Original Experimental

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Revisiting opioid toxicity: Cellular effects of six commonly used opioids

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Abstract
Objectives – There is an ongoing opioid crisis in the United States where the illicit and non-medical use of prescription opioids is associated with an increasing number of overdose deaths. Few studies have investigated opioid-induced effects on cell viability, and comparative studies are limited. Here, we examine the toxicity of six commonly used opioids: methadone, morphine, oxycodone, hydromorphone, ketobemidone, and fentanyl with respect to mitochondrial and membrane function *in vitro*.
Methods – The opioids were tested in four different cell cultures: primary cortical cell cultures, human neuroblastoma SH-SY5Y cells, and both differentiated and undifferentiated neuroblastoma/glioma hybrid NG108-15 cells. The mitochondrial activity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the membrane integrity was assessed by measuring the leakage of lactate dehydrogenase. To compare the different opioids, the toxic dose (TD_{50}) was calculated.
Results – The results displayed a similar trend of opioid-reduced cell viability in all four cell cultures. The most toxic opioid was methadone, followed by fentanyl, while morphine was overall ranked as the least toxic opioid displaying little to no negative impact on cell viability. The remaining opioids varied in rank between the different cell types.
Conclusion – This *in vitro* study highlights opioid-dependent variations in toxicity across all four tested cell types, with methadone emerging as the most potent opioid.

Keywords: methadone, morphine, fentanyl, toxic effects, cell viability, mitochondrial activity

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Abbreviations

dbcAMP	Dibutyryl cAMP
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
TD_{50}	Toxic dose 50%
LDH	Lactate dehydrogenase
MOR	Mu-opioid receptor
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBM	Neurobasal medium
NMDA	*N*-methyl-D-aspartate
PCC	Primary cortical cell
RT	Room temperature
TX	Triton-X 100

1 Introduction

Inappropriate prescription of opioids, as reported from several countries, is described as one of the main reasons

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for the dramatically increased opioid use and prescription-opioid-related mortality [1]. The actual recommendation from the International Association of Pain (IASP) is to use opioids in chronic pain patients sparsely and only in exceptional cases, in restricted doses, and only over a short period of time [2]. In the United States, the overall prevalence of overdose deaths increased from 8.2 to 32.6 deaths per 100,000 standard population from 2002 to 2022 [3]. There are also worrying trends seen in the Scandinavian countries, as oxycodone prescription has increased in Denmark, Norway, and Sweden [4,5], with recent data from Sweden showing a slight increase in oxycodone-related deaths [6].

The toxic effects associated with clinically used opioids generally involve effects associated with a negative impact on cell function, cell survival, cognition, and the immune system. For instance, acute treatment with morphine induces apoptosis in primary hippocampal cells from mice [7], and in NG108-15 cells, a mouse/rat hybrid neuroblastoma/glioma cell line [8]. Similar effects of methadone are also reported, where acute treatment with high concentrations of methadone causes cell death in both human-derived neuronal SH-SY5Y cells [9] and in primary cortical rat cells [10,11], possibly through an *N*-methyl-D-aspartate (NMDA) receptor-mediated pathway [11]. Furthermore, we have previously demonstrated that 100 μM of both methadone and fentanyl negatively affect mitochondrial viability as early as 90 min after exposure [12]. In addition, there is evidence that chronic treatment with morphine inhibits neurogenesis in rat hippocampus [13], induces apoptosis in the spinal cord of rats [14], and alters the volumetric size of different regions in the brain [15]. These effects on the brain may impact higher cognitive function, such as learning and memory, which several studies with methadone have demonstrated [16–20]. Similarly, opioid-induced cell death of immune cells may lead to a suppressed immune system, which is generally observed in patients treated with opioids [21,22].

However, evidence supporting the claim that opioids may impact cognitive function or cell viability is scarce and few comparative studies using different opioids exist. The present study therefore aims to explore the effects on cell viability in a comparative *in vitro* approach following a 24-h acute treatment of six commonly used opioids; methadone, morphine, oxycodone, hydromorphone, ketobemidone, and fentanyl, in four different neuronal cell cultures.

2 Materials and methods

The following cell cultures, with varying characteristics to represent a greater variability among cells, were used: primary rat cell cultures, undifferentiated and differentiated mouse/rat NG108-15 cells, and human undifferentiated SH-SY5Y cells. All cultures were incubated at 37°C with 5% CO_2 in a humidified incubator, and 96-well plates were precoated with 50 μg/mL poly-D-lysine (Sigma Aldrich).

