Evaluation of Synthetic Cannabinoid Receptor Agonists Toxicity in Zebrafish (Danio rerio) through Self-Administration and Fishbook Assays

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Abstract

New psychoactive substances (NPS) represent an emerging public health concern, with synthetic cannabinoid receptor agonists (SCRAs) among the most prevalent classes worldwide. Although widely reported in seizures and toxicological casework, their pharmacology and toxicity remain poorly characterized. In this study, we investigated the acute toxicity and behavioral effects of two emerging SCRAs—ADB-INACA and CHO-4'Me-5'Br-FUBOXPYRA—using zebrafish (*Danio rerio*) as an *in vivo* model. Fish embryo acute toxicity (FET) and maximum tolerated concentration (MTC) assays were performed to assess lethality and sublethal effects in early developmental stages. Social behavior was evaluated at 21 days post-fertilization (dpf) using the Fishbook assay, and drug-seeking behavior was investigated in adult zebrafish through a self-administration paradigm. Both SCRAs produced low embryonic mortality across the tested range (0.001–10 μM). CHO-4'Me-5'Br-FUBOXPYRA induced sublethal effects including pericardial edema, loss of posture, and reduced heart rate at higher concentrations, whereas ADB-INACA produced mainly impaired escape responses without significant lethality. Neither compound significantly altered social interaction in juvenile zebrafish, and CHO-4'Me-5'Br-FUBOXPYRA failed to elicit reinforcing effects in adult zebrafish, in contrast to the hydrocodone positive control. Taken together, these results indicate that both SCRAs exhibit limited cannabinoid-like effects at the tested concentrations, with only modest cardiotoxic findings for CHO-4'Me-5'Br-FUBOXPYRA. This study highlights the applicability of zebrafish assays, including Fishbook and self-administration paradigms, for investigating the toxicological and behavioral properties of novel NPS.

1 INTRODUCTION

According to United Nations Office on Drugs and Crime (UNODC), new psychoactive substances (NPS) are defined as compounds that can pose a threat to public health and are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances [1, 2]. From chemical and structural modifications on traditional illicit drugs, NPS are essentially designed to mimic its effects while not being under control by either national or international legislations [3].

Also known as legal highs and research chemicals, in the last ten years, a growing worldwide epidemic of production and use of NPS has been described, considering the significant increase in both [3, 4]. The European Union Drugs Agency (EUDA) was monitoring 1000 NPS in Europe by the end of 2024, from which 47 were first reported [5]. Between 2012 and 2025, 1391 NPS were reported in 152 countries and territories by UNODC Early Warning Advisory (EWA) [1].

NPS can be grouped considering different characteristics, with pharmacological classification being the most used [6]. In addition to this classification, the UNODC EWA also applies chemical structure similarities to group NPS, and the most common groups, by effect, are stimulants, synthetic cannabinoid receptor agonists, classic hallucinogens, synthetic opioids, sedative/hypnotics, and dissociatives [1]. Between 2018 and 2024, the NPS Discovery, a program from the Center for Forensic Science Research and Education (CFSRE), identified 174 NPS for the first time in the US, including 20 in 2024 alone [7].

Synthetic cannabinoid receptor agonists (SCRA), also described as synthetic cannabinoids or cannabimimetics, are the second most detected class of NPS worldwide, only after stimulants [1, 7]. This class contain structural groups that can bind to cannabinoid receptors type 1 (CB_1) and type 2 (CB_2), leading to effects similar to delta-9-tetrahydrocannabinol (THC). However, despite structurally differing from THC, a partial CB receptor agonist, SCRA often presents more potent and efficacious binding to CB_1 and CB_2 receptors [4].

SCRA are the largest and most challenging group of NPS due to their adverse effects, limited safety profile, evolving structure, and diversity. Frequently sold as herbal mixtures named as “Spice” and “K2”, its use was popularized as a “legal” and inexpensive alternative to *Cannabis* [4, 8]. SCRA consumption has been associated with diverse severe adverse effects, such as psychosis, delirium, cardiotoxicity, seizures, acute kidney injury, and hypothermia, implicated in several deaths worldwide [4, 9, 10]. The diversity and rapid appearance of SCRA represents a challenge for clinical and forensic toxicologists, since its pharmacology, toxicity, and abuse liability pose a threat to public health [11]. In addition, because many abused SCRAs are unknown before they are detected by forensic scientists, their effects on humans are poorly or not known at all [10, 11].

Among SCRA, N-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1H-indazole-3-carboxamide (ADB-INACA) evolved as a legal alternative to controlled substances such as indazole carboxamide type SCRAs with N-alkyl chains. ADB-INACA is a tail-less precursor to other SCRAs such as ADB-BINACA (ADB-BUTINACA), and its potency is expected to be low. Since 2023, the NPS Discovery has reported nine cases of detection of ADB-INACA in drug materials, while seven reports in toxicological specimens [12–14].

