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ORIGINAL RESEARCH ARTICLE

Cytotoxicity of Oxycodone and Morphine in Human Neuroblastoma and Mouse Motoneuronal Cells: A Comparative Approach

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Abstract

Background and Objectives Oxycodone is the most commonly used opioid for the treatment of moderate to severe pain. The peak cerebrospinal fluid concentration after epidural oxycodone was reported to be 300-fold greater (0.025 mM) than when administered intravenously after gynecologic surgery. Additionally, those patients administered epidural oxycodone had lower pain scores, needed less rescue analgesics and had fewer adverse effects compared with intravenous administration. However, oxycodone neurotoxicity requires evaluation before intrathecal implementation for routine clinical use.

Methods We used two in vitro cell culture models to compare the cytotoxicity of oxycodone with that of morphine, and to study the mechanisms underlying toxicity. Human neuroblastoma cells and mouse motoneuronal cells were treated with increasing concentrations (0.0125–2 mM) of oxycodone or morphine, and were harvested at 24, 48 or 96 h. Cell cultures were evaluated with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide and resazurin reduction assays.

Results Both morphine and oxycodone decreased cell viability in a dose-dependent manner at concentrations between 0.5 and 2 mM. Morphine increased the number of apoptotic cells compared with oxycodone when assessed by flow cytometry, and transmission electron microscopy images revealed that exposure to both opioids evoked the appearance of numerous electron-dense, probable autophagic vacuoles in the cytoplasm of the cells.

Conclusions Based on these results, it seems that the cytotoxicity of oxycodone in motoneuronal cells is similar to or less than that of morphine, and occurs only at concentrations above the peak clinical concentration in the cerebrospinal fluid after epidural administration.

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Key Points

Opioids are needed for the management of severe pain, and intrathecal administration is the most effective route for opioid analgesia; however, neurotoxicity is a concern in spinal and epidural administration of medicines.

The use of oxycodone has surpassed that of morphine, and preliminary data suggest that epidural oxycodone can be highly effective and well-tolerated; however, the safety and efficacy of intrathecal oxycodone has not been established.

We have evaluated the neurotoxicity of oxycodone in two commonly used cell models. The data indicate that the neurotoxicity of oxycodone is similar to that of morphine, which is a gold standard for intrathecal opioid administration.

1 Introduction

Oxycodone is a semi-synthetic opioid agonist derived from thebaine. It is increasingly used for the treatment of moderate to severe pain. Over the last decade, oxycodone use has surpassed that of morphine, and the global consumption of oxycodone is almost twofold higher than that of morphine; in 2012 the global consumption of oxycodone amounted 82,049 kg compared with a global consumption of 45,641 kg of morphine [1]. Oxycodone is often administered intravenously or subcutaneously and, as it has a relatively high oral bioavailability of between 40 and 65 %, administration by mouth is used in patients with normal gastrointestinal function.

Opioid receptors are mainly distributed in the central nervous system and spinal cord, and thus most of the actions of opioid agonists arise from these sites. Since 1976, when Yaksh and Rudy demonstrated the direct analgesic action of opioids on the spinal cord, there has been growing interest to use intrathecal opioids in the management of severe pain [2].

Few studies have evaluated the neuraxial administration of oxycodone with conflicting results of efficacy [3–5]. Two earlier studies reported only slight amplification in analgesic efficacy of epidural oxycodone compared with intravenous administration [3, 4]; however, in our recent study of women having lower abdominal surgery, a high analgesic efficacy of epidural oxycodone was demonstrated. Patients who were administered segmental epidural oxycodone had less pain and needed less rescue pain medication compared with intravenous administration and, in this small sample, epidural oxycodone was well-tolerated. In addition, spinal pharmacokinetics were in favor of epidural administration, and the cerebrospinal fluid peak molar concentration (0.025 mM) after epidural oxycodone was 300-fold greater than when administered intravenously [5].

However, to the best of our knowledge, neurotoxicity of intrathecal oxycodone has not been established; neurotoxicity requires evaluation before implementation of intrathecal oxycodone to routine clinical practice. To gain further knowledge of the potential toxicity at cellular level, the effects of oxycodone and morphine on cell viability and ultrastructure, as well as on markers of oxidative stress and cell cycle arrest in human (SH-SY5Y) and mouse (NSC-34) motoneuronal cells, were investigated in vitro.