2.1 Primary cortical cell (PCC) cultures

All animal experiments were approved by the Uppsala Animal Ethics Committee (5.8.18-18550/2018) according to Swedish guidelines regarding animal experiments (Animal Welfare Act SFS1998:56) and the European Communities directive (86/609/EEC).

Mixed PCCs were harvested from embryonic day 17 fetuses from pregnant Wistar rats (Charles River, Sulzfeld, Germany) as described elsewhere [23]. Briefly, cortical tissue was dissected and digested using 0.2 mg/mL trypsin (Sigma-Aldrich) for 10 min at 37°C. Tissues were centrifuged at 3,200 rpm for 3 min and trypsin was inhibited using 0.52 mg/mL trypsin inhibitor (Sigma-Aldrich) prior to mechanical dissociation. The cell pellet was resuspended in neurobasal medium (NBM; Thermo Fisher Scientific, Waltham, USA) containing 0.5 mM GlutaMAX™ (Thermo-Fisher Scientific), 2% (v/v) B27 (Thermo-Fisher Scientific), 100 U/mL penicillin/streptomycin (Thermo-Fisher Scientific), and 10% (v/v) fetal bovine serum (FBS; Thermo-Fisher Scientific). Cells were counted using Countess™II Automated Cell Counter (Thermo-Fisher Scientific) and seeded at 1×10^5 cells per well in 96-well plates. Media was replaced the next day with serum-free NBM + 2% B27. Cells grew for 7 days *in vitro* with a partial media change on day 4. The cultures contained approximately 84% neurons (beta-III tubulin-positive).

2.2 NG108-15 and SH-SY5Y cell lines

The NG108-15 cell line (kind gift from Dr. Malin Jarvius, Uppsala University) is a hybrid mix of mouse neuroblastoma and rat glioma [24] and the SH-SY5Y cell line (kind gift from Dr. Anne-Lie Svensson, Uppsala University) is derived from human neuroblastoma. Both of these cell lines share properties similar to neuronal cell cultures. NG108-15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo-Fisher Scientific) supplemented with 10% (v/v) FBS, 100 U/mL penicillin/streptomycin, and 2% (v/v) Gibco® HAT supplement (Thermo-Fisher Scientific) containing 5 mM sodium hypoxanthine, 20 μM aminopterin, and 0.8 μM thymidine. SH-SY5Y cells were cultured in a minimum essential medium (Thermo-Fisher Scientific) supplemented with 10% FBS and 100 U/mL penicillin/streptomycin. Media changes

were performed every 2–3 days and cells were subcultured at approximately 80% confluency using 0.25% trypsin-EDTA (Thermo-Fisher Scientific). Prior to the start of the experiment, undifferentiated NG108-15 and SH-SY5Y cells were seeded to a 96-well plate at a density of 1.5×10^4 cells per well. The plates were placed in an incubator overnight to ensure proper adhesion to the wells. Additional 96-well plates with NG108-15 cells were cultured in differentiation media containing DMEM supplemented with 2% (v/v) FBS, 100 U/mL penicillin/streptomycin, and 1 mM dibutyryl cAMP (dbcAMP, Sigma-Aldrich) for 3 days. All experiments were performed within 10 passages.

2.3 Immunocytochemistry

For immunocytochemistry, cells were seeded to black 96-well plates. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT), permeabilized with Tween-20, and blocked using normal donkey serum (Sigma-Aldrich). To examine the mu-opioid receptor (MOR) expression in PCC, NG108-15, and SH-SY5Y cells, 1:100 of the rabbit anti-MOR (Abcam, Cambridge, USA) was added to the cells and incubated for one hour at RT. A fluorescent-conjugated secondary antibody (Alexa488, Sigma-Aldrich) was added and incubated for another hour in RT, protected from light. Lastly, cells were counterstained using nuclei marker 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Images were acquired using ImageXpress (Molecular Devices, San Jose, USA) at 20× magnification. To evaluate MOR expression, control wells containing only the fluorescent-conjugated secondary antibody were added and images were visually compared.

2.4 Opioid treatment

The opioids methadone (Sigma-Aldrich), morphine (Apoteket AB, Stockholm, Sweden), oxycodone (Sigma-Aldrich), hydromorphone (Sigma-Aldrich), ketobemidone (Apoteket AB), and fentanyl (Sigma-Aldrich) were added to the cells at 1, 10, 100, and 1,000 µM in triplicates for 24 h. Control cells were treated with media only. As a positive control for cytotoxicity, 1% Triton X-100 (TX) was added in triplicates to ensure full functionality of the cell viability assays.