N-[5-bromo-1-[(4-fluorophenyl)methyl]-4-methyl-2-oxo-3-pyridyl]cyclohexanecarboxamide (CHO-4'Me-5'Br-FUBOXPYRA, CH-FUBBMPDORA, 6TP/SGT) is a novel SCRA recent added to the recreational market to bypass the Chinese generic ban of 2021 based on its new 5-bromo-4-methylpyridin-2(1H)-one core. CHO-4'Me-5'Br-FUBOXPYRA was first identified in Europe in 2022, being subsequently found in Germany, Italy, Slovenia, Sweden, United States, Lithuania, Romania, and Spain. In 2024, the NPS Discovery has reported one case of detection of CHO-4'Me-5'Br-FUBOXPYRA in toxicological specimen, after its first US identification in drug material in 2023 [15, 16].

Human research targeting NPS is limited due to safety and ethical concerns, and animal models have been utilized for these types of studies. Recently, zebrafish (*Danio rerio*) models have been increasingly utilized in toxicological studies involving NPS [17]. The aim of our study was to investigate the acute toxicity of ADB-INACA and CHO-4'Me-5'Br-FUBOXPYRA in both zebrafish embryos and larvae and evaluate the effects of SCRA on social interaction on juvenile larvae and its potential to led to self-administration in adult zebrafish. To the best of our knowledge, this is the first study to assess the impact of SCRAs on social interaction using an *in vivo* model, while also providing evidence of their effects in a zebrafish self-administration assay.

2 MATERIAL AND METHODS

2.1 STANDARDS AND REAGENTS

Reference materials of ADB-INACA and CHO-4'Me-5'Br-FUBOXPYRA were obtained from Cayman Chemical (Ann Arbor, MI, USA). Dimethyl sulfoxide (DMSO) and hydrocodone hydrochloride 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).

2.2 DRUG PREPARATION

For each SCRA, a stock solution (SS) at 4 mg/mL was prepared in DMSO. For CHO-4'Me-5'Br-FUBOXPYRA, a DMSO SS at 10 mg/mL was also prepared. Working solutions (WS) at 10 and 100 µg/mL were individually prepared by diluting the SS in ultrapure water for both SCRAs. For CHO-4'Me-5'Br-FUBOXPYRA, a 2.5 µg/mL WS was also prepared in ultrapure water to be used in the self-administration assay. WS from both drugs at 10 and 100 µg/mL were used for acute toxicity and fishbook experiments, by diluting it in ultrapure water to achieve concentrations from 0.002 µM to 20 µM.

2.3 ANIMAL HUSBANDRY

All animal husbandry and experiment protocols were approved by and carried out in accordance with the Institutional Animal Care and Use Committee University of Utah. Fertilized eggs were collected from group mating of TuAB zebrafish (*Danio rerio*) and embryos were raised in E3 medium at 28°C during the first five days for embryo and larval acute toxicity assays. For the fishbook assays, from 5 to 7 dpf, larvae were transferred into nursery tanks and raised at 28°C on a 14-hour on/10-hour off light cycle until 21 dpf. Adults from EkkWill strain zebrafish (*Danio rerio*) (EkkWill Waterlife Resources) were used for the self-administration assay.

2.4 FISH EMBRYO ACUTE TOXICITY (FET) TEST

The FET test, described in the Test Guideline (TG) 236 from the Organization for Economic Co-operation and Development (OECD), allows the evaluation of the acute toxicity of chemical compounds during embryonic development of zebrafish [18]. Newly fertilized eggs are exposed to the targeted substance for 96h. 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 [18, 19].

A FET test was performed to evaluate the acute toxicity of both SCRAs in zebrafish embryonic life-stage. For that, TuAB fertilized eggs were collected soon after natural spawning and transferred to Petri dishes. By transferring one embryo per well in 96-well plates, 16 embryos were used per concentration. For each compound, five concentrations were tested, ranging from 0.001 µM to 10 µM, in addition to a negative control group. Exposures occurred between 0 and 4 dpf, by adding 100 µL of the corresponding WS to a

well containing 100 µL of E3 medium and one embryo. For the negative control group, 100 µL of ultrapure water containing 0.2% DMSO (v/v) was added instead of the compound.

Evaluations were performed every 24h until 4 dpf using stereoscopic microscope (Leica, Wetzlar, Germany). 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 every 24h. Other changes were also observed as indicators of sublethal effects. Additionally, at 3 dpf, the heartbeats of five larvae from each group were recorded for 20 seconds and subsequently extrapolated to 60 seconds to determine the heart rate per minute. Finally, at 4 dpf each viable larva was gently touched in the tail using a 20 µL micropipette tip to evaluate the escape response. For all evaluations, a threshold of 30% was applied, and effects were considered relevant only when observed in more than 30% of the individuals.

2.5 MAXIMUM TOLERATED CONCENTRATION (MTC) TEST

Similarly to the embryo acute toxicity evaluation, a maximum tolerated concentration (MTC) test was performed to evaluate the toxicity of ADB-INACA and CHO-4'Me-5'Br-FUBOXPYRA in zebrafish larval stage. For that, TuAB fertilized eggs were collected soon after natural spawning and transferred to Petri dishes. At 5 dpf, larvae were transferred per well into 96-well plates, with 16 larvae per concentration. For each compound, five concentrations were tested, ranging from 0.001 µM to 10 µM, in addition to a negative control group. Exposures occurred between 5 and 8 dpf, by adding 100 µL of the corresponding WS to a well containing 100 µL of E3 medium and one larva. For the negative control group, 100 µL of ultrapure water containing 0.2% DMSO (v/v) was added instead of the compound.