2 Materials and Methods

2.1 Chemicals

3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), resazurin sodium salt and RNA-ase were obtained from Sigma-Aldrich (Helsinki, Finland); oxycodone and morphine were both purchased from Leiras Takeda Oy (Helsinki, Finland); Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and gentamicin were obtained from Lonza (Verviers, Belgium); anti-p21WAF1/Cip1 (12D1) antibodies, anti-β-actin, and anti-rabbit-immunoglobulin (Ig) G were provided by Cell Signaling Technology (Danvers, MA, USA); anti-heme oxygenase antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); ECL^{TM} prime Western blotting reagents and anti-mouse IgG horseradish peroxidase (HRP)-labeled antibody were provided by Amersham BioSciences (Buckinghamshire, UK); polyvinylidene difluoride (PVDF) membranes were provided by Millipore Laboratories, Inc. (Espoo, Finland); and the 10 mm culture plates were obtained from Sarstedt Inc. (Newton, MA, USA) and the 48-well plates were obtained from Nunc A/S (Roskilde, Denmark).

2.2 Cell Culture and Treatment

Human neuroblastoma cells (SH-SY5Y) were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). The cells were maintained in DMEM supplemented with heat-inactivated FBS (9 %) and gentamicin (10 μg/ml) at 37 °C in a 5 % CO_2 95 % air-humidified incubator. In the experiments, the cells were seeded at densities that allowed the untreated cells to reach

a nearly confluent state at the end of the experiments. The cells were treated with increasing concentrations of oxycodone or morphine after 48 h of plating in fresh medium and were harvested at 24, 48 or 96 h after treatment. Oxycodone and morphine were diluted in culture medium, and the medium was changed every second day.

Mouse motoneuronal cells, NSC-34 (kindly provided by Prof. Jari Koistinaho, A.I. Virtanen Institute, University of Eastern Finland) [6], were cultured in DMEM supplemented with 10 % FBS as well as 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5 % CO_2 95 % air-humidified incubator.

2.3 Cell Viability

The viability of the cultured SH-SY5Y cells was determined by the MTT reduction assay described previously by Pesonen et al. [7] In brief, the cells were seeded in 48-well plates ($4–5 \times 10^4$/well) and exposed to various concentrations (0.0125–2 mM) of oxycodone or morphine for 48 h. After exposure, the medium was changed to an MTT-containing (0.5 mg/ml) medium and the incubation continued at 37 °C for 2 h. Thereafter, the medium was removed and formazan crystals that had formed were solubilized with SDS-HCl buffer (pH 4.7). Absorbance was measured at 570 nm using an EL_x 800 plate reader (Biotek Instruments Inc., Winooski, VT, USA).

The viability of NSC-34 cells was determined by the resazurin reduction assay and was carried out with four replicates. Resazurin is a non-toxic and non-fluorescent blue reagent that is reduced to fluorescent and pink-colored resorufin by viable cells. Briefly, the cells were incubated with 10 μM resazurin diluted in culture medium at 37 °C for 2 h. The absorbance was measured at 590 nm (Wallac Victor2 1420 Multilabel Counter; PerkinElmer, MA, USA)

2.4 Flow Cytometric Analysis

Flow cytometric analysis after PI staining was performed as previously described by Nuutinen et al. [8]. Briefly, the SH-SY5Y cells were trypsinized and collected together with detached cells from the medium, and centrifuged at 1500 rpm for 5 min. The pellet was resuspended in phosphate-buffered saline (PBS) and fixed with ethanol (70 %) at +4 °C for 24 h. Thereafter, the cells were centrifuged at 1600 rpm and ethanol was decanted and the pellet resuspended in PBS containing RNA-ase (0.15 mg/ml), and incubated at +50 °C for 1 h. The cells were then centrifuged and the pellet resuspended in PBS containing PI (8 μg/ml) and incubated +37 °C for 2 h. The stained cells were analyzed by FACS Canto II equipment (BD-FACSDiva, version 6.1.3; BD Biosciences, San Jose, CA, USA).

