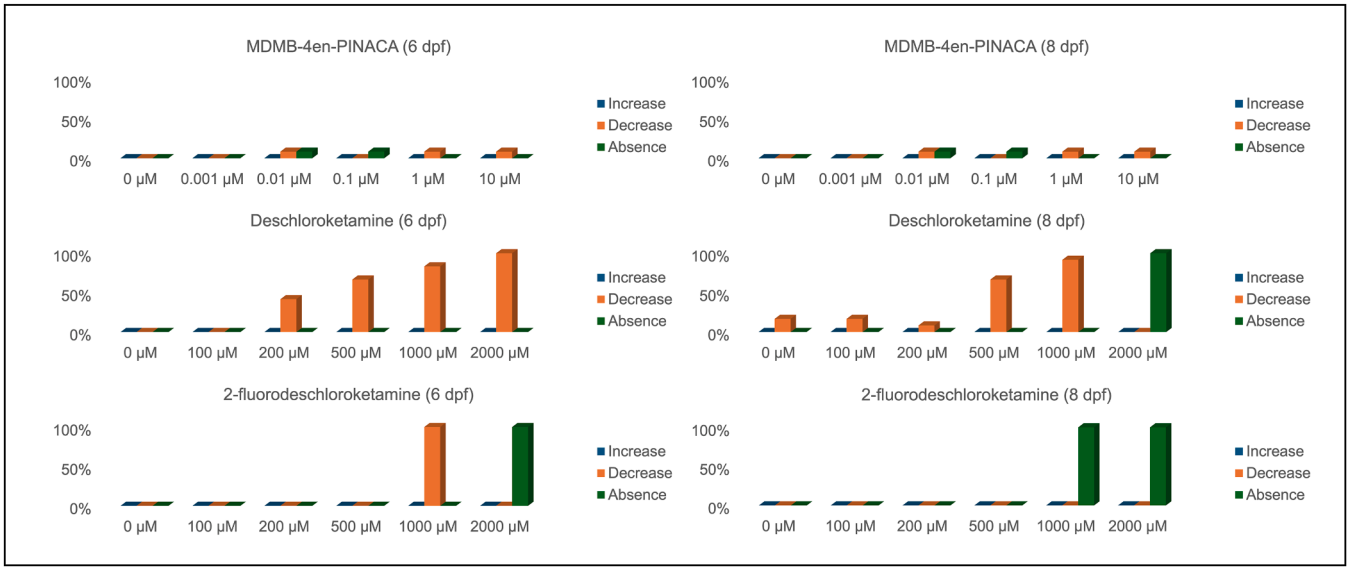

Fig. 5. Touch response observed for the MDMB-4en-PINACA, deschloroketamine (DCK), and 2-fluorodeschloroketamine (2F-DCK) for zebrafish larvae at six and eight days post-fertilization (dpf).

drug in five dpf larvae. Hoyberghs *et al.* (2024) compared the metabolites of anti-epileptics drugs in zebrafish, analyzing both one dpf embryos and five dpf larvae [57]. Their findings demonstrated increased metabolic activity in larvae compared to embryos. Similarly, Le Fol *et al.* investigated the biotransformation of estrogenic contaminants in four dpf zebrafish larvae and adults, reporting a quantitatively more

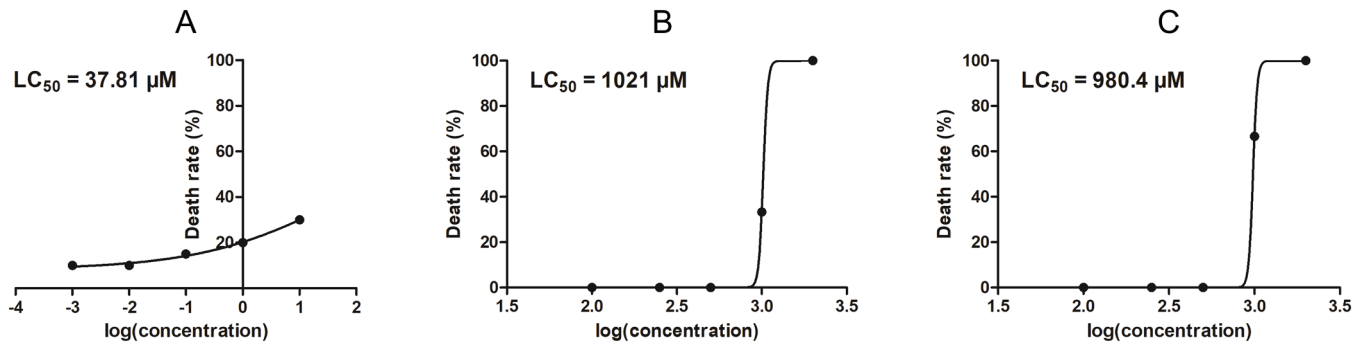

Fig. 6. Mean Lethal Concentration Calculation (LC_{50}) for Fish Embryo Acute Toxicity (FET) test with MDMB-4en-PINACA (A), Maximum Tolerated Concentration (MTC) test with deschloroketamine (DCK) (B), and MTC with 2-fluorodeschloroketamine (2F-DCK) (C).

extensive metabolism in adults than in larvae [58].

