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Toxicity and behavioural effects of ocfentanil and 2-furanylfentanyl in zebrafish larvae and mice

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ABSTRACT

The introduction of the so-called New Psychoactive Substances represents a problem of global concern due to several factors, including multiplicity of structures, poorly known activity, short half-life in the market, lack of pure standards etc. Among these problems, of the highest relevance is also the lack of information about metabolism and adverse effects, which must be faced using simple and low-cost animal models. On these grounds, the present work has been carried out on 5 days post fertilization zebrafish (*Danio rerio*) larvae in comparison with adult mice (*Mus musculus*). Ocfentanil and 2-furanylfentanyl were administered at different concentrations to zebrafish larvae (1, 10 μM) and mice (0.1, 1, 6, 15 mg/kg). The behavioural assay showed a decrease in basal locomotor activity in zebrafish, whereas in mice this effect was evident only after the mechanical stimulus. Larva extracts and mice urine were analysed by using liquid chromatography coupled to high resolution mass spectrometry to identify the metabolic pathways of the fentanyl analogs. For 2-furanylfentanyl, the most common biotransformations observed were hydroxylation, hydration and oxidation in zebrafish larvae, whereas mice produced mainly the dihydrodiol metabolite. Hydroxylation was the major route of metabolism for ocfentanil in zebrafish larvae, while in mice the O-demethylated derivative was the main metabolite. In addition, a study was conducted to evaluate morphological effects of the two drugs on zebrafish larvae. Malformations were noticeable only at the highest concentration of 2-furanylfentanyl, whereas no significant damage was observed with ocfentanil. In conclusion, the two animal models show similarities in behavioral response and in metabolism, considering the different biological investigated.

1. Introduction

The emergence of the so-called New Psychoactive Substances (NPS) is a global phenomenon of great concern, also in forensic and clinical toxicology. The vast majority of adverse clinical effects and social hazards linked to the use and diffusion of NPS is still unknown, posing a significant challenge to the policies of contrast, prevention and treatment policies (Baumann et al., 2018; Huestis et al., 2017). In such a complex situation, a major problem hindering a pharmaco-toxicological knowledge of these compounds lies in the difficulty of setting up adequate animal models to study drug effects and metabolism fast enough to cope with the continuous introduction of new products, most of which quickly disappear. This is mainly related to the complex, tedious and time-consuming procedures required to carry out appropriate pharmacodynamics, pharmacokinetics and metabolism studies in the traditional animal models. Among NPS, in the recent time, special awareness is given to the class of Novel Synthetic Opioids (NSO), generally categorized as fentanyl analogs (e.g., 2-furanylfentanyl, ocfentanil, acetylfentanyl), as well as to newly emerging non-fentanyl compounds (e.g., U-47700, AH-7921, mitragynine) (Garneau et al.,

Abbreviations: NPS, New Psychoactive Substances; ZL, Zebrafish Larvae; NSO, New Psychoactive Opioids; Fu-F, 2-furanylfentanyl; QTOF, Quadrupole Time Of Flight; 4 ANPP, 4-anilino-*N*-phenethylpiperidine; dpf, days post fertilization; FW, fish water.

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2020). These compounds were initially developed as possible alternatives to fentanyl, to achieve better therapeutic indices and higher potency (Allibe et al., 2018). Although thousands of fentanyl analogs were synthesised, only three of them have been approved for medical use. On the contrary, a number of synthetic opioids have appeared in the illicit market, sold as stand-alone products, as adulterants of heroin or included into counterfeit prescription pills (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2017). As a consequence, since 2012 thousands of fatalities caused by NSO have been reported worldwide (Goggin et al., 2017; Prekupec et al., 2017; United Nations Office on Drugs and Crime (UNODC), 2017).

Ocfentanil (N-(2-fluorophenyl)− 2-methoxy-N-[1-(2-phenylethyl) piperidin-4-yl]acetamide) is a synthetic opioid structurally related to fentanyl, differing from it because of an extra fluorine atom on the *o*-position of the aniline moiety and a methoxy instead of a methyl group. It was first produced in the early 1990 s with the aim of creating a potent naloxone-reversible opioid with less cardiovascular effects and respiratory depression. Ocfentanil is sold in a white granular or brown powder, which is available either as free base or as hydrochloric acid salt (Misailidi et al., 2018). Ocfentanil potency is approximately 2.5 times higher that of fentanyl (Misailidi et al., 2019). A study regarding dose-dependent pharmacological effects in humans was conducted by Fletcher et al. in 1991, however no conclusion about its benefits over fentanyl was drawn (Fletcher et al., 1991). To date ocfentanil has not been approved for any medical use.

Two-furanylfentanyl (N-phenyl-N-[1-(2-phenylethyl)piperidin-4-yl] furan-2-carboxamide) (or Fu-F) is a synthetic opioid with a structure similar to fentanyl, differing only in the replacement of the propionyl group for a furan ring (Kanamori et al., 2021). The above-mentioned modifications grant sevenfold higher potency over fentanyl (Wilde et al., 2019). Fu-F was first described in 1958 and, to date, it has not been approved for any medical purpose (Goggin et al., 2017; Huang et al.., 1987). Fu-F has been available in the European Union since June 2015, having been identified in 16 Member States and in Norway. In the United States this opioid appeared in December 2015. Several acute intoxications have been reported (Slovenian National Forensic Laboratory, 2016; Varshneya et al., 2022) and in 2018 Fu-F was included in schedule I of the Controlled Substances Act (Drug Enforcement Administration, 2018; Kanamori et al., 2021). Fu-F is commonly sold as a powder but other forms such as liquid, tablets, or nasal spray are also available in the market (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2017).

To study in-vivo effects and metabolism of these substances, overcoming the difficulties related to the use of traditional animal models, our research group has recently joined a research trend proposing ZL as particularly suitable for this purpose. Indeed, ZL present several advantages, such as small dimensions (roughly 4 mm long), high production (200–300 eggs per day) and fast development. These unique features are also extremely useful in terms of experimental time, since it is possible to obtain many replicates in a short time. Lastly, it is important to point out that in Europe zebrafish embryos are not considered laboratory animals until 5 dpf, i.e. the independent feeding stage, thus no specific authorizations are required. (Ali et al., 2011; de Souza Anselmo et al., 2018).

In the last decades, zebrafish has been used as a complementary animal model to study behavioural effects and toxicity of drugs, including fentanyl analogs. Indeed, in a study conducted by Varshneya and co-authors, fentanyl analogs showed significant dose-dependent hyperlocomotion in mice (Varshneya et al., 2019, 2021). In contrast, hypoactive behaviour was highlighted by Kirla et al. for zebrafish larvae (Kirla et al., 2021). Moreover, the morphological effect of fentanyl (Cooman et al., 2021) and fentanyl derivatives (Kirla et al., 2021) at different concentration levels has been recently investigated providing evidence of lethal and sublethal malformations in embryos on the 4th day post fertilization (dpf) after 24 h of exposure. However, up to date information regarding the effect and metabolism of 2-furanylfentanyl and ocfentanil is still limited (Watanabe et al., 2017).