2.5 Mitochondrial function assay

The mitochondrial function was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MTT is metabolized to a purple formazan product in active mitochondria and acts as a marker for mitochondrial function. After 24 h of opioid treatment, 1 mg/mL MTT (Sigma-Aldrich) was added to each well and incubated for 30 min prior to lysing the cells with dimethyl sulfoxide. The absorbance of the formazan product was quantified using a plate reader (FLUOstar Omega, Ortenberg, Germany) at 570 nm.

2.6 Membrane integrity assay

The membrane integrity was assessed using the lactate dehydrogenase (LDH) assay. Briefly, LDH is a cytosolic enzyme that is released from cells during membrane damage and therefore acts as a marker for membrane integrity. After 24 h of opioid treatment, 50 µL media from each well was transferred to a new empty 96-well plate. A cytotoxicity detection kit (Sigma-Aldrich) was prepared according to instructions from the manufacturer, added to each well, and incubated for 30 min at RT, protected from light. The reaction mix metabolizes LDH to a red formazan product, correlating to the amount of LDH present in cell media. To quantify the levels of membrane integrity, the absorbance of the red formazan product was detected at 492 nm using a plate reader.

2.7 Statistical analysis

Statistical analysis was conducted in GraphPad Prism (v.9.3.1). PCCs harvested from one individual rat, and cells from one NG108-15 or SH-SY5Y passage were each considered one culture (n = 1). Results were normalized to the percent of control to account for culture variability. Data were analyzed by non-linear regression to obtain a best-fit value of the toxic dose 50% (TD_{50}) for each opioid, the dose causing severe dysfunction in 50% of the cell population. Data are presented as mean log TD_{50} ± standard deviation and mean TD_{50} in table format, and graph data points are presented as mean ± standard error of the mean (SEM). The calculated TD_{50} values were used to rank the toxicity of the different opioids.

3 Results

3.1 Immunocytochemistry

In all tested cell types, PCC cultures, NG108-15, and SH-SY5Y cell line, a clear MOR intensity was detected in the cell cytoplasm (Figure 1). These results confirm that the MOR is expressed in all tested cell types.

3.2 Mitochondrial function

The results of the calculated best-fit TD_{50} value from each opioid treatment and cell type (as assessed using the MTT assay) are presented in Table 1. For PCC cultures, undifferentiated and differentiated NG108-15 cells, and the SH-SY5Y cells, methadone treatment had the lowest calculated TD_{50} in comparison to the other opioids. Fentanyl treatment had the second lowest TD_{50} value followed by ketobemidone or oxycodone, depending on cell type. Morphine had the highest TD_{50} values of all the opioids in PCC cultures, differentiated NG108-15 cells, and SH-SY5Y cells and can be considered to induce little to no damage to the mitochondrial function. For undifferentiated NG108-15 cells, oxycodone had the highest TD_{50} value, although very similar to morphine. The nonlinear regression curves of the two opioids with the highest TD_{50} values (methadone and fentanyl), as well as the opioid with the lowest TD_{50} (morphine), are illustrated in Figure 2 while graphs of the other opioids (ketobemidone, oxycodone, and hydromorphone) are illustrated in Figure S1, Supplementary material.

3.3 Membrane integrity

The opioid treatments induced low cytotoxicity and the nonlinear regression curves were ambiguous, and as a result, the TD_{50} values calculated from this assay were not applicable. Thus, the data from this assay are not included in the ranking of opioids and no further statistical analysis was performed. For a calculated mean percentage of cytotoxicity for control, 100 and 1,000 µM, see Table S1, Supplementary material.