It is plausible that MDMB-4en-PINACA has been fully metabolized, resulting in products that do not exhibit significant toxicity to cause notable effects. This hypothesis suggests that the reduction in observed effects in the larvae may be more related to the metabolization process rather than activity at CB_1 receptors. In fact, as reported by Gu *et al.* (2022), 22 metabolites were identified after exposing adult zebrafish to 1 µg/mL MDMB-4en-PINACA for 24 h showing that this model is highly capable of metabolizing this substance [53]. Several other studies demonstrate the metabolism of other SCRA by zebrafish [18,19,37,40, 53,59,60].

Ketamine, a nonbarbiturate dissociative anesthetic, is considered a noncompetitive NMDA receptor antagonist. By its dissociative action, as well as the partial agonism of opiate µ-receptors, ketamine is well used in clinical procedures as an anesthetic and for managing pain [61]. Regarding the NMDA antagonistic action, ketamine can control symptoms of depression and suicidal ideation. Several endogenous effects are observed by ketamine use, such as elevated levels of glutamate and brain-derived neurotrophic factor (BDNF) in the brain. Notably, ketamine could interact with sigma receptors as well as cholinergic, opioid, and monoaminergic systems [61,62]. The human NMDA receptor subunits are encoded by seven genes: *GRIN1*, four *GRIN2*, and two *GRIN3*. These genes encode, respectively, GluN1, GluN2A-D, and GluN3A-B. All known NMDA receptors are obligatory heterotetramers sets of subunits that form a central ion channel pore with a striking resemblance to an inverted potassium channel, being composed of two copies of *GluN1* and *GluN2* [63–65].

Cox *et al.* (2005) studied the presence of NMDA receptor subunits in zebrafish embryos and larvae, showing that the subtypes NMDAR1 and NMDAR2 are present after one dpf, through the detection of their genes (NR, or glutamate ionotropic receptor N-methyl-D-aspartate – GRIN) [66]. Information regarding GRIN gene expression in zebrafish embryos, larvae, and adults has already been described in The Zebrafish Information Network (ZFIN), which was consulted in March 2024. *GRINAA* is the earliest gene expressed in the zygote stage, followed by *GRIN2AA* in the cleavage stage. During the blastula period, in addition to *GRINAA* and *GRIN2AA*, the expression of *GRIN1A* begins but is not sustained in the gastrula stage, when *GRINAA*, *GRINAB*, *GRIN2AA*, *GRIN2AB*, and *GRIN2DA* are expressed. At the segmentation stage, *GRIN1A* is expressed again, as well as GRINAA, *GRINAB*, *GRIN1B*, *GRIN2AA*, *GRIN2AB*, *GRIN2DA*, and *GRIN2DB*. At hatching time, the genes expressed are *GRINAA*, *GRINAB*, *GRIN1A*, *GRIN1B*, *GRIN2AA*, *GRIN2AB, GRIN2BB*, *GRIN2DA*, and *GRIN2DB*. Regarding larval stage, *GRINAA*, *GRINAB*, *GRIN1A*, *GRIN1B*, *GRIN2AA*, *GRIN2AB*, *GRIN2BA*, *GRIN2BB*, *GRIN2CB*, *CRIN2DA*, and *GRIN2DB* are expressed. Surprisingly, at the juvenile stage, only *GRIN2BB* expression was described, whereas in adulthood, only *GRIN1A* and *GRIN1B* were detected. Information related to each gene expression and the stages of zebrafish development are displayed in Table 1 [67].

Since DCK and 2F-DCK are ketamine derivatives from the β-keto-arylcyclohexamines subclass, they are expected to share the same mechanism of action as ketamine, acting as NMDA receptor antagonists, but with greater potency than most NPS [68]. Studies have shown that some effects of these drugs in humans comprise neurological and cardiovascular disorders, consciousness impairment, agitation, abnormal behavior, potentially leading to fatal outcomes [69,70]. Moreover, Kim *et al.* studied the effects of DCK in mice demonstrating not only increased locomotor activation and stereotypy but also rewarding and positive

Table 1
NMDA genes expression in zebrafish stages of life [67]. Zebrafish stages such as blastula, segmentation, pharyngula, hatching, and larval (highlighted in light gray) are those that expresses at least one variation of *GRIN1* and one of *GRIN2*, possibly leading to the expression of proteins such as GluN1 and GluN2A-D, which are essential for NMDA receptor expression.

Gene	Stage of expression									
	Zygote	Cleavage	Blastula	Gastrula	Segmen-tation	Pharyn-gula	Hatching	Larval	Juvenile	Adult
	0–0.74 hpf	0.75–2.24 hpf	2.25–5.24 hpf	5.25–10.32 hpf	10.33–23.99 hpf	24–47.99 hpf	48–71.99 hpf	72 hpf - 29.99 dpf	30–89.99 dpf	90–730 dpf
GRIN1A			X		X	X	X	X		X
GRIN1B						X	X	X		X
GRIN2AA		X	X	X		X	X	X		
GRIN2AB				X	X	X	X	X		
GRIN2BA								X		
GRIN2BB							X	X	X	
GRIN2CB								X		
GRIN2DA				X	X	X	X	X		
GRIN2DB						X	X	X		
grin3a								X		

hpf hours post-fertilization; dpf days post-fertilization.

reinforcing effects, similar to ketamine [71]. Despite the remarkable importance of these drugs, there are no available data in the literature regarding their exposure in the zebrafish model.