2.5 Transmission Electron Microscopy

After exposure, the cells were fixed with 25 mg/ml glutaraldehyde in phosphate buffer (pH 7.4), and post-fixed in 10 mg/ml osmium tetraoxide (OsO_4) for 3 h. The cells were then dehydrated with increasing concentrations of ethanol and embedded in Epon LX-112 resin (Ladd Research Industries Inc., Williston, VT, USA). The blocks were sectioned into ultrathin slices and double stained with uranyl acetate and lead citrate. The samples were examined with a transmission electron microscope (JEM-2100F; Jeol Ltd, Tokyo, Japan).

2.6 Electrophoresis and Immunoblotting Analysis

Immunoblotting was carried out using the whole-cell proteins. In isolation of the protein fractions, the cells were washed with ice-cold PBS and scraped from the plates (on ice) in cold PBS, centrifuged at 1300 rpm for 10 min, and the pellets suspended in radioimmunoprecipitation assay (RIPA)-like lysis-buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1 % SDS, 0.5 % NP40, 2 mM dithiothreitol, and protease and phosphatase inhibitors). The suspensions were incubated on ice for 30 min and centrifuged at 13,000 rpm for 15 min. Supernatants were collected and used for analysis. Protein concentrations of the fractions were measured with the Bradford method using protein assay reagent obtained from BioRad Laboratories Inc. (Philadelphia, PA, USA).

The cell protein fractions were subjected to 100–150 mg/ml sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to PVDF membranes, as described previously [9]. p21 protein was analyzed using anti-p21 antibodies (1:1000) and heme oxygenase-1 (HO1) anti-heme oxygenase (1:500) as primary antibodies, and anti-rabbit or anti-mouse antibodies as the secondary antibodies (1:2000). Densitometric analysis of protein bands was carried out using the QuantityOne® program (BioRad Laboratories Inc., Hercules, CA, USA), and the data were normalized by β-actin, which was used as the loading control. The Western blot analysis was carried out from three independently performed cell exposure experiments.

2.7 Statistics

Results obtained using SH-SY5Y cells are expressed as the mean (standard deviation) or a percentage of the control cells, as appropriate. For EC_{50} values, 95 % confidence intervals (CI) were calculated. Six independent experiments with SH-SY5Y cells containing six replicates per concentration were performed, and the overall significance between groups and concentrations was calculated using a

linear mixed effect model. Group differences were tested using an independent samples t test in different concentration points. To calculate EC_{50} for both compounds, concentrations were log10-transformed, and analyzed by non-linear regression analysis using the following equation:

$$y = \text{minimum} + \frac{\text{maximum} - \text{minimum}}{1 + 10^{(\text{LogEC50} - x) * \text{hillslope}}}$$

using a constraint of $y = 0$ for the minimum. Non-linear curves were fitted for exposures and controls separately, and Student's t test was used to compare log EC_{50} parameters. Statistical analysis were performed using IBM SPSS Statistics for Windows version 22.0. (IBM Corporation, Armonk, NY, USA), and non-linear regression analysis was performed using R version 3.1.1 for Windows (R Foundation for Statistical Computing, Vienna, Austria). A p value <0.05 was considered as significant.

3 Results

Both morphine and oxycodone decreased SH-SY5Y cell viability in a dose-dependent manner, as analyzed by the MTT assay. A statistically significant decrease was seen using a 0.5 mM concentration of both opioids, and the EC_{50} was similar in the two groups—0.82 mM (95 % CI 0.78–0.87) for morphine and 0.81 mM (95 % CI 0.78–0.85) for oxycodone ($p = 0.7$, $n = 6$. However, significant difference was noted between the two compounds ($p = 0.015$) as cell viability was significantly less with morphine with the two highest concentrations (1.5 mM, $p < 0.001$; and 2 mM, $p = 0.018$) than with oxycodone (Fig. 1).