4 Discussion

This study reveals differences in opioid-induced cell viability among methadone, morphine, oxycodone, hydromorphone,

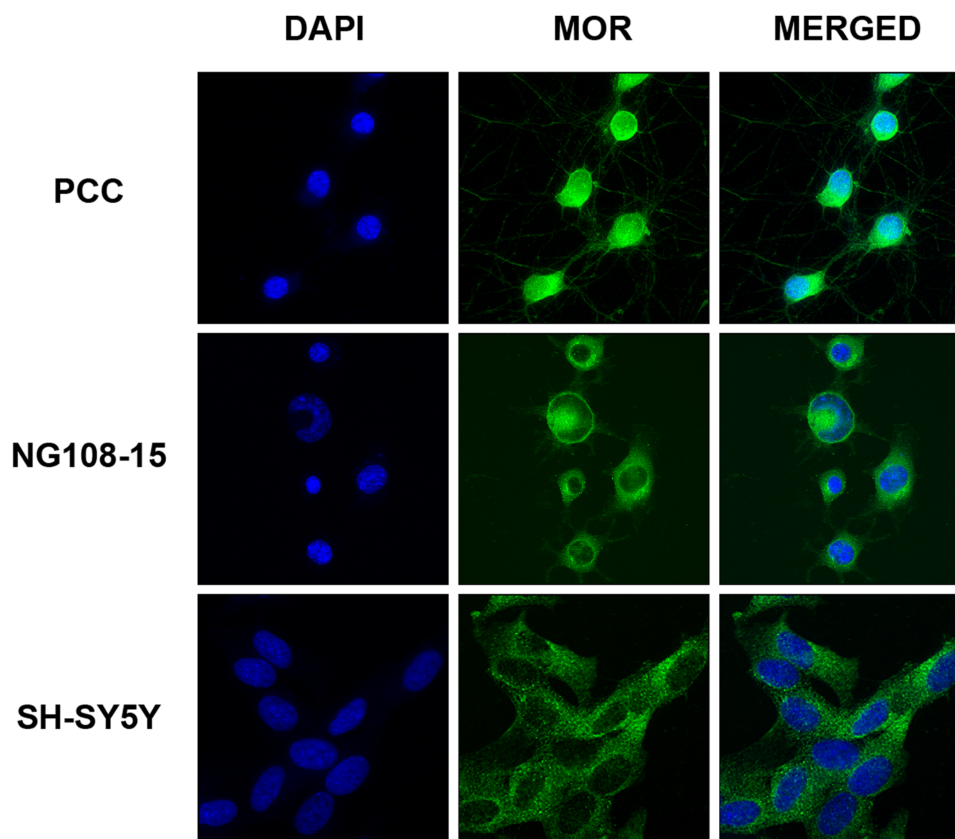

Figure 1: MOR immunocytochemistry. PCC cultures, NG108-15, and SH-SY5Y cells were exposed to a primary antibody targeting the MOR and visualized using a fluorescent-conjugated secondary antibody (Alexa 488). Cells were counterstained with the nuclei marker DAPI. Images were acquired using ImageXpress (Molecular Devices) mounted with a 20× objective and display a clear expression of the MOR in the cell cytoplasm in all tested cell types.

Table 1: Calculated best-fit values of mean log TD_{50} ± standard deviation and mean TD_{50} after nonlinear regression analysis of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolism in percentage of control (n = 4–5) after 24 h opioid treatment with methadone, fentanyl, oxycodone, hydromorphone, ketobemidone, and morphine in PCC, undifferentiated and differentiated NG108-15 cells, and SH-SY5Y cells

Cell type	Opioid	Mean log TD_{50} ± SD	Mean TD_{50}
PCC cultures	Methadone	−3.76 ± 0.05	174 μM
	Fentanyl	−3.54 ± 0.10	292 μM
	Oxycodone	−2.95 ± 0.13	1116 μM*
	Ketobemidone	−2.88 ± 0.13	1311 μM*
	Morphine	−2.27 ± 0.56	5382 μM*
	Hydromorphone	N/A**	N/A**
Undifferentiated NG108-15	Methadone	−4.25 ± 0.13	56 μM
	Fentanyl	−3.94 ± 0.10	115 μM
	Ketobemidone	−3.52 ± 0.13	300 μM
	Hydromorphone	−3.15 ± 0.10	701 μM
	Morphine	−2.94 ± 0.08	1142 μM*
	Oxycodone	−2.94 ± 0.11	1158 μM*
Differentiated NG108-15	Methadone	−4.55 ± 0.09	28 μM
	Fentanyl	−4.03 ± 0.16	94 μM
	Ketobemidone	−3.72 ± 0.11	189 μM
	Oxycodone	−3.27 ± 0.20	538 μM
	Hydromorphone	−3.09 ± 0.15	806 μM
	Morphine	−2.19 ± 0.88	6490 μM*
Undifferentiated SH-SY5Y	Methadone	−4.18 ± 0.10	66 μM
	Fentanyl	−3.67 ± 0.10	213 μM
	Ketobemidone	−3.52 ± 0.15	300 μM
	Oxycodone	−3.46 ± 0.14	350 μM
	Hydromorphone	−3.13 ± 0.09	735 μM
	Morphine	−2.99 ± 0.12	1016 μM*