On these grounds, the present work aims at providing new information on the behavioral effect and metabolism of 2-furanylfentanyl and ocfentanil using ZL in comparison with a well-known animal model such as mice. The ZL model was also used to evaluate morphological effects of the two drugs.

2. Materials and methods

2.1. Zebrafish model

2.1.1. Animal husbandry

Zebrafish experiments were performed at the Centre for Experimental Research (CIRSAL) of the University of Verona in accordance with the Italian and European Legislations (Directive 2010/63/EU) with permission of the Animal Welfare Body of the University of Verona. Larvae utilized in the experiments were the offspring of AB-strain wild-type adults, bred according to standard protocols (Kimmel at al, 1995) at a constant temperature of 28 (± 1) °C and tanks were kept to a 14:10-h light-dark cycle. Maintenance 3,5-lt tanks housed mixed sex groups with a maximum density of 17 exemplars. Adult zebrafish were fed four times a day, twice with *Artemia* nauplii and twice with dry food (*Zmsystems,* Winchester, UK). For reproduction, pairs of adult zebrafish were transferred into a breeding cage: the two breeders were divided by a transparent partition until the following day, when the partition was removed allowing spawning. Embryos were collected and raised in a Petri dish in fish water medium (1 mM calcium sulphate, 1.2 mM sodium bicarbonate and 0.02 % *(w/v)* Instant Ocean).

2.1.2. Reagents and chemicals

Standards of 2-furanylfentanyl and ocfentanil were provided from Comedical (Comedical, Trento, Italy) as methanolic solutions. Appropriate amounts of these solutions were spiked in fish water medium to obtain the final concentration of 1 μM and 10 μM. As a result of the dilution of the pure standard, the 1 μM and 10 μM solutions contained 0.3 % and 3.3 % of methanol, respectively. Thus, two different vehicles were prepared, the first being composed of fish water and 0.3 % methanol (vehicle 1), the second one (vehicle 2) containing 3.3 % of methanol.

Methanol and acetonitrile were purchased from *VWR Chemicals* (Fontenay-Sous-Bois, France), while formic acid 98 % for LC-MS from *Merck KGaA* (Darmstadt, Germany). Sodium bicarbonate and calcium sulphate used for fish water were purchased from *Merck KGaA* (Darmstadt, Germany) and Instant Ocean from Tecniplast (Varese, Italy); 48-well plates were provided by Sarstedt (Numbrecht, Germany). MilliQ water was obtained from a model PureLab Chorus 1 Complete water purification system (*Elga Veolia* Lane End, High Wycombe, UK).

2.1.3. Morphological effects of 2-furanylfentanyl and ocfentanil in zebrafish larvae

A total of seventy zebrafish larvae (ZL), deriving from different hatchings in two distinct days, underwent a 24 h-incubation at 28 °C on the fourth day post fertilization. ZL were placed in 48-well plates with one larva per well and 1 ml fish water containing the two fentanyl analogs at two concentration levels (1 and 10 μM). Two replicates of each assay were performed on two different days. Overall, for behavioural tests 14 larvae (n = 7 for each replicate) were used for each of the following: vehicle, 2-furanylfentanyl and ocfentanil 1 μM and 10 μM. Afterwards, larvae were anaesthetised by using ice and then monitored using a model DM2500 microscope connected with a model ICC50W camera (Leica Microsystem Vertrieb GmbH, Wetzlar, German). Larvae were evaluated for their morphological defects including yolk sac edema (YSE), pericardial edema (PE), body axis (AXIS), trunk length (TRUN), caudal fin (CFIN), pectoral fin (PFIN), pigmentation (PIG), jaw (J) and somite (SOMI) deformities according to the abnormalities reported by Noyes (Noyes et al., 2015) and also shown in larvae exposed to

increasing concentrations of fentanyl (Pesavento et al., 2022).

2.1.4. Behavioural assay

2-furanylfentanyl and ocfentanil standards utilized for ZL behavioural assay were diluted in a solution of fish water medium; final tested doses were 1 and 10 μM. These concentrations were selected according to our previous study regarding the maximum tolerated concentrations of fentanyl in zebrafish (Pesavento et al., 2022). Moreover, in order to evaluate a potential solvent effect, control larvae were exposed to 0.3 % and 3.3 % methanol solution (vehicle 1 and 2, respectively). On the fifth day after fertilization, larvae were individually placed in 48-well plates filled with 1 ml of fish water (FW) medium and treatment, either 2-furanylfentanyl, ocfentanil or vehicle control, just before behavioural test. The multi-well plate was arrayed into an infrared backlit plate holder in the observation chamber of DanioVision (Noldus, Wageningen, Netherlands), where larvae were exposed to a white light for 120 min of acclimation. The temperature was set at 28 °C. Subsequently, larvae underwent three series of a rapid change of illumination from darkness to a strong illumination (i.e., 10-min light off and 10-min light on). In total the behavioural test consisted of 180 min recording for each experimental replicate. EthoVision 17 XT video tracking software calculated each subject's activity as the total distance moved in each minute of the recording. The number of replicates and of larvae for each treatment was the same as for the morphological assay, with the addition of n = 14 larvae in fish water medium, used as control.

In order to evaluate the acute effect of the studied NSOs in ZL, a previously validated paradigm (Achenbach et al., 2018) was conducted for each drug. Larvae locomotor activity was measured by means of Ethovision XT software, providing the total distance travelled by each subject in every minute of the recording. Firstly, raw data were analysed in total to assess the drug effect over time, then the assay was divided into two main phases, namely *habituation* (1–120 min) and *stimulation (*120–180 min). Furthermore, the *stimulation* phase consisted in three replicates of a 10-minute period of darkness followed by 10 min of light, considering that in normal conditions larvae tend to increase locomotor activity in darkness and to decrease it after a light stimulus (Kalueff, 2017; MacPhail et al., 2009). Lastly, experimental data were split according to the two concentration levels i.e., 1 μM (0.33 % methanol) and 10 μM (3.3 % methanol) with the purpose of assessing the potential influence of the vehicle (water and methanol).

2.2. Mouse model

2.2.1. Animal husbandry

Ninety-six adult Male ICR (CD-1®) mice (about 12 weeks old) weighing 30–35 g (Centralised Preclinical Research Laboratory, University of Ferrara, Italy) were group housed (5 mice per cage; floor area per animal was 80 cm2; minimum enclosure height was 12 cm), exposed to a 12:12-h light-dark cycle (light period from 6:30 AM to 6:30 PM) at a temperature of 20–22 °C and humidity of 45–55 % and were provided ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. The experimental protocols performed in the present study were in accordance with the U.K. Animals (Scientific Procedures) Act of 1986 and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU). Experimental protocols were approved by the Italian Ministry of Health (licence n. 335/2016-PR) and by the Animal Welfare Body of the University of Ferrara. According to the ARRIVE guidelines, all possible efforts were made to minimize the number of animals used and to reduce the animals' pain and discomfort.