Evaluations were performed every 24h until 8 dpf using stereoscopic microscope. Signs of acute toxicity, such as locomotor impairment (hypoactivity, absence/decrease of touch-response, shaking, and loss of posture – defined as the larva lying on its side or in an abnormal orientation instead of maintaining an upright position), deformations, lack/decrease of heartbeat, or death were observed. The MTC is defined as the highest concentration tested in which no signs of toxicity are noticed [20, 21]. Additionally, every day, each viable larva was gently touched in the tail using a 20 µL micropipette tip, to evaluate the escape response. The 30% threshold was also applied for MTC assay.

2.6 FISHBOOK ASSAY

The fishbook assay was first described in 2022 by Geng and collaborators, consisting in a scalable and fully automated assay system in which a three-dimensional (3D) printed test arena is used [22]. Each arena is a long rectangular lane made by nontransparent material and divided into three parts by two transparent windows: (i) the longer middle part, that serves as the test compartment; (ii) a smaller end-compartment that contains a live fish as social stimulus; (iii) and another smaller end-compartment that is left empty. To ensure throughput, 44 arenas are grouped together and a telecentric lens–based imaging system is used to enable simultaneously image of all 44 [22, 23].

In each assay, 20 dpf TuAB zebrafish were collected from nursery tanks and transferred to deep petri dishes containing 40 mL of E3 medium mixed with rotifers and 5 µL of AmQuel (Kordon) to remove harmful ammonia excreted by fish. Fifteen fish were sorted into each dish. Dishes were incubated at 28°C in a larvae incubator overnight. On the next day, at 21 dpf, each dish was fed with rotifers in the morning. After 90 min of feeding, a 10 µM WS of ADB-INACA or CHO-4'Me-5'Br-FUBOXPYRA was individually added to each dish. For the negative control group, DMSO was used instead of the drug. After 30 min of drug exposure, the fishbook assay was conducted. For that, test subjects were individually placed inside each test chamber. Zebrafish visual access to social stimulus zone and to the empty compartment was temporarily blocked by two 3D printed white comb-like structures placed in front of each compartment. Once all test subjects were placed into the test arena array, the array was placed inside the imaging station, and the combs were removed. After a 5-min acclimation period, a 10-min test session was recorded. For ADB-INACA and CHO-4'Me-5'Br-FUBOXPYRA, exposure groups were composed by n = 22 and n = 40 individuals, respectively, while negative control groups for each assay were composed by n = 20 and n = 41 individuals, respectively.

Videos were streamed through the software Bonsai and were analyzed in real time during recording. The frame-by-frame x and y coordinates of each fish relative to its own test compartment were exported as a CSV file. Data were analyzed using custom scripts (Python) to calculate social scores. Social score was defined as a fish's average y-axis position for all frames. All social scores have values between − 1 and 1, in which higher social score values demonstrate a shorter average distance between a test fish and a social stimulus fish, which suggests a stronger social preference, while lower values demonstrate a shorter average distance between a test fish and the empty zone.

2.7 SELF-ADMINISTRATION ASSAY

To promote the discovery of novel drugs that may significantly reduce opioid self-administration, Bossé and collaborators (2017) have developed an assay in young adult zebrafish, modeled after well-established paradigms used in rodents [24]. Zebrafish are well suited to model addiction-related behaviors, as their reward system carries striking similarities with humans and other vertebrates. Fish also engage in reward-associated behaviors, such as conditioned place preference [24, 25].

Drug-seeking behavior was evaluated using a custom test arena, comprising a plastic tray with enclosed submersible square platforms connected to a larger water reservoir. The system was equipped with a pump and illuminated with a warm white light source. A Raspberry Pi minicomputer and two infrared cameras monitored the two platforms, designated as "active" and "inactive". When a fish crossed the "active" platform, the minicomputer detected the movement and activated a 12V peristaltic pump, which released the solution of interest through a small silicone tube attached to the side of the platform and connected to an external bottle. Conversely, when the fish crossed the "inactive" platform, no solution was released, but the number of crossings were also recorded. In the self-administration assay developed by the authors, fish were trained to trigger the delivery of a hydrocodone solution by swimming across the "active" platform. A recirculation system avoided hydrocodone diffusion to the opposite corner of the apparatus, where the "inactive" platform (not paired with drug release) was

located. This paradigm resulted in robust opioid seeking within five days of training, as measured by the number of triggering events on the active platform, as compared to the inactive platform.

Here, three-month-old EkkWill zebrafish were used to investigate the effects of CHO-4’Me-5’Br-FUBOXPYRA, consisting in five days of food conditioning, followed by five days of drug treatment with the SCRA or hydrocodone (as positive control). A negative control group was also employed, delivering water at the “active” platform. For food conditioning, six groups of n = 15–17 fish were used, performing daily sessions of 50 min for each group. At the end of each day, fish were re-grouped in two large tanks containing approximately 50 animals each. For the five days of drug treatment, the six groups were allocated for SCRA exposure (two groups), hydrocodone exposure (two groups), or as negative control (two groups). Similarly, at the end of each day, fish from the same treatments were re-grouped in large tanks containing approximately 35 animals each. For hydrocodone exposure, of a dose of 1.5 µg from a solution of 6 mg/L was released in the system at each triggering in the “active” platform. Similarly, for SCRA exposure, a dose of 0.625 µg from a solution of 2.5 mg/L was released every triggering event.