Our embryo evaluation results support the findings of Cox *et al.* (2005) showing the absence of the NMDA receptors until one dpf in zebrafish since fewer effects were observed during this period and also the possibility of DCK and 2F-DCK acting at these receptors. Additionally, regarding the potential of NMDA receptors expression at the blastula stage, no significant effects were observed, corroborating the fact that this receptor is continuously expressed only after the segmentation stage.

After one dpf, the expression of NMDA receptors initiates, and, subsequently, we observed an increase in both, sublethal and lethal effects, even in low frequency. In the larvae study, the toxicity profile was different. Several sublethal effects such as loss of posture, shaking behavior, and reduced response to touch were observed. Additionally, lethal endpoints were also noticed, resulting in 100 % mortality for both drugs after eight dpf. Some of these effects could be compared to those reported in humans and could emphasize not only the toxicity of ketamine derivatives in advanced life stages of zebrafish but also that the uncompleted development of NMDA receptors in embryos may have influenced the less toxic effects noticed in early life stages.

Based on the results obtained in this study, we estimated the LC_{50} for MDMB-4en-PINACA in zebrafish embryos and for DCK and 2F-DCK in zebrafish larvae. Although both ketamine derivates led to 100 % mortality in zebrafish larvae, there was a considerable difference in the ranges evaluated. The LC_{50} for MDMB-4en-PINACA was approximately 26 times lower than for the ketamine derivatives, once again, highlighting the severity of intoxications caused by this drug as well as by SCRA. However, LC_{50} comparisons between different tests should be interpreted cautiously, as they may reflect differences in experimental design including exposure duration, developmental stage, and evaluated endpoints.

Furthermore, with the MTC studies, it is also possible to establish a safe concentration to work with each of these three drugs. This is defined as the highest drug concentration without significant effects observed during each time evaluated. For six dpf, 1 μM, 2000 μM, and 1000 μM for MDMB-4en-PINACA, DCK, and 2F-DCK, respectively. At eight dpf, 10 μM, 1000 μM, and 500 μM for MDMB-4en-PINACA, DCK, and 2F-DCK, respectively. Further studies must be developed considering the metabolic evaluation of these drugs and also the toxicity of their metabolites in zebrafish embryos and larvae. Also, molecular clarifications of the data here discussed could be a reliable tool to fully comprehend the mechanism of action of these NPS.

By using zebrafish for the investigation of NPS and applying the FET and MTC assays, we present a practical, cost-effective, ethical, and high-throughput approach for toxicity screening. Both the methodologies employed in our study aligns with the 3Rs principle (Replacement, Reduction, and Refinement), mainly by reducing the use of mammalian models in toxicology assays, as well as supporting the use of more humane and sustainable testing strategies. Our findings contribute with toxicological data that can enhance the understanding the risks associated with NPS consumption, specifically MDMB-4en-PINACA, DCK, and 2F-DCK, and further the promotion of risk assessments and regulatory policies for these substances. Furthermore, this study provides valuable data both for forensic and clinical toxicology, offering insights that may assist in drug monitoring efforts, intoxication cases, and the development of future regulations aiming NPS exposure management. While our study primarily focuses on evaluating the acute toxicity of selected NPS using zebrafish as an *in vivo* model, it is important to acknowledge that the presence of these substances in wastewater and the environment may pose broader ecological and health risks, and further investigations regarding these implications should be pursued.

5. Conclusions

The emergence of new psychoactive substances (NPS) over the last two decades has posed a significant health issue. Among these, the SCRA and ketamine derivatives classes include different substances with several cases already reported in the literature, such as MDMB-4en-PINACA, DCK, and 2F-DCK. In this study we evaluated the acute toxicity of these three NPS both in embryos and larvae, reinforcing the applicability of this model for assessing NPS toxicity. Additionally, our study identified distinct morphological and behavioral effects induced by these NPS. Specifically, embryonic exposure to MDMB-4en-PINACA resulted in significant developmental abnormalities, including lack of heartbeat, lack of somite formation, pericardial edema, and yolk edema. Larval exposure to the ketamine derivatives (DCK and 2F-DCK) resulted in pronounced behavioral alterations, such as decreased touch response and shaking behavior. Moreover, with the MTC studies, it is also possible to establish a safe concentration to investigate deeper each of these three drugs using zebrafish embryos and larvae. Finally, we estimated the LC_{50} for MDMB-4en-PINACA in zebrafish embryos and for DCK and 2F-DCK in zebrafish larvae, highlighting the severity of intoxications caused by SCRA.

It is important to acknowledge the limitations of this study. The protocol was based on a single experimental replicate, which may limit the reproducibility of the findings. While the sample size ($n = 20$ per group) was adequate for statistical analyses, future studies should incorporate additional replicates to enhance robustness and validate the observed effects. In addition, LC_{50} comparisons between different tests (FET and MTC) should be interpreted with caution, as differences in exposure duration, developmental stage, and evaluated endpoints may influence the results. While these comparisons provide valuable insights into relative toxicity, future research should aim to standardize exposure conditions across different zebrafish life stages to ensure a more direct comparability of toxic effects.