Microscopy of the cells after oxycodone treatments revealed cytoplasmic vacuoles at higher concentrations
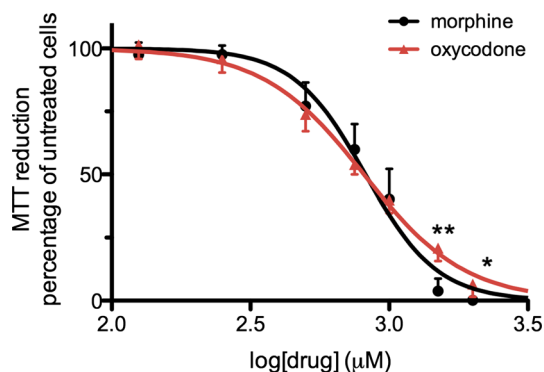

Fig. 1 The effect of morphine and oxycodone on SH-SY5Y cell viability. The cells were exposed to various concentrations of oxycodone or morphine, and cell viability was determined by MTT assay and expressed as a percentage of untreated cells. * Indicates $p = 0.018$ and ** indicates $p < 0.001$ between the two groups. *MTT* 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide

(1–2 mM), whereas the highest concentration (2 mM) of morphine caused de-attachment of the cells and massive cell death (Fig. 2). Similar cytoplasmic vacuoles and cell death were not seen in the control cultures. Transmission electron microscopy of SH-SY5Y cells showed appearance of many electron-dense vacuoles that contained cytoplasmic material such as ribosomes and degradation products after oxycodone exposure (Fig. 3a). These vacuoles resemble autophagosome/lysosome structures and were observable at 0.5–1 mM concentrations of oxycodone. Only a few similar vacuoles were seen after exposure to the same concentrations of morphine. Instead, several swollen mitochondria were seen, indicating mitochondria damage by morphine exposure (0.25–1 mM) (Fig. 3b). The highest concentration of oxycodone or morphine (2 mM) increased a number of empty (electron-lucent) vacuoles, suggesting fluid accumulation into the cells and membrane disturbances (Fig. 3b, d).

To study whether cytotoxicity would be associated with apoptosis or cell cycle arrest, control and opioid-treated SH-SY5Y cells were analyzed using flow cytometer after PI staining (Table 1). Exposure of the cells to morphine (0.5–2 mM) increased the number of sub-G1-phase cells representing apoptotic cells from 3 % up to 50 %, whereas the increase of apoptotic cells by the same concentrations of oxycodone was less, i.e. from 3 % up to 13 %. In control cultures, approximately 2 % of the cells were in the sub-G1 phase, and approximately 69 % in the G1/G0 phase.

Next, the expression of markers for cell cycle arrest (p21 protein [10]) and oxidative stress (HO1 [11]), was evaluated by immunoblotting after exposure of SH-SY5Y cells to various concentrations of oxycodone or morphine (Fig. 4). Both opioids increased the amount of p21 protein in a dose-dependent manner over the control levels at 24 and 48 h of exposure. The highest increase by oxycodone was approximately fourfold over the control level, whereas the highest increase by morphine was approximately tenfold over the control level. The other marker protein, HO1, showed weak increase only at lower concentrations of oxycodone (0.25–0.5 mM) after 48 h of exposure (data not shown).

Mouse motoneuronal cells, NSC-34, show a similar sensitivity to both morphine- and oxycodone-induced toxicity than human neuroblastoma cells on the resazurin reduction assay. Only the highest concentrations (2 mM)—and no concentrations of 0.25 mM or lower—clearly decreased NSC-34 cell viability after 96 h of treatment.