2.8 STATISTICAL ANALYSIS

Graphs were generated using Excel, GraphPad Prism, or Python. Statistical analyses were performed using two-tailed Student’s t-tests for comparisons between two groups and one-way analysis of variance (ANOVA) for comparisons involving more than two groups. Significance levels (P values) were reported accordingly.

3 RESULTS

3.1 ACUTE TOXICITY TESTS

For FET experiments, both SCRAs produced low embryo mortality at the evaluated concentration range. The only endpoint observed was embryo coagulation in less than 30% of the embryos considering all timepoints. Sublethal effects were noticed only for CHO-4’Me-5’Br-FUBOXPYRA considering the threshold: pericardial edema (10 µM, 2 dpf; 10 µM, 4 dpf) and loss of posture (10 µM, 3 dpf). Regarding escape-response, all concentrations above 0.1 µM reduced the response in zebrafish larvae with 4 dpf for CHO-4’Me-5’Br-FUBOXPYRA in more than 30% of the individuals, while for ADB-INACA only at 0.01, 1, and 10 µM. Additionally, at 3 dpf, the highest concentration of exposure (10 µM) for CHO-4’Me-5’Br-FUBOXPYRA reduced the heart rate in a significant difference (p-value < 0.01) in comparison with the other concentrations and with the non-exposed groups. ADB-INACA did not produced significant effects in this evaluation. Data for FET evaluations are presented in Fig. 1.

For the MTC of CHO-4'Me-5'Br-FUBOXPYRA, considering the threshold, the absence of escape-response was noticed at 6 (1 and 10 µM), 7 (all exposed groups), and 8 dpf (0.01, 0.1, 1, and 10 µM). For ADB-INACA, absence of escape-response was also noticed at 6 (1 and 10 µM), 7 (0.1 and 10 µM), and 8 dpf (10 µM). Furthermore, loss of posture was detected at 7 dpf (CHO-4'Me-5'Br-FUBOXPYRA, 10 µM) and at 8 dpf (ADB-INACA, 10 µM). Data for MTC results are presented in Fig. 2.

** p-value < 0.01*

3.2 FISHBOOK ASSAY

By performing the Fishbook experiments, it was possible to notice that both CHO-4'Me-5'Br-FUBOXPYRA and ADB-INACA did not achieve significant effects in the social score average when compared to negative control groups in 21 dpf juvenile zebrafish larvae. The average social scores for ADB-INACA and its negative control group were 0.484 and 0.424, respectively, while for CHO-4'Me-5'Br-FUBOXPYRA and negative control group, 0.658 and 0.623, respectively. Each compound was tested on a separate day, together with its respective negative control group. Fishbook data are presented in Fig. 3.

3.3 SELF-ADMINISTRATION ASSAY

Food conditioning led to data consistent with previous data from self-administration assays [24, 25]. During the days, a slightly increase in the number of triggering events in the active platform was noticed, demonstrating that the fish learned to go to the active platform to receive food (data not shown). Regarding the five days of treatment with hydrocodone, despite a slight reduction in the number of triggering events for the active platform at day four, fish sought for the opioid during the days, in contrast to what happened to SCRA treatment, in which the number of triggering events in both platforms were similar. Data for the self-administration assay are reported in Fig. 4.

4 DISCUSSION

An increase in the production and use of NPS has been described worldwide and SCRAs are the second most detected class of NPS worldwide, only after stimulants [1, 7]. This class contains structural groups capable of binding to cannabinoid receptors CB_1 and CB_2, producing effects similar to those of THC. However, SCRAs often exhibit more potent and efficacious binding to these receptors [4]. SCRA consumption has been associated with diverse severe adverse effects, implicated in several deaths worldwide [4, 9, 10].

Human studies on NPS remain scarce due to ethical and safety constraints, leading to the use of animal models for such investigations and, recently, zebrafish have emerged as a widely employed model in toxicological research involving NPS [17]. Specifically, for SCRA, zebrafish were mostly applied for the investigation of drug metabolism of different compounds [26–30]. However, some studies also evaluated the acute toxicity in embryos or larvae [20, 29–33]. Here, we were able to investigate the acute toxicity of SCRA in both zebrafish embryos and larvae, as well to evaluate the effects of SCRA on social interaction on juvenile larvae and its potential to lead to self-administration in adult zebrafish.