Ethical approval

All experiments described in this study were reviewed and approved by the Ethical Committee for Animal Research for the Universidade Estadual de Campinas (6096–1/2022 and 6096–1(A)/2023).

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CRediT authorship contribution statement

Costa Jose Luiz: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Maurer-Morelli Claudia Vianna:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Peterson Randall Theodore:** Writing – review & editing, Writing – original draft. **Fais Viviane Cristina:** Methodology, Investigation, Formal analysis, Conceptualization. **Godoi Alexandre Barcia de:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Rodrigues Leonardo Costalonga:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Leonardo Costalonga Rodrigues reports financial support was provided

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2025.102018.

Data availability

The data underlying this article are available in the article.

References

[1] E. Marchei, S. Graziano, M.R. Varì, A. Minutillo, V. Aquilina, S. Pichini, M. C. Rotolo, NPS–EQA PART I: four years' experience in external quality assessment program in Italy for classical and new psychoactive substances analysis in oral fluid, J. Pharm. Biomed. Anal. (2024) 116084, https://doi.org/10.1016/j.jpba.2024.116084.

[2] EMCDDA, New psychoactive substances – the current situation in Europe (European Drug Report 2023), European Drug Report 2023: Trends and Developments 2022 (2023) 1–7. ⟨https://www.emcdda.europa.eu/publications/european-drug-report/2023_en⟩.

[3] UNODC, Current NPS Threats, United Nations Office on Drugs and Crime VI (2023). ⟨https://www.unodc.org/res/scientists/ewa/Current_NPS_Threats_VI.pdf⟩.

[4] S. Graziano, S. Zaami, R. Tittarelli, S. Pichini, M. Sciotti, P. Barretta, F.P. Busardò, E. Marinelli, A. Tini, G. Scaravaggi, E. Buscaglia, E. Brambilla, M. Cavallo, C. A. Locatelli, R. Pacifici, M.R. Varì, The complex and constantly evolving public health threat of new psychoactive substances in Italy: addressing the main functions of a national observatory of drugs, Ann. Ist. Super. Sanita 57 (2021) 144–150, https://doi.org/10.4415/ANN_21_02_06.

[5] J. Meijer, M. Lamoree, T. Hamers, J.-P. Antignac, S. Hutinet, L. Debrauwer, A. Covaci, C. Huber, M. Krauss, D.I. Walker, E.L. Schymanski, R. Vermeulen, J. Vlaanderen, An annotation database for chemicals of emerging concern in exposome research, Environ. Int 152 (2021) 106511, https://doi.org/10.1016/j.envint.2021.106511.

[6] M. Pourchet, L. Debrauwer, J. Klanova, E.J. Price, A. Covaci, N. Caballero-Casero, H. Oberacher, M. Lamoree, A. Damont, F. Fenaille, J. Vlaanderen, J. Meijer, M. Krauss, D. Sarigiannis, R. Barouki, B. Le Bizec, J.-P. Antignac, Suspect and non-targeted screening of chemicals of emerging concern for human biomonitoring, environmental health studies and support to risk assessment: from promises to challenges and harmonisation issues, Environ. Int 139 (2020) 105545, https://doi.org/10.1016/j.envint.2020.105545.

[7] R. Gerona, NPS pharmacology and toxicology. Designer Drugs, Elsevier, 2024, pp. 65–98, https://doi.org/10.1016/B978-0-12-811764-4.00013-6.

[8] Nacionalni Forenzicni Laboratorij, Analytical Report: MDMB-4en-PINACA, 2018.

[9] EMCDDA, Initial report on the new psychoactive substance methyl 3,3-dimethyl-2-(1-(pent-4-en-1-yl)-1H- indazole-3-carboxamido)butanoate (MDMB-4en-PINACA), European Union (2020). ⟨https://www.drugsandalcohol.ie/33289/1/emcdda-initial-report-MDMB-4en-PINACA⟩.

[10] The Center for Forensic Science Research & Education, Q4 2023 Trend Reports, 2023.

[11] T.B. Rodrigues, M.P. Souza, L. de Melo Barbosa, J. de Carvalho Ponce, L.F. N. Júnior, M. Yonamine, J.L. Costa, Synthetic cannabinoid receptor agonists profile in infused papers seized in Brazilian prisons, Forensic Toxicol. 40 (2022) 119–124, https://doi.org/10.1007/s11419-021-00586-7.

[12] UNODC, Phencyclidine-type substances, n.d. ⟨https://www.unodc.org/LSS/SubstanceGroup/Details/6bf165ed-82e7-47e0-9eaa-daacc42d99cd⟩.

[13] K. Štefková-Mazochová, H. Danda, W. Dehaen, B. Jurásek, K. Šíchová, N. Pinterová-Leca, V. Mazoch, B.H. Krausová, B. Kysilov, T. Smejkalová, L. Vyklický, M. Kohout, K. Hájková, D. Svozil, R.R. Horsley, M. Kuchař, T. Páleníček, Pharmacokinetic, pharmacodynamic, and behavioural studies of deschloroketamine in Wistar rats, Br. J. Pharm. 179 (2022) 65–83, https://doi.org/10.1111/bph.15680.