4 Discussion

Neuraxial opioids are highly effective analgesics due to direct uptake into the spinal cord and cerebrospinal fluid [5]. In neuraxial analgesia, safety is an issue as medicines
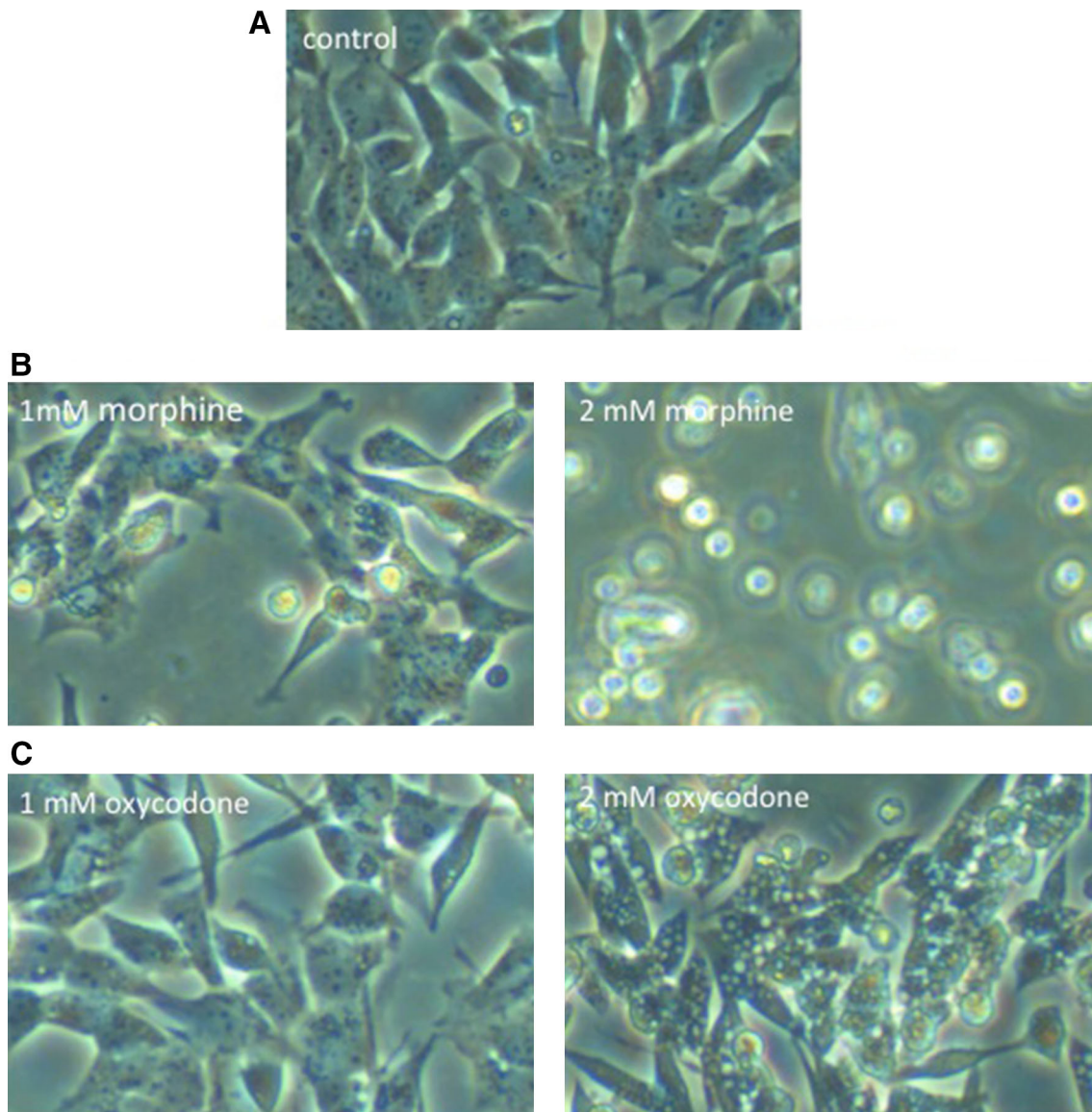
Fig. 2 Light microscopy of untreated and opioid-treated SH-SY5Y cells. **a** Untreated SH-SY5Y cells; **b** cells treated with various concentrations of morphine (1 and 2 mM); and **c** cells treated with various concentrations of oxycodone (1 and 2 mM). Magnification ×100

are administered into a close proximate of neural structures, and thus screening for potential neurotoxicity should be performed for all compounds intended for epidural or spinal administration [12]. Since its first neuraxial application in the late 1970s, morphine has been the gold standard for spinally administered opioids, and morphine is one of two opioids, along with sufentanil, that has been approved for intrathecal delivery in most countries [13]; however, this does not imply or rule out that morphine is an ideal drug for neuraxial administration. The highest concern with intraspinal morphine is the risk of both early and delayed respiratory depression after cephalad spread in the cerebrospinal fluid [14]. Other opioids are also administered intrathecally, although many of them, like fentanyl, are neither approved for intrathecal administration nor formally evaluated for neurotoxicity; however, extensive clinical experience with fentanyl is used to ensure the safety of intrathecal administration [15].