The two SCRAs studied here were ADB-INACA and CHO-4'Me-5'Br-FUBOXPYRA and both are few described in the literature. Monti and collaborators (2025) [14] investigated the *in vitro* CB_1 receptor activity of some SCRAs, including tail-less precursors such as ADB-INACA and MDMB-INACA, demonstrating only minimal CB_1 receptor activity when compared to final products. For ADB-INACA,

concentration-response curves failed to reach a clear plateau, not allowing accurate potency estimation, while MDMB-INACA displayed very low efficacy with EC_{50} values exceeding 2000 nM for both assays performed [14]. These weak activities align with previous reports on the low CB_1 receptor potency of such precursors. Deventer and collaborators (2023) [34] investigated the impact of halogen substitution on the pharmacological activity of tail-less SCRAs. Specifically, (S)-ADB-INACA was evaluated at CB_1 and CB_2 receptors. At CB_1, no full concentration–response curve could be obtained, and only partial activity was observed, with a maximal receptor activation of 251% at 100 μM. Compared with its brominated analog (S)-ADB-5'Br-INACA, (S)-ADB-INACA displayed a slight rightward shift of the curve, indicating reduced potency. At CB_2, both compounds exhibited similar potencies (EC_{50} values of 164 and 156 nM, respectively), although (S)-ADB-INACA was slightly less efficacious (E_{max} 121%) [34]. Despite its low influence on CB_1 receptor, ADB-INACA was able to attenuate mechanical and cold allodynia in rats with paclitaxel-induced peripheral neuropathy (PIPN), with better analgesic effects on PIPN than MDMB-INACA and ADB-HINACA and similarly to MDA-19 [12]. In our study, ADB-INACA acute toxicity was investigated from 0.001 to 10 μM, resulting in low embryo or larval mortality. The most pronounced effect observed was absence of escape-response for embryos at 0.01, 1, and 10 μM and for larvae at 0.1, 1, and 10 μM. In the Fishbook assay, ADB-INACA was investigated at 10 μM, also resulting in an absence of significant effects in the social score average when compared to negative control group. Collectively, these data support the hypothesis that tail-less precursors, although structurally related to potent SCRAs, are unlikely to result in significant cannabinoid-like effects at physiologically relevant concentrations.

Regarding CHO-4'Me-5'Br-FUBOXPYRA, Deventer and collaborators (2025) [15] investigated the metabolism and the potential to activate CB_1 and CB_2 of this SCRA, leading to the identification of four hydroxylated metabolites and resulting in a limited activation potential for both receptors [15]. In our study, CHO-4'Me-5'Br-FUBOXPYRA acute toxicity was investigated from 0.001 to 10 μM, resulting only in sublethal effects as pericardial edema (10 μM, 2 dpf; 10 μM, 4 dpf) and loss of posture (10 μM, 3 dpf). Absence of escape-response was also observed for all concentrations above 0.01 μM considering all evaluations. Additionally, the exposure to 10 μM reduced the heart rate in a significant difference (p-value < 0.01). Similarly to ADB-INACA, CHO-4'Me-5'Br-FUBOXPYRA could not promote significant effects in the social score average at 10 μM on the Fishbook assay. Finally, in the self-administration assay, in contrast to hydrocodone treatment, fish exposed to 0.625 μg of CHO-4'Me-5'Br-FUBOXPYRA did not exhibit drug-seeking behavior throughout the experimental period, demonstrating an absence of reinforcing effects associated with this SCRA. Altogether, the results from our work and those reported in the literature demonstrate that although this SCRA has a limited potential to activate cannabinoid receptors, to impact social behavior, or to generate reinforcing effects, some important cardiac effects can be noticed at higher doses, such as pericardial edema and reduced heart rate.

Despite our meaningful results, there are a few limitations to this study, including the evaluation of only two SCRAs, the use of acute rather than chronic exposures, and the absence of complementary molecular or neurochemical assessments. Nevertheless, the zebrafish model was determined to be

highly appropriate for the investigation of NPS. In particular, the addition of the Fishbook assay for social behavior and the self-administration paradigm for drug-seeking behavior expands the range of zebrafish-based approaches available for assessing the toxicological and behavioral consequences of new psychoactive substances. These assays were found to be feasible, reproducible, and translationally relevant and can be applied to a larger number of emerging substances. Collectively, they offer a valuable means of rapid toxicological surveillance, bridging the gap between preclinical toxicology and public health needs.

5 CONCLUSIONS

NPS intoxication is an important phenomenon and SCRAs are among the most prevalent classes reported in seizures or biological determinations. With FET and MTC tests, the acute toxicity for both drugs was evaluated at embryo and larval zebrafish stages, demonstrating low mortality and general absence of important effects in the evaluated range. After SCRA exposure, social behavior was considered normal when compared to non-exposed larvae. Furthermore, with self-administration assays, no notable effects on drug-seeking behavior were observed following the exposure to CHO-4'Me-5'Br-FUBOXPYRA in adult zebrafish. Summarizing, considering the tests here developed, our data suggest that both ADB-INACA and CHO-4'Me-5'Br-FUBOXPYRA did not achieve significant outcomes. However, cardiac effects for CHO-4'Me-5'Br-FUBOXPYRA were observed, including pericardial edema at FET evaluations and reduced heart rate. Additionally, our work demonstrates that zebrafish experiments such as Fishbook and the self-administration assay can be successfully applied for investigating the effects of NPS.

Declarations

Data Availability

The data supporting the findings of this study are included within the article. Additional materials generated during the experiments, such as raw images and video files, are available from the corresponding author upon reasonable request.