[14] G. Frison, L. Zamengo, F. Zancanaro, F. Tisato, P. Traldi, Characterization of the designer drug deschloroketamine (2-methylamino-2-phenylcyclohexanone) by gas chromatography/mass spectrometry, liquid chromatography/high-resolution mass spectrometry, multistage mass spectrometry, and nuclear magnetic resonance, Rapid Commun. Mass Spectrom. 30 (2016) 151–160, https://doi.org/10.1002/rcm.7425.

[15] A. Moghimi, S. Rahmani, R. Zare, M. Sadeghzadeh, Synthesis of 2-(2-fluorophenyl)-2-methylamino-cyclohexanone as a new ketamine derivative, Synth. Commun. 44 (2014) 2021–2028, https://doi.org/10.1080/00397911.2014.885053.

[16] EMCDDA, European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). EMCDDA-Europol 2015 Annual Report on the implementation of Council Decision 2005/387/JHA, Europol (2016) 9–15. ⟨http://www.emcdda.europa.eu/system/files/publications/4724/TDAN17001ENN_PDFWEB.pdf⟩.

[17] World Health Organization, Critical Review Report: 2-fluorodeschloroketamine, 2023 8–9. ⟨https://cdn.who.int/media/docs/default-source/46th-ecdd/2-fdck_46th-ecdd-critical-review_public-version.pdf?sfvrsn= 5c6e5f45_1⟩.

[18] P.A. Markin, A. Brito, N.E. Moskaleva, F. Tagliaro, M.R. La Frano, M.V. Savitskii, S. A. Appolonova, Short- and long-term exposures of the synthetic cannabinoid 5F-APINAC induce metabolomic alterations associated with neurotransmitter systems and embryotoxicity confirmed by teratogenicity in zebrafish, Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 243 (2021) 109000, https://doi.org/10.1016/j.cbpc.2021.109000.

[19] H. Hei, J. fen Wang, W. fang Zhang, Y. yan Wang, Duo-qi Xu, Study of the in vitro and in vivo metabolism of a novel synthetic cannabinoid PX-2 in human liver microsomes and zebrafish, Int J. Mass Spectrom. 456 (2020), https://doi.org/10.1016/j.ijms.2020.116388.

[20] E. Fan, Z. Xu, J. Yan, F. Wang, S. Sun, Y. Zhang, S. Zheng, X. Wang, Y. Rao, Acute exposure to N-Ethylpentylone induces developmental toxicity and dopaminergic receptor-regulated aberrances in zebrafish larvae, Toxicol. Appl. Pharm. 417 (2021) 115477, https://doi.org/10.1016/j.taap.2021.115477.

[21] J. Cachat, P. Canavello, M. Elegante, B. Bartels, P. Hart, C. Bergner, R. Egan, A. Duncan, D. Tien, A. Chung, K. Wong, J. Goodspeed, J. Tan, C. Grimes, S. Elkhayat, C. Suciu, M. Rosenberg, K.M. Chung, F. Kadri, S. Roy, S. Gaikwad, A. Stewart, I. Zapolsky, T. Gilder, S. Mohnot, E. Beeson, H. Amri, Z. Zukowska, R. D. Soignier, A.V. Kalueff, Modeling withdrawal syndrome in zebrafish, Behav. Brain Res. 208 (2010) 371–376, https://doi.org/10.1016/j.bbr.2009.12.004.

[22] L.J.M. Kily, Y.C.M. Cowe, O. Hussain, S. Patel, S. McElwaine, F.E. Cotter, C. H. Brennan, Gene expression changes in a zebrafish model of drug dependency suggest conservation of neuro-adaptation pathways, J. Exp. Biol. 211 (2008) 1623–1634, https://doi.org/10.1242/jeb.014399.

[23] G.D. Bossé, R.T. Peterson, Development of an opioid self-administration assay to study drug seeking in zebrafish, Behav. Brain Res. 335 (2017) 158–166, https://doi.org/10.1016/j.bbr.2017.08.001.

[24] W. Li, B. Song, Z. Zeng, Z. Yang, F. Li, S. He, J. Tong, Y. Chen, C. Zhang, D. Wang, Z. Li, W. Xiong, Exploring micro(nano)plastics toxicity from an environmental management perspective: Zebrafish as a vital bridge for assessing potential human health risks, J. Environ. Manag. 373 (2025) 123934, https://doi.org/10.1016/j.jenvman.2024.123934.

[25] M. Ferrante, P. Rapisarda, A. Grasso, C. Favara, G. Oliveri Conti, Glyphosate and environmental toxicity with "One Health" approach, a review, Environ. Res 235 (2023) 116678, https://doi.org/10.1016/j.envres.2023.116678.

[26] S. Elliott, R. Sedefov, M. Evans-Brown, Assessing the toxicological significance of new psychoactive substances in fatalities, Drug Test. Anal. 10 (2018) 120–126, https://doi.org/10.1002/dta.2225.