Oxycodone is structurally related to morphine, and our hypothesis was that cytotoxicity of oxycodone should be similar to that of morphine, and this was the case. Based on the data, the neurotoxic potential between morphine and oxycodone in vitro with two cell lines of neurological origin are well comparable. However, based on the effects on mitochondria toxicity, a signal that occurred at the lowest concentration, and cell cycle arrest potency, morphine proved to be slightly more potent than oxycodone. The safety factor for morphine after a single administration
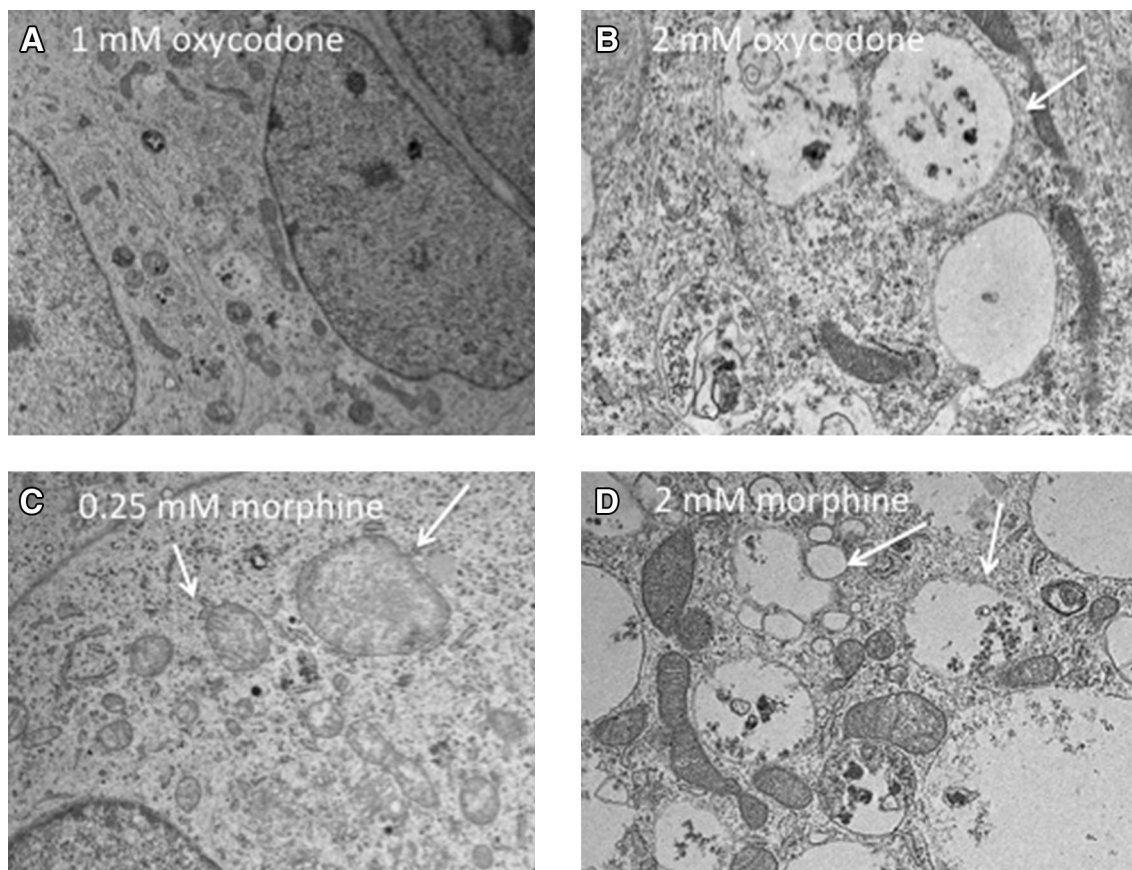
Fig. 3 Transmission electron micrographs of SH-SY5Y cells. **a** Cells treated with oxycodone (1 mM) for 24 h. A number of autophago-some-lysosome vacuoles can be seen in the cytoplasm of the cells; mitochondria are intact (magnification ×2000). **b** Cells treated with oxycodone (2 mM). Empty, electron-lucent vacuoles are seen in the cytoplasm. **c** Swollen mitochondria in the cytoplasm of cells treated with morphine (0.25 mM). **d** Increasing empty vacuoles in the cytoplasm of SH-SY5Ycells treated with morphine (2 mM). Magnification ×5000

Table 1 The effect of morphine or oxycodone on the cell cycle of neuroblastoma cells[a]