*Calculated theoretical value, mean TD_{50} exceeds highest concentration used.
**Calculated TD_{50} is not applicable due to ambiguous curve fit.

ketobemidone, and fentanyl. Both mitochondrial function and membrane integrity were assessed as markers for cell viability. Interestingly, the membrane integrity assay showed lower levels of cytotoxicity than the mitochondrial activity assay, and no stable TD_{50} value could be calculated. This suggests that acute opioid exposure more effectively reduces mitochondrial function compared to membrane integrity. There was a toxic effect of methadone at higher concentrations, but it was difficult to compare methadone with the different opioids.

Methadone treatment induced a higher degree of toxicity when compared to all the other opioids studied. In the mitochondrial function assay, the calculated TD_{50} value for methadone was the lowest in all four tested cell types, followed by fentanyl as the second lowest. The ranking of the other opioids (hydromorphone, morphine, ketobemidone, oxycodone) varied between the different cell types, but the highest TD_{50} value was found for oxycodone in undifferentiated NG108-15 and for morphine in the other three cell types. The overall ranking of the ability to decrease mitochondrial function in all different cell types was methadone > fentanyl > ketobemidone > oxycodone > hydromorphone > morphine. Since the four tested cell cultures originate from either rat, mouse, or human, and a similar toxicity profile was observed across the four cell cultures for the different opioids, this indicates that these opioid-induced effects occur in cells of both rodent and human origin.

All of the opioids in the present study display affinity to the MOR [25,26], which was confirmed in this study to be expressed in NG108-15, SH-SY5Y, and PCC cultures using immunocytochemistry. However, the MOR binding K_i-values do not correlate with the degree of induced toxicity between the treatments. The affinity ranking, as described by Volpe and coworkers (excluding ketobemidone), is as follows: hydromorphone (0.3654 nM) > morphine (1.168 nM) > fentanyl (1.346 nM) > methadone (3.378 nM) > oxycodone (25.87 nM) [25]. The affinity K_i-values for ketobemidone were not reported in this study but it is known that ketobemidone has a lower binding affinity for the MOR than morphine [27]. Interestingly, both morphine and hydromorphone display high affinity to the MOR but induce little to no effect on cell viability. This suggests that the cause of the opioid-induced toxicity demonstrated in the present study is not mediated via the MOR, but rather through other signaling pathways. The delta- or kappa-opioid receptor may be involved, but similar to the MOR, both morphine and hydromorphone display high affinity to these opioid receptors as well, in comparison to the other opioids [28–30].

Interestingly, methadone and ketobemidone, two of the most toxic opioids in this study, differ from the other opioids in regard to binding properties, as they are non-competitive antagonists to the NMDA receptor with K_i-values of 0.85 and 26 μM, respectively [31]. It is likely that these opioids induce cell damage via the NMDA receptor when used in higher concentrations. We have previously reported that the opioid receptor antagonist naloxone is unable to prevent methadone-induced mitochondrial dysfunction and that the methadone-induced damage is associated with upregulated mRNA expression of NMDA receptor subunits [11] indicating that methadone-induced toxicity is linked to the NMDA receptor. Furthermore, activation of the NMDA receptor is suggested to be associated with opioid dependence and neuronal toxicity as prolonged treatment with morphine induces apoptosis via activation of the NMDA receptor in opioid-tolerant mice [14]. It is possible that regulation of the NMDA receptor plays a key role in opioid-induced effects on cell viability, as seen from the high doses of methadone treatment in this study, and that