[27] P. Brandeburová, I. Bodík, I. Horáková, D. Žabka, S. Castiglioni, N. Salgueiro-González, E. Zuccato, V. Špalková, T. Mackuľak, Wastewater-based epidemiology to assess the occurrence of new psychoactive substances and alcohol consumption in Slovakia, Ecotoxicol. Environ. Saf. 200 (2020) 110762, https://doi.org/10.1016/j.ecoenv.2020.110762.

[28] R. Bade, D. Nadarajan, E.M. Driver, R.U. Halden, C. Gerber, A. Krotulski, W. Hall, J.F. Mueller, Wastewater-based monitoring of the nitazene analogues: first detection of protonitazene in wastewater, Sci. Total Environ. 920 (2024), https://doi.org/10.1016/j.scitotenv.2024.170781.

[29] D. Nadarajan, J. O'Brien, S. Cresswell, B. Kele, J. Mueller, R. Bade, Application of design of experiment for quantification of 71 new psychoactive substances in influent wastewater, Anal. Chim. Acta 1321 (2024), https://doi.org/10.1016/j.aca.2024.343036.

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

[31] T. Braunbeck, B. Kais, E. Lammer, J. Otte, K. Schneider, D. Stengel, R. Strecker, The fish embryo test (FET): origin, applications, and future, Environ. Sci. Pollut. Res. 22 (2014) 16247–16261, https://doi.org/10.1007/s11356-014-3814-7.

[32] W.A. de Barros, C. da, S. Nunes, J.A. da, C.R. Souza, I.J. dos, S. Nascimento, I. M. Figueiredo, T.M. de Aquino, L. Vieira, D. Farias, J.C.C. Santos, Â. de Fátima, The new psychoactive substances 25H-NBOMe and 25H-NBOH induce abnormal development in the zebrafish embryo and interact in the DNA major groove, Curr. Res Toxicol. 2 (2021) 386–398, https://doi.org/10.1016/j.crtox.2021.11.002.

[33] K.T. Kirla, K.J. Groh, M. Poetzsch, R.K. Banote, J. Stadnicka-Michalak, R.I. L. Eggen, K. Schirmer, T. Kraemer, Importance of toxicokinetics to assess the utility of zebrafish larvae as model for psychoactive drug screening using meta-chlorophenylpiperazine (mCPP) as example, Front Pharm. 9 (2018) 1–12, https://doi.org/10.3389/fphar.2018.00414.

[34] K.T. Kirla, C. Erhart, K.J. Groh, J. Stadnicka-Michalak, R.I.L. Eggen, K. Schirmer, T. Kraemer, Zebrafish early life stages as alternative model to study 'designer drugs': concordance with mammals in response to opioids, Toxicol. Appl. Pharm. 419 (2021) 115483, https://doi.org/10.1016/j.taap.2021.115483.

[35] A. Kurach, B. Chłopaś-Konowałek, M. Budzyńska, P. Zawadzki, A. Szpot, Boguszewska-Czubara, Etazene induces developmental toxicity in vivo Danio rerio and in silico studies of new synthetic opioid derivative, Sci. Rep. 11 (2021) 1–11, https://doi.org/10.1038/s41598-021-03804-9.

[36] V.B. Maphanga, K. Skalicka-Wozniak, B. Budzynska, A. Skiba, W. Chen, C. Agoni, G.M. Enslin, A.M. Viljoen, Mesembryanthemum tortuosum L. alkaloids modify anxiety-like behaviour in a zebrafish model, J. Ethnopharmacol. 290 (2022) 115068, https://doi.org/10.1016/j.jep.2022.115068.