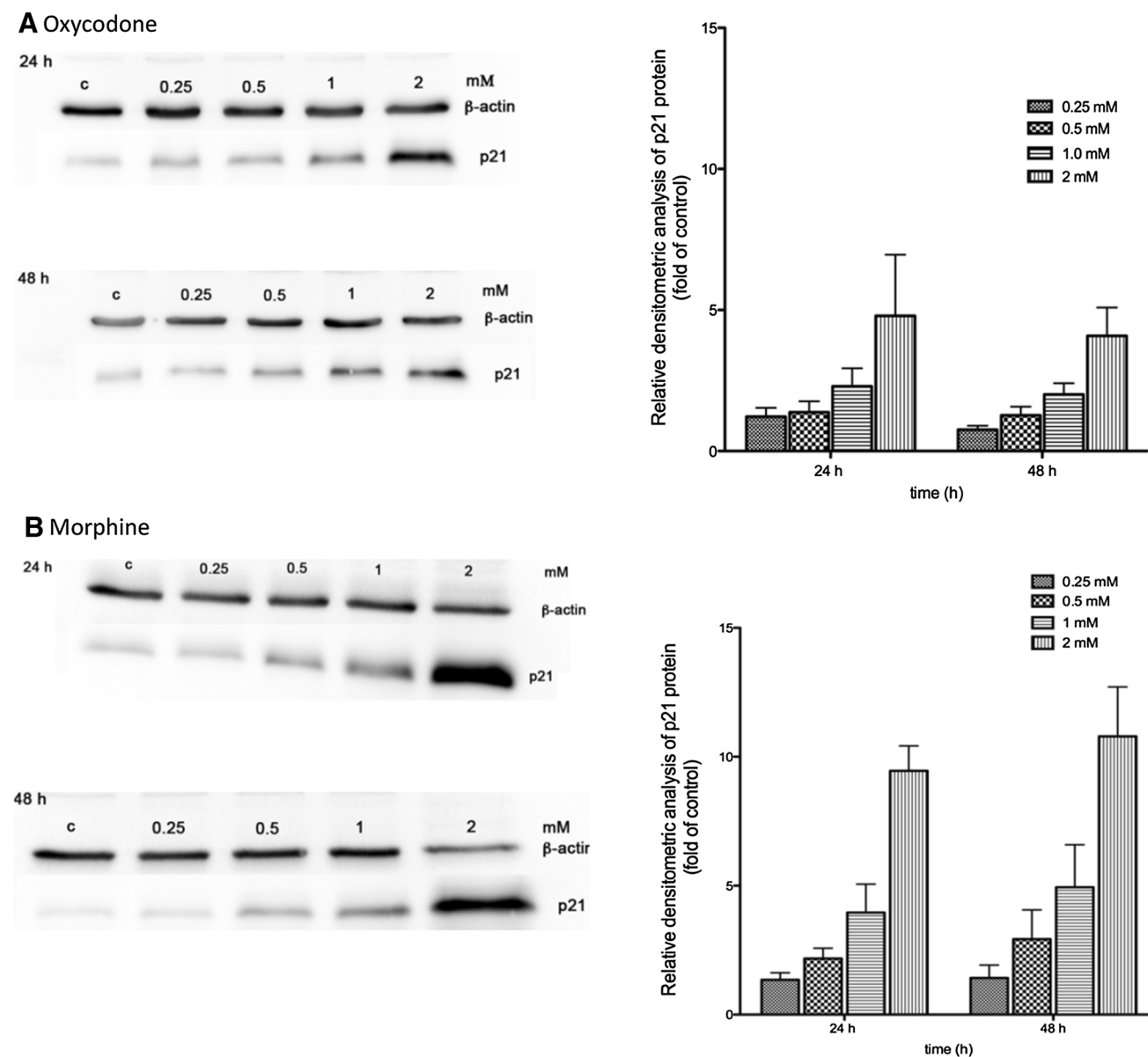| Cell phase	Control	Morphine [mM]			Oxycodone [mM]		
		0.5	1	2	0.5	1	2
SubG1	2.1 (1.2)	3.4 (1.2)	13.7 (0.2)	50.1 (6.7)	3.1 (0.4)	5.7 (1.1)	13.8 (4.5)
G1/G0	69.8 (4.8)	74.2 (1.3)	74.9 (1.8)	29.7 (0.3)	70.1 (2.4)	75.3 (6.0)	73.0 (3.9)
S	11.6 (3.1)	9.2 (0.9)	5.0 (0.6)	8.4 (3.0)	10.1 (1.0)	8.3 (2.9)	6.1 (0.6)
G2/M	15.9 (3.5)	12.8 (4.0)	6.3 (1.8)	9.5 (5.3)	15.9 (1.0)	10.6 (1.9)	7.0 (1.5)