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Author Information

Contributions

LC Rodrigues was responsible for conceptualization, methodology, formal analysis, investigation, writing – original draft, writing – review & editing, visualization; M Muhsen was responsible for

conceptualization, methodology, validation, formal analysis, investigation; SR Aliabadi was responsible for conceptualization, methodology, validation, formal analysis, investigation; T Zhang was responsible for conceptualization, methodology, validation, formal analysis, investigation; CV Maurer-Morelli was responsible for conceptualization, writing – original draft, writing – review & editing, visualization, supervision; RT Peterson was responsible for conceptualization, methodology, validation, formal analysis, resources, review & editing, supervision, project administration, funding acquisition; JL Costa was responsible for conceptualization, validation, resources, writing – review & editing, visualization, supervision, project administration, funding acquisition.

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Competing Interests

Authors declare no conflicts of interest.

Ethics approval

All animal husbandry and experimental procedures were approved by and carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Utah.

Consent to participate

Not applicable, as this study did not involve human participants.

Consent to publish

Not applicable, as this study did not involve human participants.

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Figures

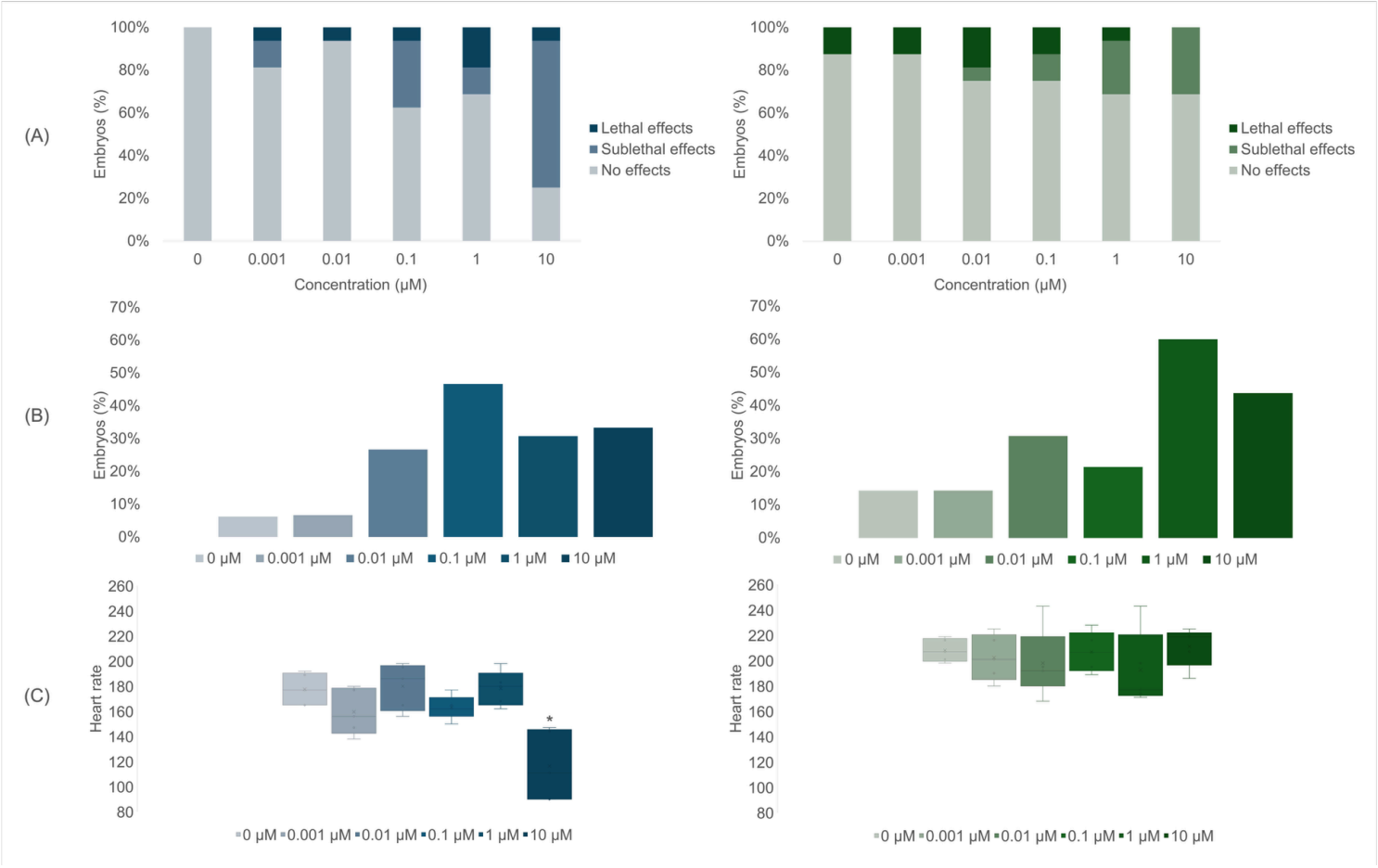

Figure 1

Fish embryo acute toxicity (FET) test for CHO-4'Me-5'Br-FUBOXPYRA (blue) and ADB-INACA (green). (A) Evaluation of lethal, sublethal, and absence of effects at the end of the evaluation period – 4 days post-fertilization (dpf) (n=16/group); (B) absence of escape-response at 4 dpf (n=13-16/group); and (C) heart rate evaluation at 3 dpf (n=5/group).