[37] L. Wagmann, F. Frankenfeld, Y.M. Park, J. Herrmann, S. Fischmann, F. Westphal, R. Müller, V. Flockerzi, M.R. Meyer, How to study the metabolism of new psychoactive substances for the purpose of toxicological screenings—a follow-up study comparing pooled human liver S9, HepaRG cells, and Zebrafish Larvae, Front. Chem. 8 (2020) 1–15, https://doi.org/10.3389/fchem.2020.00539.
[38] T.M. Gampfer, L. Wagmann, Y.M. Park, A. Cannaert, J. Herrmann, S. Fischmann, F. Westphal, R. Müller, C.P. Stove, M.R. Meyer, Toxicokinetics and toxicodynamics of the fentanyl homologs cyclopropanoyl-1-benzyl-4′-fluoro-4-anilinopiperidine and furanoyl-1-benzyl-4-anilinopiperidine, Arch. Toxicol. 94 (2020) 2009–2025, https://doi.org/10.1007/s00204-020-02726-1.
[39] Y.M. Park, M.R. Meyer, R. Müller, J. Herrmann, Drug administration routes impact the metabolism of a synthetic cannabinoid in the zebrafish larvae model, Molecules 25 (2020), https://doi.org/10.3390/molecules25194474.
[40] L.H.J. Richter, J. Herrmann, A. Andreas, Y.M. Park, L. Wagmann, V. Flockerzi, R. Müller, M.R. Meyer, Tools for studying the metabolism of new psychoactive substances for toxicological screening purposes – a comparative study using pooled human liver S9, HepaRG cells, and zebrafish larvae, Toxicol. Lett. 305 (2019) 73–80, https://doi.org/10.1016/j.toxlet.2019.01.010.
[41] K. Gu, S. Qin, Y. Zhang, W. Zhang, G. Xin, B. Shi, J. Wang, Y. Wang, J. Lu, Metabolic profiles and screening tactics for MDMB-4en-PINACA in human urine and serum samples, J. Pharm. Biomed. Anal. 220 (2022), https://doi.org/10.1016/j.jpba.2022.114985.
[42] S.Y. Li, W.J. Shi, D.D. Ma, J.G. Zhang, Z.J. Lu, X.B. Long, X. Liu, C.S. Huang, G. G. Ying, Effects of new psychoactive substance esketamine on behaviors and transcription of genes in dopamine and GABA pathways in Zebrafish Larvae, Bull. Environ. Contam. Toxicol. 112 (2024), https://doi.org/10.1007/s00128-024-03883-0.
[43] L. Xu, H. Yan, Y. Tang, Y. Liu, P. Xiang, T. Hang, In vitro and in vivo metabolic study of three new psychoactive β-keto-arylcyclohexylamines, J. Anal. Toxicol. 48 (2024) 217–225, https://doi.org/10.1093/jat/bkae020.
[44] L.M. Félix, A.M. Vidal, C. Serafim, A.M. Valentim, L.M. Antunes, S. Campos, M. Matos, S.M. Monteiro, A.M. Coimbra, Ketamine-induced oxidative stress at different developmental stages of zebrafish (Danio rerio) embryos, RSC Adv. 6 (2016) 61254–61266, https://doi.org/10.1039/C6RA08298J.
[45] L.M. Félix, C. Serafim, A.M. Valentim, L.M. Antunes, M. Matos, A.M. Coimbra, Apoptosis-related genes induced in response to ketamine during early life stages of zebrafish, Toxicol. Lett. 279 (2017) 1–8, https://doi.org/10.1016/j.toxlet.2017.07.888.
[46] C. Santos, A.M. Valentim, L. Félix, J. Balça-Silva, M.L. Pinto, Longitudinal effects of ketamine on cell proliferation and death in the CNS of zebrafish, Neurotoxicology 97 (2023) 78–88, https://doi.org/10.1016/j.neuro.2023.05.008.
[47] K.M. Jung, D. Piomelli, Lipids: The endocannabinoid system, Encyclopedia of Biological Chemistry: Third Edition 2, 2021. 776–784. https://doi.org/10.1016/B978-0-12-809633-8.21365-4.
[48] E. Benarroch, Endocannabinoids in basal ganglia circuits: implications for Parkinson disease, Neurology 69 (2007) 306–309, https://doi.org/10.1212/01.wnl.0000267407.79757.75.
[49] J.M. Baldwin, The probable arrangement of the helices in G protein-coupled receptors, EMBO J. 12 (1993) 1693–1703, https://doi.org/10.1002/j.1460-2075.1993.tb05814.x.
[50] C.S. Lam, S. Rastegar, U. Strähle, Distribution of cannabinoid receptor 1 in the CNS of zebrafish, Neuroscience 138 (2006) 83–95, https://doi.org/10.1016/j.neuroscience.2005.10.069.
[51] L.H. Antonides, A. Cannaert, C. Norman, N. NicDáeid, O.B. Sutcliffe, C.P. Stove, C. McKenzie, Shape matters: the application of activity-based in vitro bioassays and chiral profiling to the pharmacological evaluation of synthetic cannabinoid receptor agonists in drug-infused papers seized in prisons, Drug Test. Anal. 13 (2021) 628–643, https://doi.org/10.1002/dta.2965.
[52] A.J. Krotulski, A. Cannaert, C. Stove, B.K. Logan, The next generation of synthetic cannabinoids: detection, activity, and potential toxicity of pent-4en and but-3en analogues including MDMB-4en-PINACA, Drug Test. Anal. 13 (2021) 427–438, https://doi.org/10.1002/dta.2935.
[53] K. Gu, S. Qin, Y. Zhang, W. Zhang, G. Xin, B. Shi, J. Wang, Y. Wang, J. Lu, Metabolic profiles and screening tactics for MDMB-4en-PINACA in human urine and serum samples, J. Pharm. Biomed. Anal. 220 (2022) 114985, https://doi.org/10.1016/j.jpba.2022.114985.