2.2.2. Drug preparation and dose selection

Two-furanylfentanyl and ocfentanil were purchased from LGC standards (LGC Standards S.r.l., Sesto San Giovanni, Milan, Italy) and were dissolved in a saline solution (0.9 % NaCl), which was also used as the vehicle. Drugs were administered by intraperitoneal (i.p.) injection of a volume of 4 μL/g. The range of doses of drugs tested (0.1–15 mg/kg i.p.) was chosen based on our previous studies (Bilel et al., 2022; Pesavento et al., 2022).

2.2.3. Spontaneous locomotion test

A single mouse was placed in a square plastic cage (60 ×60 cm) located in a sound- and light-attenuated room, and changes induced by 2-furanylfentanyl and ocfentanil in motor activity were monitored for 310 min (Bilel et al., 2020; Ossato et al., 2018) by means of an ANY-maze video-tracking system (Ugo Basile, application version 4.99 g Beta). Four mice were monitored at the same time in each experiment. The system calculated the distance travelled (m) every 15 min for a maximum of 310 min. In order to avoid the problem of distinguishing whether the immobility of the mouse was due to the action of opioids agonist or to the normal resting behaviour of the animal in the open field test, mice were mechanically stimulated when they were completely stationary (Pesavento et al., 2022). At minute 225, each mouse was gently touched on its back three consecutive times with a plastic stick with a rounded tip. In the analysis of spontaneous locomotion in the open field test for each treatment (vehicles or 4 different 2-furanylfentanyl or ocfentanil doses, 0.1, 1, 6 and 15 mg/kg) 8 mice were used (total mice used: 80).

2.3. Identification and analysis of metabolites

A series 1260 HPLC (Agilent Technologies, Waldbronn, Germany) in tandem with a 6540 Accurate-Mass QTOF (Agilent Technologies, Palo Alto, CA, USA) was used for the present study. The chromatographic separation was performed on a Zorbax Eclipse XDB (2.1 × 150 mm, 5 μm particle size, Agilent Technologies). The composition of the mobile phases was as follows: formic acid 0.1 % as phase A and acetonitrile with 0.1 % formic acid as phase B. In all experiments the flow rate was set at 500 μL/min and injection volume was 5 μL. Samples were eluted with a linear gradient from 2 % to 95 % B of solvent B lasting 12 min; the final conditions (95 % B) were kept for 3 min and then the starting conditions (2 % B) were restored in 1 min and kept for 7 min, to allow system re-equilibration. The QTOF-MS was performed using a Jet Stream electrospray ion source (Agilent Technologies) operating in positive ionization mode. Source parameters were gas temperature 325 °C, gas flow 8 L/min, nebulizer pressure 30 psi, sheath gas temperature 360 °C, sheath gas flow 12 L/min, VCap 3750 V, nozzle voltage 500 V and fragmentor voltage 150 V. For continuous mass calibration, the following reference ions were used: purine 121.0508 [M + H] + and HP-921 = hexakis(1 H,1 H,3 H-tetrafluoropropoxy) phosphazine 922.0097 [M + H] + . Data acquisition was performed in full scan mode in the mass range of 100–1000 *m/z*. Fragmentations were obtained applying a collision energy of 20 or 10 eV on selected ions. MassHunter Acquisition software and the Qualitative Analysis version B.04.00 (Agilent Technologies) were used for data handling.

2.3.1. Zebrafish larvae: in vivo identification of metabolites

After the behavioural assay, the larvae were euthanized with ice. Afterwards, *treated* and *control* larvae were collected separately and snap-frozen at − 80 °C and stored until the extraction. ZL on the fifth day post fertilization were lyophilized using Savant™ DNA SpeedVac™ Concentrator Kits (Thermo Scientific, Waltham, Massachusetts, USA) for 60 min and then extracted with 50 μL of methanol, shaken for 2 min and centrifuged at 12,054 g for 5 min with Microfuge Lite centrifuge (Beckman Coulter, Ca, USA). Lastly, the supernatant obtained was transferred in an autosampler vial and injected into the instrument (Gampfer et al., 2020).

2.3.2. Mice: in vivo identification of metabolites

Mice were individually placed inside metabolic cages (Ugo Basile SRL, Gemonio, Varese, Italy) with free access to water and food. Overall, in the urine collection studies for each treatment (vehicle and 15 mg/kg

2-furanylfentanyl or ocfentanil) 8 mice were used (total mice used: 24).

For the identification of metabolites, urines were collected after the injection of vehicle, 2-furanylfentanyl or ocfentanil at 15 mg/kg doses in a time interval of 0–5 h. Urine samples were kept at − 80 °C until analysis. After dilution (1:1000) in autosampler vials with ultrapure water, samples were directly injected into the LC-QTOF.

2.4. Statistics

The statistical analysis was performed using GraphPad Prism software (Version 8). Normal response distributions were verified using Shapiro-Wilk normality test. Afterwards for ZL a two-way ANOVA test followed by a Dunnett's multiple comparison test were conducted to evaluate the effect over time of the two drugs at the lowest concentrations (1 μM). However, for the highest concentrations (10 μM) a two-way ANOVA and a Tukey post-hoc test were used. Moreover, Kruskall-Wallis and Dunn's tests were assessed to measure significant differences ($p < 0.0001$) between the three phases of the behavioural test compared to the vehicle (habituation, light off, light on). To evaluate the effects of 2-furanylfentanyl or ocfentanil in mice at different doses over time compared to the vehicle, a two-way ANOVA followed by a Bonferroni test for multiple comparisons was performed. Furthermore, the comparison of the effects induced by 2-furanylfentanyl or ocfentanil on the response to mechanical stimulation of locomotion was conducted using a two-way ANOVA followed by a Bonferroni test for multiple comparisons. Finally, a Student's t-test was used to determine statistical significance ($p < 0.05$) between the different groups (see motor changes after mechanical stimulation).

3. Results

3.1. Morphological effects on ZL of 2-furanylfentanyl and ocfentanil

A specific study was conducted to test acute morphological effects of 2-furanylfentanyl and ocfentanil on ZL. In 2-furanylfentanyl-treated larvae, the concentration of 1 μM did not induce morphological changes in ZL. On the other hand, the highest concentration (10 μM) caused a yolk sac edema in 62.5 % of cases, which is noticeable as a swollen abdomen in Fig. 1. In addition, skin pigmentation in larvae treated with 2-furanylfentanyl was darker than the control larvae. On the contrary, ZL treated with ocfentanil at the two concentrations tested (1 and 10 μM) did not show any malformations.

3.2. Behavioral assay in ZL

As depicted in Fig. 2 Panel A, the statistical analysis showed a significant effect of treatment ($F_{2738} = 444$, $p < 0.0001$), time ($F_{17,738} = 292.6$, $p < 0.0001$) and time x treatment interaction ($F_{34,738} = 34.38$, $p < 0.0001$). Indeed, there was a significant decrement in larvae locomotion under the effect of the two synthetic opioids in the *habituation* phase (time point 120), whereas only 2-furanylfentanyl declined the movement during the *stimulation* phase (120–180 min). The above-mentioned effect was more pronounced at the highest concentration for 2-furanylfentanyl: in Fig. 2 Panel B, the two-way ANOVA showed a significant effect of treatment ($F_{2684} = 5086$, $p < 0.0001$), time ($F_{17,684} = 40.23$, $p < 0.0001$) and time x treatment interaction ($F_{34,684} = 71.91$, $p < 0.0001$). Overall, 2-furanylfentanyl dramatically inhibited the activity of ZL.