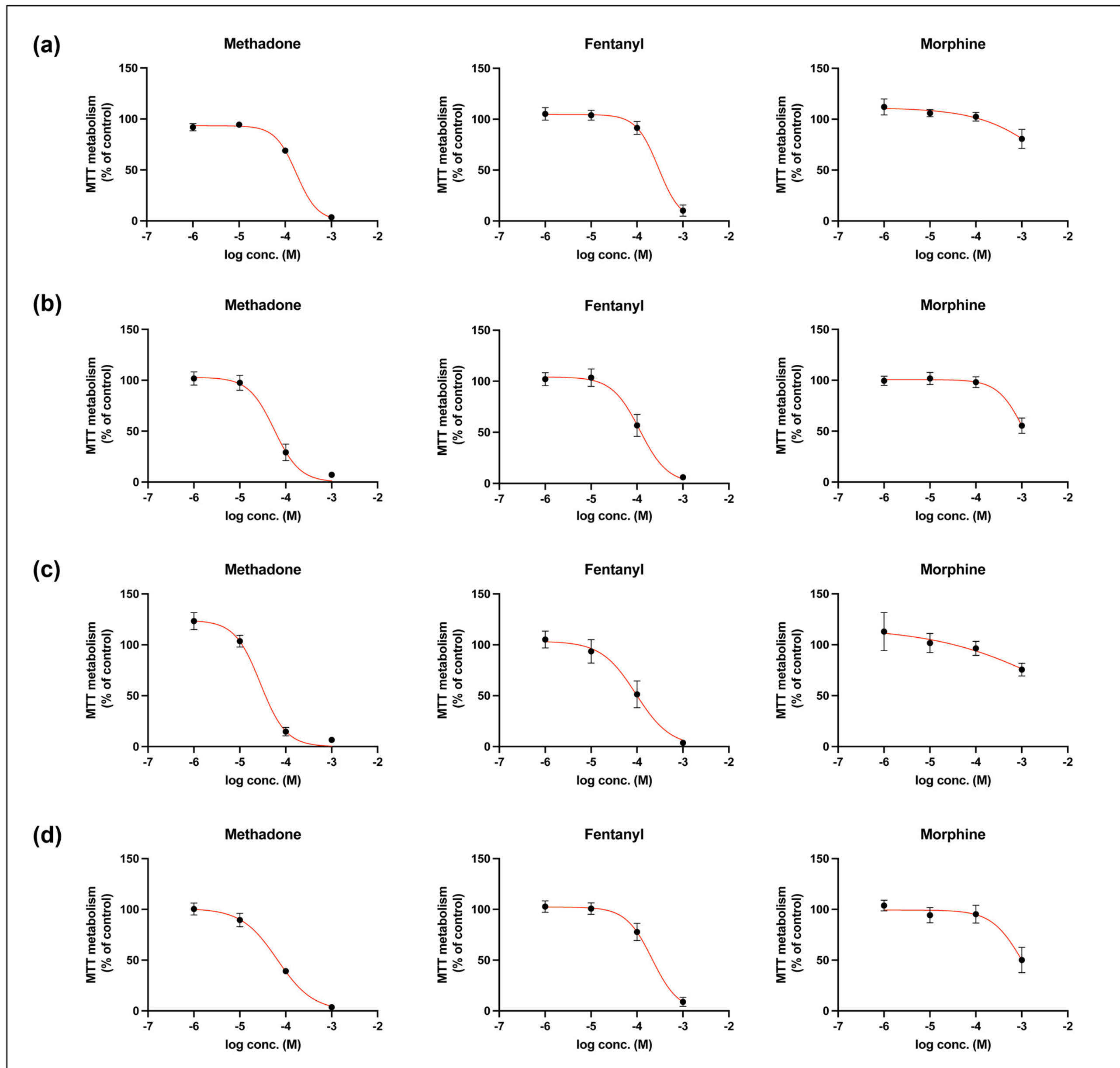

Figure 2: The effect of opioid treatment on mitochondrial activity. The level of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolism after 24 h treatment with the opioids methadone, fentanyl, and morphine in concentrations 1, 10, 100, and 1,000 µM was determined in (a) PCC cultures, (b) undifferentiated NG108-15 cells, (c) differentiated NG108-15 cells, and (d) undifferentiated SY-SY5Y cells. Data were normalized to percentage of control and nonlinear regression analysis was performed to determine the best-fit TD_{50} value for each opioid treatment. The nonlinear fit is shown in red and measured data points are shown in black as mean ± SEM from 4 to 5 different cultures (*n* = 4–5).

this can occur in opioid-tolerant patients consuming opioids long-term.