[54] S. Liu, H. Lai, Q. Wang, R. Martínez, M. Zhang, Y. Liu, J. Huang, M. Deng, W. Tu, Immunotoxicity of F 53B, an alternative to PFOS, on zebrafish (Danio rerio) at different early life stages, Sci. Total Environ. 790 (2021) 148165, https://doi.org/10.1016/j.scitotenv.2021.148165.
[55] Q. Li, P. Wang, L. Chen, H. Gao, L. Wu, Acute toxicity and histopathological effects of naproxen in zebrafish (Danio rerio) early life stages, Environ. Sci. Pollut. Res. 23 (2016) 18832–18841, https://doi.org/10.1007/s11356-016-7092-4.
[56] M.N. Calienni, D.A. Feas, D.E. Igartúa, N.S. Chiaramoni, S. del V. Alonso, M. J. Prieto, Nanotoxicological and teratogenic effects: a linkage between dendrimer surface charge and zebrafish developmental stages, Toxicol. Appl. Pharm. 337 (2017) 1–11, https://doi.org/10.1016/j.taap.2017.10.003.
[57] J. Hoyberghs, A. Coppens, C. Bars, C. Van Ginneken, K. Foubert, S. Van Cruchten, Assessing developmental toxicity and non-CYP mediated biotransformation of two anti-epileptics and their human metabolites in zebrafish embryos and larvae, Curr. Res Toxicol. 7 (2024) 100186, https://doi.org/10.1016/j.crtox.2024.100186.
[58] V. Le Fol, F. Brion, A. Hillenweck, E. Perdu, S. Bruel, S. Aït-Aïssa, J.-P. Cravedi, D. Zalko, Comparison of the in vivo biotransformation of two emerging estrogenic contaminants, BP2 and BPS, in Zebrafish Embryos and Adults, Int J. Mol. Sci. 18 (2017) 704, https://doi.org/10.3390/ijms18040704.
[59] W. Xinze Liu, P. Liu, T. Xiang, Y. Hang, L. Shi, H. Yue, Yan, Metabolism of ADB-4en-PINACA in Zebrafish and rat liver microsomes determined by liquid chromatography–high resolution mass spectrometry, J. Anal. Chem. 77 (2022) 1036–1046, https://doi.org/10.1134/S1061934822080184.
[60] A. Morales-Noé, F.A. Esteve-Turrillas, S. Armenta, Metabolism of third generation synthetic cannabinoids using zebrafish larvae, Drug Test. Anal. 14 (2022) 594–603, https://doi.org/10.1002/dta.3195.
[61] S.B. Rosenbaum, V. Gupta, P. Patel, J.L. Palacios, Ketamine (2024) 1–12.
[62] C.F. Zorumski, Y. Izumi, S. Mennerick, Ketamine: NMDA receptors and beyond, J. Neurosci. 36 (2016) 11158–11164, https://doi.org/10.1523/JNEUROSCI.1547-16.2016.
[63] E. Karakas, H. Furukawa, Crystal structure of a heterotetrameric NMDA receptor ion channel, Science 344 (1979) (2014) 992–997, https://doi.org/10.1126/science.1251915.
[64] K.B. Hansen, F. Yi, R.E. Perszyk, H. Furukawa, L.P. Wollmuth, A.J. Gibb, S. F. Traynelis, Structure, function, and allosteric modulation of NMDA receptors, J. Gen. Physiol. 150 (2018) 1081–1105, https://doi.org/10.1085/jgp.201812032.
[65] S.F. Traynelis, L.P. Wollmuth, C.J. McBain, F.S. Menniti, K.M. Vance, K.K. Ogden, K.B. Hansen, H. Yuan, S.J. Myers, R. Dingledine, Glutamate receptor ion channels: structure, regulation, and function, Pharmacol. Rev. 62 (2010) 405–496, https://doi.org/10.1124/pr.109.002451.
[66] J.A. Cox, S. Kucenas, M.M. Voigt, Molecular characterization and embryonic expression of the family of N-methyl-D-aspartate receptor subunit genes in the zebrafish, Dev. Dyn. 234 (2005) 756–766, https://doi.org/10.1002/dvdy.20532.
[67] Y.M. Bradford, C.E. Van Slyke, L. Ruzicka, A. Singer, A. Eagle, D. Fashena, D. G. Howe, K. Frazer, R. Martin, H. Paddock, C. Pich, S. Ramachandran, M. Westerfield, Zebrafish information network, the knowledgebase for Danio rerio research, Genetics 220 (2022), https://doi.org/10.1093/genetics/iyac016.
[68] S. Mestria, S. Odoardi, G. Biosa, V. Valentini, G. Di Masi, F. Cittadini, S. Strano-Rossi, Method development for the identification of methoxpropamine, 2-fluoro-deschloroketamine and deschloroketamine and their main metabolites in blood and hair and forensic application, Forensic Sci. Int 323 (2021) 110817, https://doi.org/10.1016/j.forsciint.2021.110817.
[69] M.H.Y. Tang, T.C. Li, C.K. Lai, Y.K. Chong, C.K. Ching, T.W.L. Mak, Emergence of new psychoactive substance 2-fluorodeschloroketamine: toxicology and urinary analysis in a cluster of patients exposed to ketamine and multiple analogues, Forensic Sci. Int. 312 (2020), https://doi.org/10.1016/j.forsciint.2020.110327.
[70] T. Gicquel, C. Richeval, V. Mesli, A. Gish, F. Hakim, R. Pelletier, R. Cornez, A. Balgairies, D. Allorge, J. Gaulier, Fatal intoxication related to two new arylcyclohexylamine derivatives (2F-DCK and 3-MeO-PCE), Forensic Sci. Int 324 (2021) 110852, https://doi.org/10.1016/j.forsciint.2021.110852.
[71] J.M. Kim, B. Han, H.K. Min, J. Yun, Y.H. Kim, S.O. Choi, M.S. Kang, Rewarding and reinforcing effects of two dissociative-based new psychoactive substances, deschloroketamine and diphenidine, in mice, Pharmacol. Biochem Behav. 213 (2022), https://doi.org/10.1016/j.pbb.2022.173333.