As per the lowest drug concentration (1 μM), in the *habituation* phase both fentanyl analogs reduced the total distance travelled when compared to the vehicle. There was a significant increase in activity in the *light off*- phase compared to *habituation*, whereas movement in the *light on*-phase declined equally in treated and control larvae (Fig. 2; Panel C). Conversely, 2-furanylfentanyl at the highest concentrations (10 μM), reduced and flattened the total distance travelled overall, while ocfentanil 10 μM showed a decrease in locomotion compared to the vehicle only in the *habituation* phase (Fig. 2; Panel D).

3.2.1. Behavioural assay in mice

Spontaneous locomotion in mice was significantly affected by systemic administration of 2-furanylfentanyl (Fig. 3; Panel A: significant effect of treatment ($F_{4700} = 31.27$, $p < 0.0001$), time ($F_{19,700} = 42.22$, $p < 0.0001$) and time x treatment interaction ($F_{76,700} = 7.118$, $p < 0.0001$)) and ocfentanil (Fig. 3; Panel C: significant effect of treatment ($F_{4700} = 58.98$, $p < 0.0001$), time ($F_{19,700} = 47.80$, $p < 0.0001$) and time x treatment interaction ($F_{76,700} = 9.917$, $p < 0.0001$)) in the 0.1–15 mg/kg range of doses. 2-furanylfentanyl and ocfentanil at 1 mg/kg rapidly increased the total distance travelled in mice, and the effect lasted up to 90 min. By increasing the dose, both compounds at 6 mg/kg transiently reduced spontaneous locomotion at 30 min minutes after injection, while they enhanced locomotion from about 60 to 150–180 min. The opioid agonists at the highest dose (15 mg/kg) significantly inhibited the total distance travelled in the first 30 min, while they facilitated locomotion up to 150 min (Fig. 3; Panel A and C). The effects of 2-furanylfentanyl and ocfentanil were no longer evident

Fig. 1. Morphological effects on zebrafish larvae. ZL treated with ocfentanil 10 μM did not show any malformations. 62.5 % of larvae treated with 10 μM 2-furanylfentanyl have two distinct signs of morphological modifications, i.e. hyperpigmentation and a swollen abdomen.

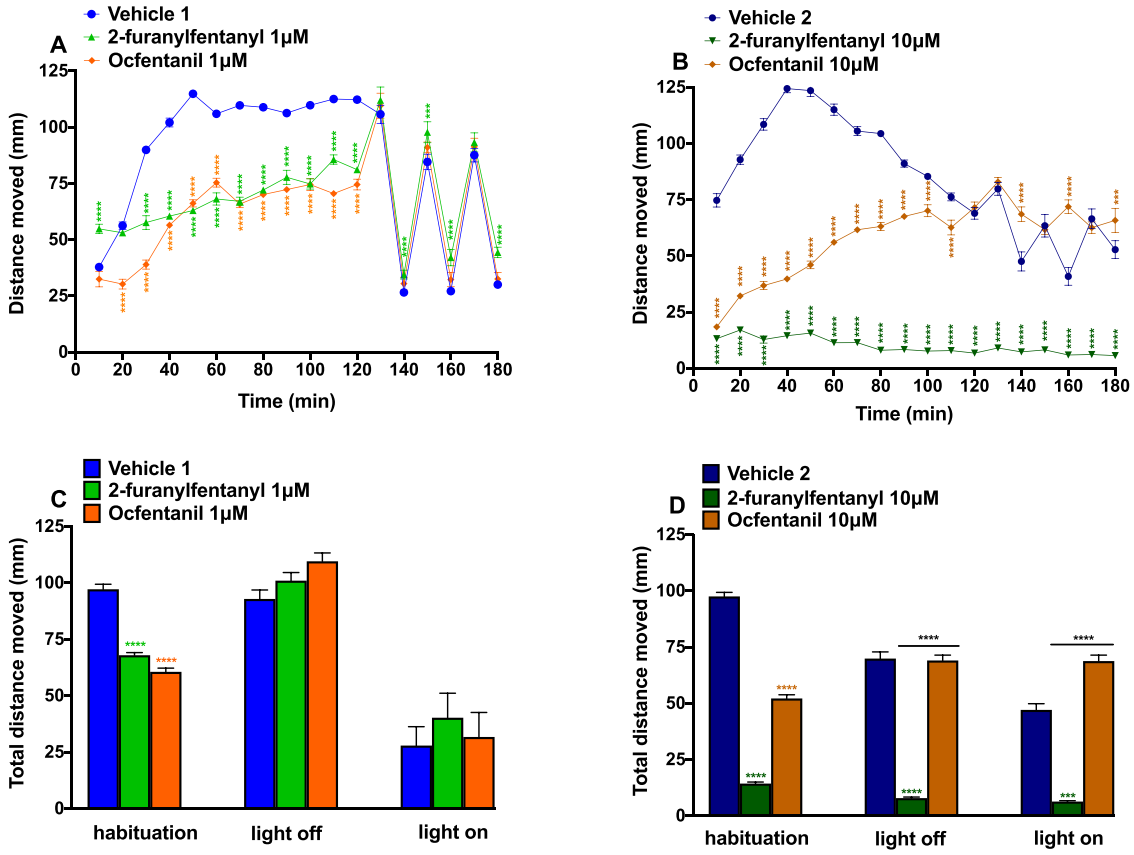

Fig. 2. Behavioural assay on zebrafish larvae. Behavioral response to 2-furanylfentanyl and ocfentanil at the two-concentration level of 1 μM (Panel A, C) and 10 μM (Panel B, D) in ZL. Vehicle 1 and 2 consisted of fish water containing 0.3 % and 3.3 % of methanol, respectively. (A-B) *Light on* time interval: minute 0–120,130–140,150–160,170–180; *light off* time interval: minute 120–130,140–150,160–170. Number of larvae treated: vehicle 1 n = 14, vehicle 2 n = 14, 2-furanylfentanyl 10 μM n = 14, 2-furanylfentanyl 1 μM n = 14, ocfentanil 10 μM n = 14, ocfentanil 10 μM n = 14. Data are expressed as the mean ± SEM of total distance travelled (mm) in the unit of time (10 min). Statistical analysis was performed by two-way ANOVA test followed by a Dunnett's multiple comparison test in Panel A, while in Panel B a two-way ANOVA followed by Tukey's multiple comparison test were performed. (C-D) Data are expressed as the mean ± SEM of the total distance travelled. The bar chart was split in three different conditions namely *habituation*, *light off* and *light on*. Kruskall-Wallis and Dunn's multiple comparison tests were performed to evaluate significant differences within groups and experimental phases. * **p < 0.001, * ** *p < 0.0001.

after 180 min and the spontaneous locomotion of mice was similar in 2-furanylfentanyl- ocfentanil- and vehicle-treated animals at 225 min (Fig. 3; Panel A and C). To reveal a potential effect of 2-furanylfentanyl and ocfentanil under these experimental conditions, the motor activity of the mouse was stimulated mechanically (as described in materials and methods).