** p-value < 0.01*

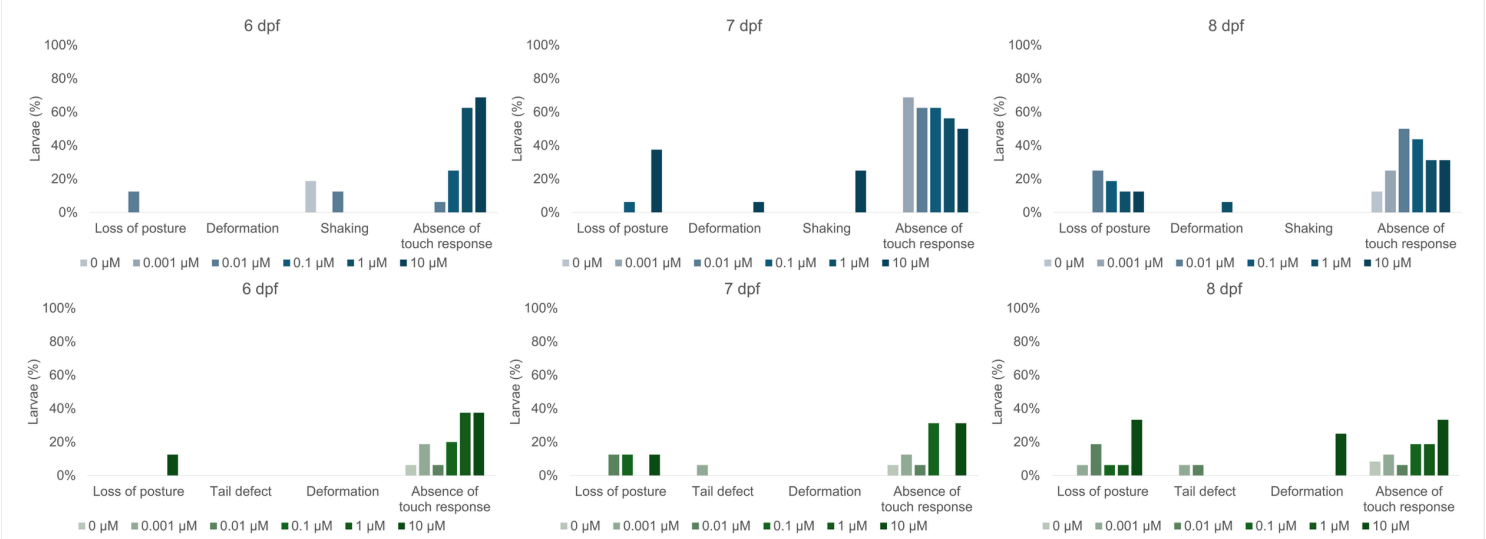

Figure 2

Maximum tolerated concentration (MTC) test for CHO-4'Me-5'Br-FUBOXPYRA (blue) and ADB-INACA (green). Evaluation of lethal and sublethal effects and absence of escape-response at 6, 7, and 8 days post-fertilization (dpf) (n=16/group).