The results from the present study further confirm the *in vitro* toxicity profile of various opioids. Methadone has previously been shown to induce mitochondrial and membrane damage [10], induce necrosis [9], and upregulate pro-apoptotic proteins [16]. A recent study demonstrated impaired memory function as well as increased astrogliosis and decreased number of neurons in the hippocampus of male rats treated with methadone [32]. Thus, decreased cellular function may be one contributing factor to the reported methadone-induced cognitive dysfunction as seen in both rodents and humans [16–19]. Additionally, previous literature suggest that fentanyl induces cellular toxicity and decreases cell viability [33–35] which is further confirmed in this study. There are few studies describing the cell viability effects of

oxycodone, hydromorphone, and ketobemidone. However, oxycodone has been reported to reduce cell viability in the human neuroblastoma cell line SH-SY5Y [36]. On the contrary, a recent study demonstrated that oxycodone did not affect mitochondrial function [37], and furthermore, using human hepatoma cells, oxycodone treatment did not impact membrane integrity [38]. A similar ambiguity with regard to oxycodone was seen in this study as the opioid seems to be more toxic in the human SH-SY5Y cells compared to the other cell types. A previous comparative study using morphine, fentanyl, and hydromorphone, among others, revealed a similar outcome as the present study whereas fentanyl was more effective in inducing cytotoxicity compared with morphine and hydromorphone [39]. To the best of our knowledge, there have been no published reports on the effects of ketobemidone on cell viability, most likely because this opioid is not commonly used outside the Scandinavian countries. However, there are reports that ketobemidone exerts immunomodulating effects as the opioid has been shown to inhibit chemokines associated with inflammation [40].

The main advantage of the present pre-clinical study is the comparison of various opioids tested in four cell types with different origins, including human and rat. Methadone was associated with the highest degree of toxicity, independent of the cell type used. The cell cultures used in the present study seem to have different vulnerabilities to opioid-induced toxicity, as the TD_{50} values obtained vary based on the type of cell culture used. Overall, the TD_{50} values, as calculated from the mitochondrial function assay, were lower in the cell lines, NG108-15 and SH-SY5Y, in comparison to the PCC cultures. This is likely due to the greater variation in the proportions of different cell types in PCC cultures, in contrast to the uniform cell types present in the cell lines. This is not surprising given that PCC cultures are more biologically relevant in comparison to cell lines as they also contain other cell types that are normally present in the brain. As both the NG108-15 and SH-SY5Y cells originate from neuroblastoma, this indicates that neurons are more sensitive to opioid-induced damage compared to other cell types present in the PCC cultures. Using different types of cell cultures and high opioid concentrations enabled us to detect differences between the opioid treatments and it is possible that some of these effects occur in the brain following the chronic use of opioids in humans.

The present study contains data that are limiting the interpretation of the results, particularly in relation to clinical relevancy. For instance, the TD_{50} values demonstrated in the present study are considered to be very high and it is unlikely that these concentrations correspond to the opioid levels in the brain following oral or parental administration in a clinical setting. For instance, the $C_{\max}$ of methadone in the plasma of patients overdosing on methadone has been reported to reach as high as 4,000 ng/mL, which corresponds to approximately 12 μM [41]. However, it is important to consider that the effects observed in the present study result from an acute 24-h opioid treatment. The obtained TD_{50} values would likely be lower if the opioids were administered repeatedly for a longer time. It is difficult to maintain cells for long-term *in vitro*, and therefore difficult to mimic the opioid administration of a traditional chronic user without compromising the general health of the cultured cells, which in turn also would impact the outcome of the results. Thus, these results need to be further evaluated *in vivo* to confirm the clinical relevance. Another important aspect to consider is that the human-derived cell line SH-SY5Y in the present study was used in a non-differentiated state and thus, the clinical significance of the results from the SH-SY5Y cells needs to be evaluated accordingly. However, as the same trend with regard to opioid-induced toxicity was observed in the PCC cultures and differentiated NG108-15 cells, which are more physiologically relevant, it is likely that these effects occur also in differentiated SH-SY5Y cells.

In conclusion, the present *in vitro* study reveals opioid-dependent differences in toxicity which is confirmed in all four tested cell types. Methadone was the most potent opioid with regard to opioid-induced mitochondrial damage, followed by fentanyl and ketobemidone. The impact of morphine and hydromorphone on cell viability was less pronounced and thus the least toxic to the cell cultures. Oxycodone was also ranked as one of the least toxic opioids in the assays examined. The results from this comparative study warrant further investigation *in vivo* as aside from the problems associated with addiction and mortality, opioids may impact higher cognitive functions in long-term opioid users.

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Artificial intelligence/Machine learning tools: Not applicable.

Supplementary Material: This article contains supplementary material (followed by the link to the article online).

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