Mechanical stimulation of the mouse promoted a transient but significant increase in the motor activity of the vehicle-treated animal at 225 min (Fig. 3; Panel B and D) while the groups of animals treated with 2-furanylfentanyl and ocfentanil showed a persistence of the inhibitory effect of the opioid on motor activation.

In fact, mechanically stimulated motor activity was completely reduced in the group treated with 1–15 mg/kg 2-furanylfentanyl ($F_{9,\ 70}$ = 11.73; P < 0.0001; Fig. 3; Panel B). Ocfentanil partially prevented (at 1 mg/kg) and completely reduced (at 6 and 15 mg/kg) the mechanically stimulated motor activity in mice ($F_{9,\ 70}$ = 19.22; P < 0.0001; Fig. 3; Panel D). The observed discrepancies between the two compounds at the dose of 1 mg/kg could be related to differences in their pharmacokinetics in response to various motor tests (Bilel et al., 2022).

3.3. Identification and analysis of metabolites

The analysis of 2-furanylfentanyl and ocfentanil metabolites was conducted using LC-HRMS acquiring data in full-scan mode. The identification of metabolites was performed by considering accurate mass (± 5 ppm), retention times and MS/MS spectra.

The current study identified a total of six metabolites of 2-furanylfentanyl (Table 1). The corresponding metabolic pathway is shown in Fig. 4. On the bases of the relative amount of peak area, in larvae extracts the main metabolites were nor-furanylfentanyl (M1) and hydroxy-furanylfentanyl (M2), resulting from the hydroxylation on the phenethyl group and the corresponding conjugated form (M2′). Other minor metabolites detected were M3, obtained from N-oxidation on the piperidine ring, hydroxy-methoxyfuranylfentanyl (M4) and dihydrodiol metabolite (M5), resulting from hydration on the furan ring. In contrast with ZL, only dihydrodiol metabolite and M2′ were identified in mice urine.

On the other hand, seven ocfentanil metabolites were detected in the two animal models, resulting from hydroxylation or/and demethylation of the parent drug (Table 2). The mono-hydroxylation occurred in three different positions in larvae, i.e., at the phenyl ring of the phenethyl moiety (M1), in β-position of the ethyl linker (M2) and at the piperidine ring (M3). On the contrary, in mice urine only M4, resulting from hydroxylation in ω-position, was detected. O-desmethyl ocfentanil (M5), corresponding to the loss of methyl of the methoxy group, was also identified in zebrafish larvae as well as in mice urine, representing in the latter the most abundant metabolite along with its conjugated form (M5′). Another hydroxylation occurred in ZL at the fluorophenyl moiety of M5, forming M6 (hydroxy-O-desmethyl-ocfentanil) (Fig. 5). Strangely enough, two of the most common biotransformations described in fentanyl and in fentanyl analogs, namely N-dealkylation and the formation of 4-ANPP (flouro-despropionylfentanyl), were not detected in the two *in vivo* models tested.

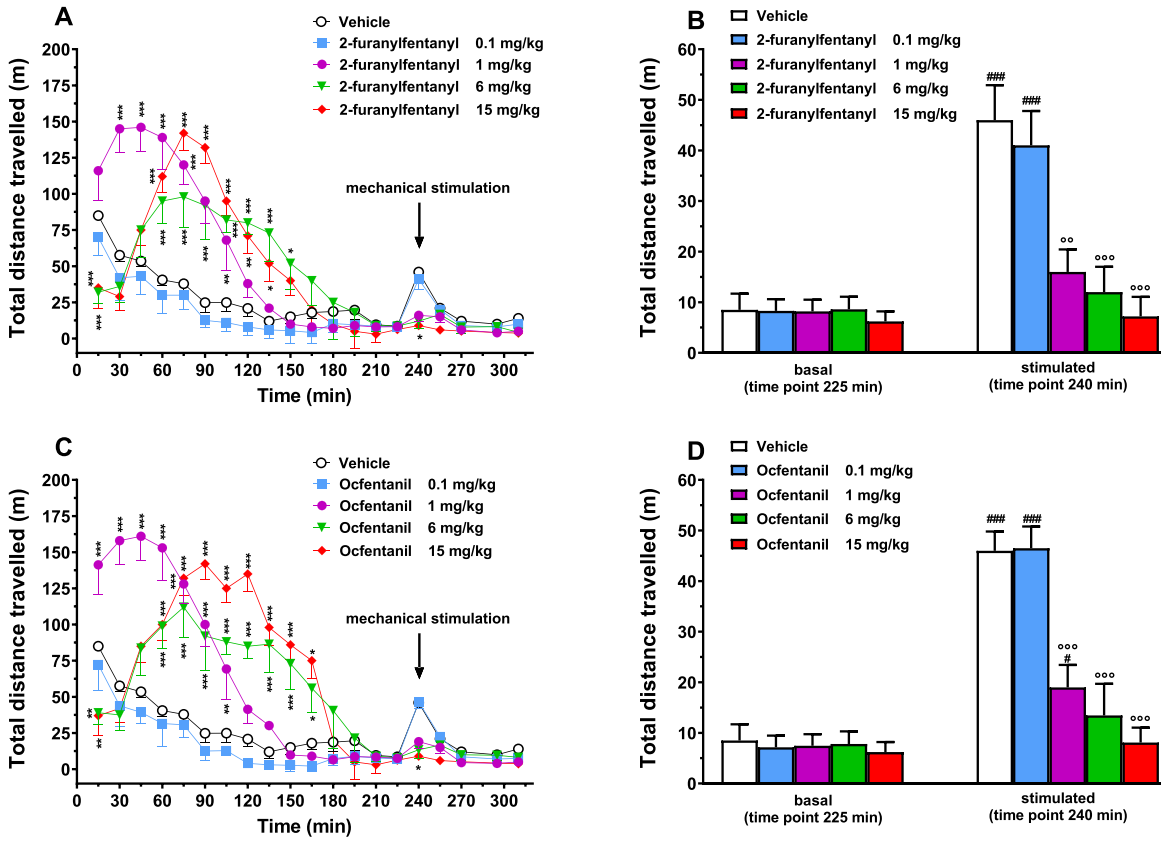

Fig. 3. Behavioural effects on mice. Effect of the systemic administration of 2-furanylfentanyl (0.1–15 mg/kg i.p.; Panel A, B) and ocfentanil (0.1–15 mg/kg i.p.; Panel C, D) on the total distance travelled by the mouse. Data are expressed as metres travelled and represent the mean ± SEM of 8 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the dose response curve of each time point (Panel A, C) and by one-way ANOVA followed by Tukey's test for multiple comparisons for the dose response curve of basal (225 min) versus stimulated responses (240 min; Panel B, D). *p < 0.05, * *p < 0.01, * **p < 0.001 versus vehicle; #p < 0.05, ###p < 0.001 versus basal; °°p < 0.01, °°°p < 0.001 versus stimulated responses.