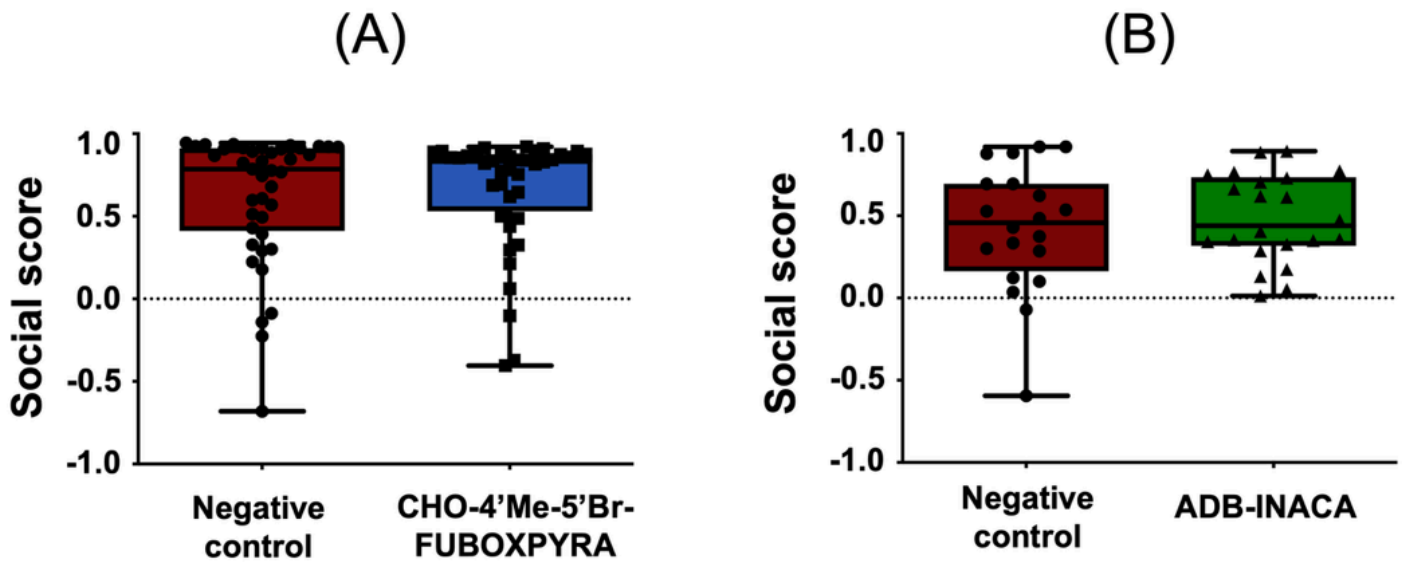

Figure 3

Fishbook assay. Boxplots from the comparison of social score obtained for negative groups and (A) CHO-4'Me-5'Br-FUBOXPYRA (n=40-41/group) and (B) ADB-INACA (n=20-22/group). In each boxplot, the box encloses data points from the 25th to 75th percentile, the horizontal line marks the median value, and the lines above and below the box reach data points with the maximum and minimum values.

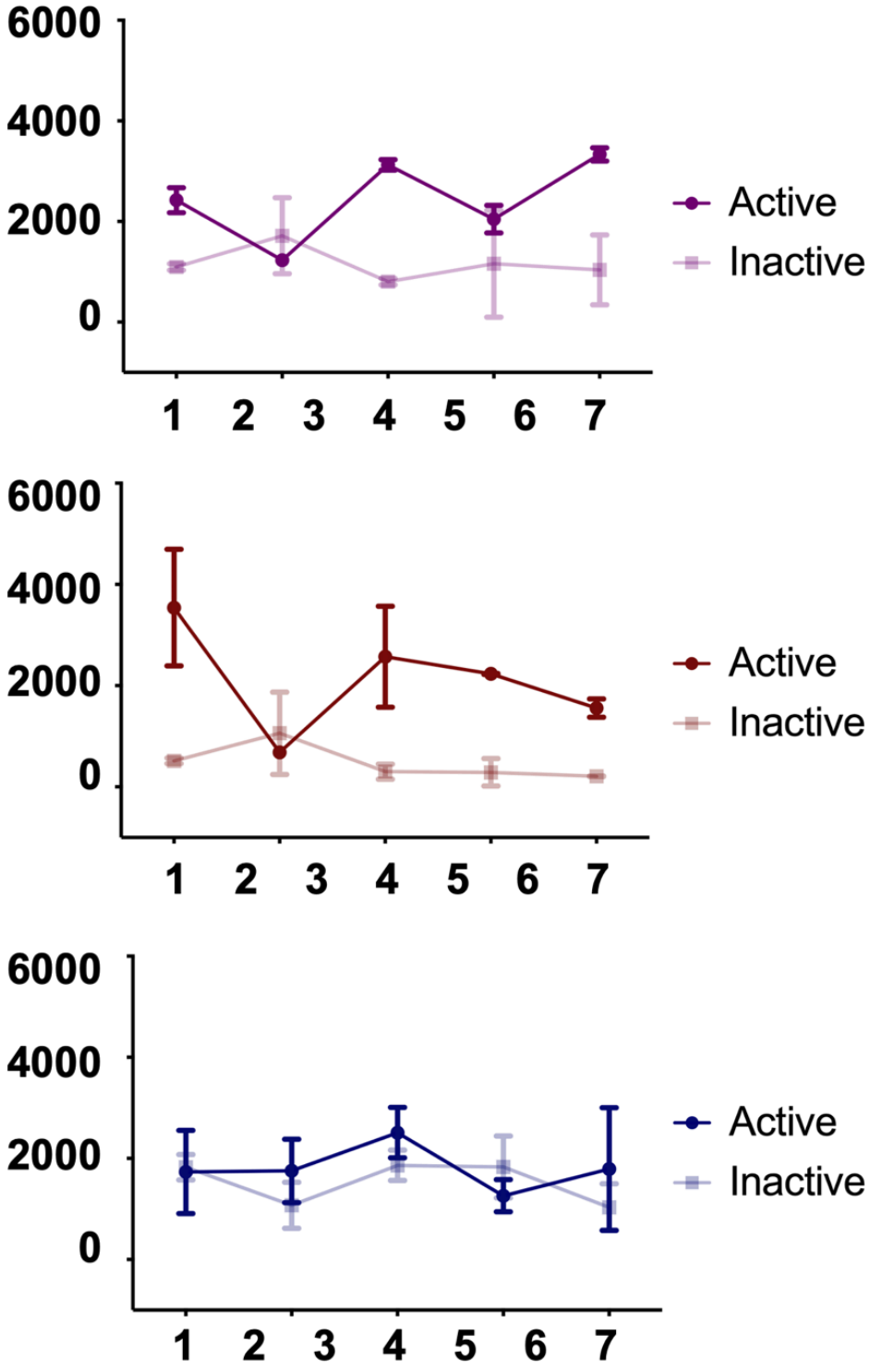

Figure 4

Self-administration assay. Comparison of the number of triggering events for active and inactive platforms for hydrocodone (purple), water (red), and CHO-4'Me-5'Br-FUBOXPYRA (blue) treatments. For each treatment, two groups of n=15-17 fish were used.

Supplementary Files

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- floatimage1.png