Table 1
List of 2-furanylfentanyl metabolites tentatively identified in zebrafish larvae and mice with the proposed fragmentation spectra. The metabolites are rated from + to + ++ + according to their peak areas (n.d.: not detected).

Compounds			Zebrafish Larvae (Extract)			Mice (Urine)		
Name	*Chemical formula*	*Precursor ion*	*RT*	*Major products*	*%*	*RT*	*Major products*	*%*
2-furanylfentanyl	$C_{24}H_{26}N_2O_2$	375.2068	8.6	188.1558 105.0703	+++	8.6	188.1467 105.0722	+++
M1	$C_{16}H_{18}N_2O_2$	271.1384	7.0	188.0633 170.1110	++	n.d.		
M2	$C_{24}H_{26}N_2O_3$	391.2016	7.9	204.1391 121.0653	++	n.d.		
M3	$C_{24}H_{26}N_2O_3$	391.2016	8.7	105.0681 283.1390	+	n.d.		
M4	$C_{25}H_{29}N_2O_4$	421.2122	7.9	234.1485 151.0754	+	n.d.		
M5	$C_{24}H_{28}N_2O_4$	409.2124	7.9	188.1380 105.0653	+	8.0	188.1438 105.0701	+++
M2'	$C_{30}H_{24}N_2O_9$	567.2337	7.4	391.1900 204.1320	++	7.5	391.1649 204.1181	++

4. Discussion

The present study reports for the first time a comparison of the effect of two potent fentanyl analogs, i.e. 2-furanylfentanyl and ocfentanil, on the locomotor activity and metabolism in two different animal models, namely ZL and mice. The results are discussed taking also into consideration those previously obtained with fentanyl in similar studies (Kirla et al., 2021; Pesavento et al., 2022).

4.1. Locomotor activity and morphology in ZL

Overall, larvae locomotor activity was impaired after the treatment with the two opioids in both habituation and stimulation phase. In particular, at the lowest concentration (1μM), both 2-furanylfentanyl and ocfentanil decreased the locomotor activity of ZL. A significant dose-dependent reduction in activity due to fentanyl and fentanyl analogs at a comparable concentration was also recently reported in a similar behavioural study in ZL (Kirla et al., 2021; Pesavento et al., 2022).

In this study, it was also demonstrated that a high concentration (10 μM) of 2-furanylfentanyl and ocfentanil induced an inhibitory locomotor effect in ZL in habituation, light on and light off phases. Moreover, the vehicle used for higher concentration induced a slight increase of the locomotion in ZL. This effect could be related to an enhancement of the direct toxicity exerted by the two compounds (Fu et al., 2017) or may also be caused by the synergic effect of methanol present in the vehicle. To investigate the possible role of methanol, a specific study was performed. The influence of methanol on the locomotor activity in ZL was

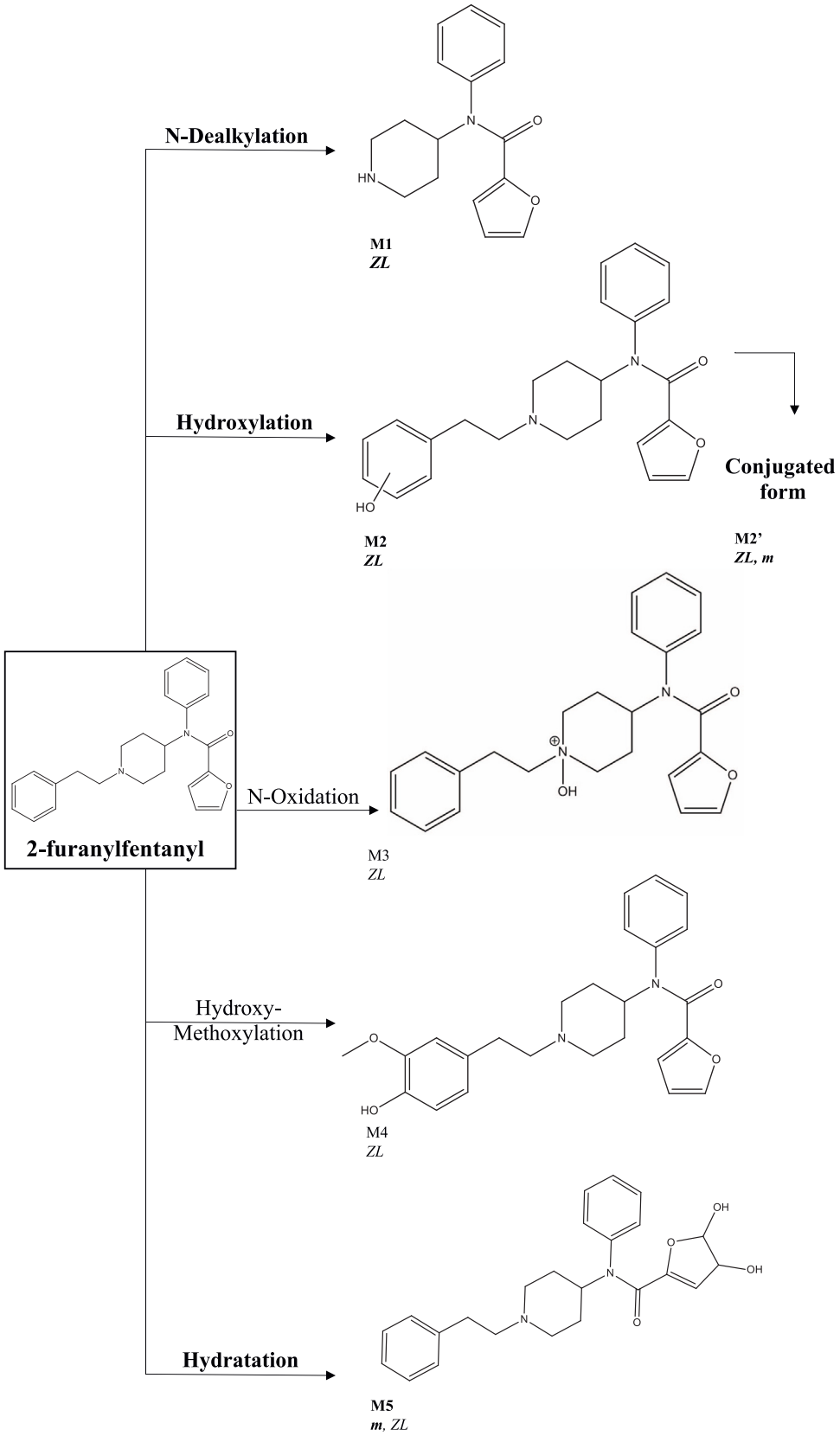

Fig. 4. . 2-furanylfentanyl metabolism. Schematic representation of proposed metabolic pathway of 2-furanylfentanyl, in mice (*m*) and zebrafish larvae (*ZL*). The main metabolic reactions and the resulting metabolites are highlighted in bold.

Table 2
List of ocfentanil metabolites tentatively identified in zebrafish larvae and mice with the proposed fragmentation spectra. The metabolites are rated from + to + ++ + according to their peak areas (n.d.: not detected).

Compounds			Zebrafish Larvae (Extract)			Mice (Urine)		
Name	*Chemical formula*	*Precursor ion*	*RT*	*Major products*	*%*	*RT*	*Major products*	*%*
Ocfentanil	$C_{22}H_{27}FN_2O_2$	371.2135	7.9	188.1504 105.0697	+++	7.9	188.1426 105.0694	+++
M1	$C_{22}H_{27}FN_2O_3$	387.2084	7.2	204.1470 121.0700	++	n.d.		
M2	$C_{22}H_{27}FN_2O_3$	387.2084	8.2	279.1510 105.0697	++	n.d.		
M3	$C_{22}H_{27}FN_2O_3$	387.2084	7.5	204.1294 186.1181	+	n.d.		
M4	$C_{22}H_{27}FN_2O_3$	387.2084	n.d.			8.7	371.2512 89.0641	+
M5	$C_{21}H_{25}FN_2O_2$	357.1978	7.6	188.1459 105.0703	++	7.6	188.1443 105.0706	+++
M6	$C_{21}H_{25}FN_2O_3$	373.21020	7.9	189.1416 105.0672	+++	n.d.		
M5'	$C_{27}H_{33}FN_2O_8$	533.2299	n.d.			7.2	357.2093 188.1499	++++

evaluated by incubating larvae with fish water containing 0, 0.3 % and 3.3 % methanol (Fig. S1). As shown in the figure, larvae seem not to be sensitive to methanol, except for a slight effect observed with methanol at 3.3 %. However, the inhibition of locomotor activity exerted by ocfentanil and 2-furanylfentanyl at the concentration of 10 μM observed in Fig. 2 is clearly higher than that related to the presence of methanol.

In ZL the effect of 2-furanylfentanyl was more significant than that induced by ocfentanil, but similar to the effect of fentanyl at the same concentration (Pesavento et al., 2022). These differences could be related to a higher toxicity of 2-furanylfentanyl compared to ocfentanil, at least at high doses. In fact, in the morphological test, 2-furanylfentanyl induced malformation in ZL at the dose of 10 μM. In particular, the administration of this drug produced abdominal swelling, which has been reported as a common sign of acute toxicity in zebrafish (Cooman et al., 2021). Moreover, it is known that the yolk sac lipophilicity provides an ideal compartment for the accumulation of hydrophobic toxicants (Sant and Timme-Laragy, 2018). Therefore, the higher hydrophobicity of 2-furanylfentanyl could increase the accumulation of this drug in the yolk sac, causing the observed morphological toxicity. On the contrary, no alterations were observed after incubation with ocfentanil or in fish water containing methanol. A further explanation could be that the malformations occurring after the administration of 2-furanylfentanyl 10 μM, could create by itselves create a motor impairment, hence creating hypolocomotion in the behaviour assay.

4.2. Locomotor activity in mice

The administration of 2-furanylfentanyl and ocfentanil in mice (0.1–15 mg/kg) induced a significant impairment in motor activity, in particular at the dose of 1 mg/kg. These data are in accordance with the recent study of Varshneya et al. (2023) reporting the effect of ocfentanil, and also with our previous studies (Pesavento et al., 2022; Bilel et al., 2022). In our previous study, 2-furanylfentanyl did not induce a stimulation of motor activity in mice at the dose of 1 mg/kg in the accelerod test. However, in the open field test the effect of 2-furanylfentanyl is facilitator. This contradiction could be related to a difference in the efficacy of the two compounds on the mu opioid receptors in response to the motor test used (Bilel et al., 2022). Indeed, a recent study by Santos et al. (2022) revealed that the hyper-locomotor effect of opioids, and in particular of fentanyl, depends on their efficacy on the mu opioid receptors (Santos et al., 2022). In addition, it was suggested that the facilitation of locomotion by opioid agonists is triggered by the activation of the mesolimbic dopaminergic system, which is regulated by the endogenous opioid system, controlling the release of dopamine in the nucleus accumbens (Di Chiara and Imperato, 1988; Matsui et al., 2014). Yet, a recent study by Torralva et al. (2020) revealed that highly potent fentanyl interacts with dopamine (D1 and D4) receptors in the low micromolar range. In addition, fentanyl interacts also with serotonin receptors (5-HT1A) at low concentrations (Martin et al., 1991; Torralva et al., 2020). On these grounds, ocfentanil and 2-furanylfentanyl could increase locomotion involving the opioid-serotonin system, as highlighted by Gurtu (1990)). Additionally, we inserted a mechanical stimulation at 240 min in the open field test to reveal potential persistence or disappearance of motor impairments in mice. Our results revealed that mechanically stimulated motor activity was completely reduced with 2-furanylfentanyl (1–15 mg/kg) while ocfentanil partially prevented (at 1 mg/kg) or completely reduced (at 6 and 15 mg/kg) mechanically stimulated motor activity in mice. As already reported, the effect of ocfentanil and 2-furanylfentanyl on motor activity (Drag test) persisted up to 5 h (Bilel et al., 2022). The differences seen between the two compounds at the dose of 1 mg/kg could be related to differences in their pharmacodynamics in response to various motor tests (Bilel et al., 2022; Santos et al., 2022). Moreover, the differences in responses to mechanically induced locomotor output could be related to a difference in the action of the two fentanyl analogs on tactile stimulation of the dorsal root ganglia system (Zhang et al., 2015) as demonstrated in the tail pinch test (Bilel et al., 2022).

4.3. Comparison of opioids locomotor effects in ZL and mice

Opioid receptor expression and activation in zebrafish have been demonstrated to be both biologically and pharmacologically comparable to that of rodents and humans (Sanchez-Simon and Rodriguez, 2008; Gonzalez-Nunez and Rodriguez, 2009). Furthermore, zebrafish opioid receptor transcripts could be detected in early development stages (before 3 h post fertilization). In a previous study, we demonstrated that, in contrast to zebrafish, the rodent model showed hyperlocomotion induced by low doses (1 mg/kg) of fentanyl (Pesavento et al., 2022). Our results confirm previous findings concerning differences in locomotor effects induced by opioids between the two models. In ZL the effect seems to be mediated by the activation of the mu opioid receptor, as highlighted by Zaig et al., (2021). Different factors can account for these differences. For instance, an important element to be considered in the comparison between behavioural effects in ZL and mice is developmental stage. Indeed, reduced swimming activity in ZL treated with fentanyl analogues could be related to a higher sensitivity of embryos to toxicants compared with adult mice (Ducharme et al., 2015). In addition, zebrafish hypomotility could be related to earlier respiratory depression compared to mice at low concentrations of fentanyl and its analogs. In particular, differently from mammalians, ZL have a complex respiratory system, which they use to move water through their gills for oxygen absorption and carbon dioxide elimination (Zaig et al., 2021). Furthermore, differences in locomotor effects induced by 2-furanylfentanyl and ocfentanil between the two animal models could be related to differences in metabolism, as discussed below.

Moreover, Kaig and his colleagues revealed that fentanyl induced respiratory depression in ZL and this effect was reversed by a high concentration (20 μM) of the mu opioid receptor antagonist naloxone (Zaig et al., 2021).

4.4. Metabolism of 2-furanylfentanyl and ocfentanil in ZL and mice

According to the metabolic assay performed with the same animal models for fentanyl (Pesavento et al., 2022), ZL and mice showed

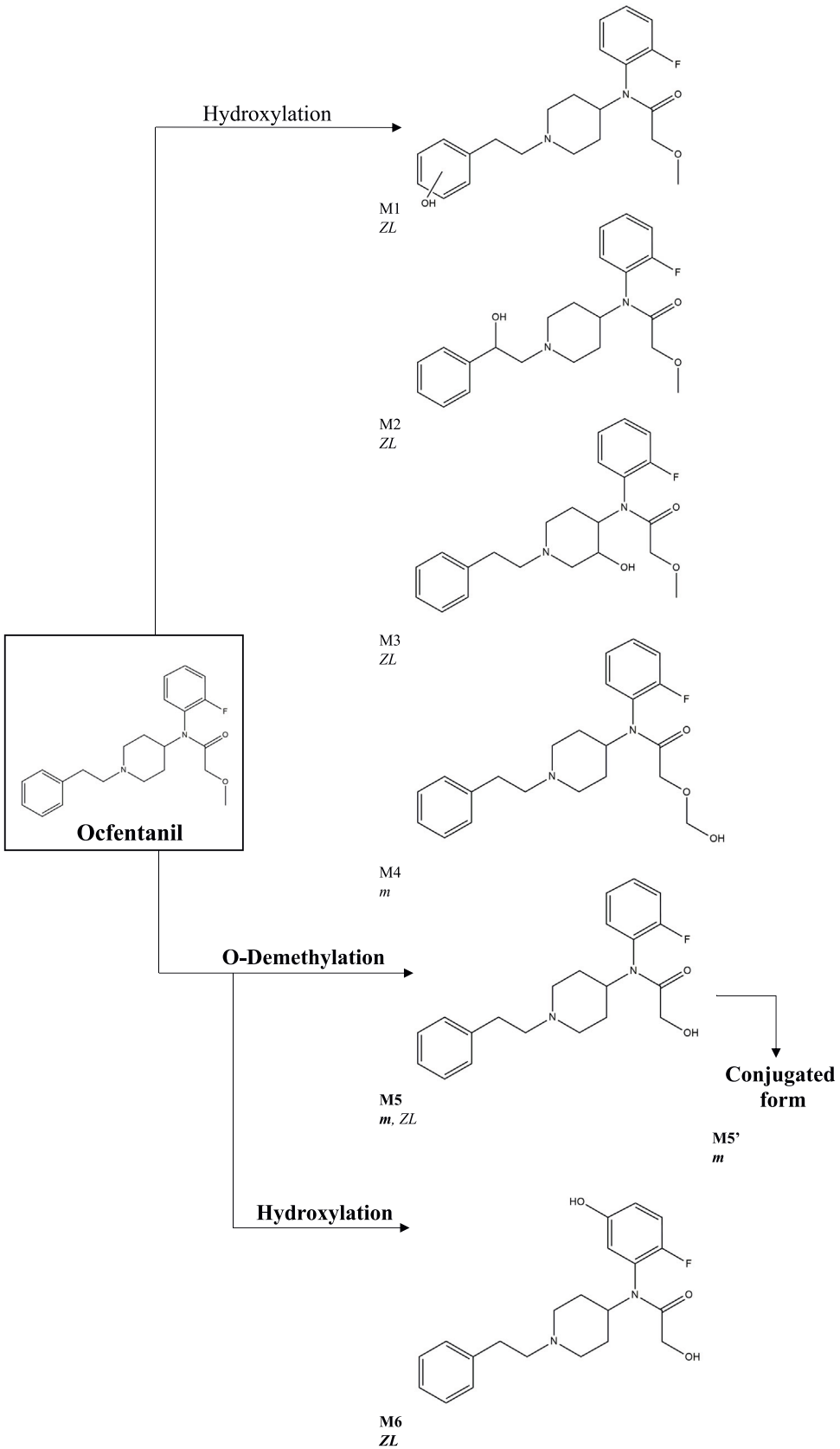

Fig. 5. Ocfentanil metabolism. Schematic representation of proposed metabolic pathway of ocfentanil, in mice (*m*) and zebrafish larvae (*ZL*). The main metabolic reactions and the resulting metabolites are highlighted in bold.

relatively similar metabolic pathways. In particular, the analysis of larvae extracts identified a large number of phase I metabolites, mostly due to hydroxylation, hydration and oxidation, for 2-furanylfentanyl. On the contrary, mice mainly seemed to produce the dihydrodiol metabolite. The major route of metabolism for ocfentanil in ZL was hydroxylation, while in mice it was o-demethylation. The two models produced phase II metabolites, i.e., glucuronide metabolites. These results are partially consistent with previous studies on 2-furanylfentanyl metabolism in different experimental models (i.e. microsomes and human urine), where the major route of metabolism was represented by hydroxylation and formation of dihydrodiol metabolite (Gaulier et al., 2017; Goggin et al., 2017; Kanamori et al., 2021; Watanabe et al., 2017). However, no 4-anilino-N-phenethylpiperidine (4-ANPP) was observed, pointing out that this common metabolite of fentanyl is not present in its analog. This difference might be due to a lack of the amidase enzyme involved in the production of 4-ANPP in ZL and in mice (Watanabe et al., 2017). On the contrary, ocfentanil results in mice and ZL are consistent with the metabolic pathway proposed by Allibe et al. (2018), in which the major route of metabolism *in vitro* and in biological samples was represented by hydroxylation, demethylation and glucuronidation, whereas 4-ANPP was not described and nor-ocfentanil was elucidated only in human liver microsomes, but not in biological samples. Our results confirm the validity of ZL for the identification of NSOs metabolites, even though they showed small differences in metabolism when compared to mice, which could be related to the different route of exposure as suggested for other opioids (Kirla et al., 2021).

5. Conclusions

The present study evaluates behavioural effects, toxicity and metabolism of 2-furanylfentanyl and ocfentanil, in ZL and mice. Consequently, in our opinion, the results prove that ZL can provide, at least in a preliminary phase, useful information on different pharmaco-toxicological aspects of NSO and consequently can be regarded as a suitable animal model for large-scale screening of the newly introduced clandestine drugs (tentatively also non-opioid), to be performed before the completion of more formal studies in mammals.

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CRediT authorship contribution statement

Bilel and **Murari:** data acquisition, Investigation, original draft preparation. **Pesavento:** Investigation, Methodology, original draft preparation. **Arfè** and **Tirri:** investigation. **Torroni:** Formal analysis. **Marti** and **Tagliaro:** Supervision, Funding acquisition. **Rossella Gottardo:** Conceptualization, original draft preparation, final editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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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.neuro.2023.01.003.

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