Data are expressed as mean (standard deviation) from two or three independent experiments

[a] The cells were exposed for 24 h, stained with propidium iodide and analyzed with flow cytometry

for mitochondrial damage is 10 if 0.025 mM [5] peak clinical concentration is the reference value, suggesting that, at the concentrations commonly used in clinical practice, the risk for neurotoxicity is low. Based on the data, morphine-induced neurotoxicity could be linked to mitochondria-mediated apoptotic processes, which could be anchored with the increased expression of the p53 and p21 proteins and cell cycle arrest. The differences in neurotoxic potential between morphine and oxycodone may

also be catalyzed by different additional mechanisms of actions that cannot be identified by the limited set of tests used in the study. However, cytotoxic reactions were identified for both compounds only at concentrations far above the peak clinical concentration (0.025 mM) in the cerebrospinal fluid after epidural administration [5], and therefore may not be a cause of concern in acute administrations; however, after repeated administration, these signals should be considered.

A Oxycodone

B Morphine

Fig. 4 Expression of p21 protein in SH-SY5Y cells after exposure to various concentrations (0.25–2 mM) of oxycodone or morphine for 24 or 48 h. Immunoblots of total cell lysate protein (20 μg) and densitometric analysis of the respective columns. The level of immunodetectable protein was analyzed by densitometry and normalized to the loading control (β-actin). Values are expressed as the fold increase relative to the control values, and each column represents the mean (standard deviation) from three independent experiments (c = control). Representative immunoblot and densitometrically analyzed columns of p21 protein after exposure of SH-SY5Ycells to various concentrations of **a** oxycodone and **b** morphine

While investigating the neurotoxicity of neuraxial compounds, it is important to estimate appropriate concentrations used for cell exposure. In a recent clinical study, the peak cerebrospinal fluid concentrations of oxycodone after epidural administration of 0.1 mg/kg body weight were between 0.0025 and 0.025 mM [5]. These in vivo concentrations are 20- to 200-fold lower than 0.5 mM of oxycodone that, in the present in vitro study, indicated some morphological signs of neurotoxicity. The mean cerebrospinal fluid concentration of morphine after epidural injection of 0.1 mg/kg body weight is 0.007 mM [16]. Thus, we concluded that the oxycodone and morphine concentrations evaluated in this study were appropriate and a certain safety margin for clinical use of oxycodone and morphine has been identified.

In this study, we used a human neuroblastoma cell line that is a neuronal-like cell line, and mouse motoneuron cell line, in order to have an anatomically relevant cell line for comparison. The toxicity of both compounds appeared to be similar in both cell lines. Cell toxicity of oxycodone has

also been evaluated in human hepatoma HepG2 cells. Incubation of HepG2 cells with an oxycodone concentration of 0.2 mM did not indicate loss of cell viability, depletion of glutathione or decreased total protein count, which might reflect higher protective metabolic capacity of hepatocytes compared with neural cells [17]. Genotoxicity of oxycodone has been evaluated in the Ames *Salmonella typhimurium* and *Escherichia coli* test, chromosomal aberration test in human lymphocytes, and the in vivo bone marrow micronucleus test in mice, indicating no mutagenic for oxycodone. Oxycodone has only been shown to be clastogenic in concentrations considerably higher than the clinical dose range. Clastogenicity has been detected in the presence of metabolic activation in the human chromosomal aberration test at 3 mM of exposure, and in the mouse lymphoma assay at doses of 0.125 mM or greater with metabolic activation and 1 mM or greater without metabolic activation. Some gene mutation has been detected in an in vitro mammalian cell test at 1 mM. Neither developmental nor reproductive toxicity has been found in rats and rabbits [18].

Recent data indicate that the spinal pharmacokinetics and pharmacodynamics of oxycodone are in favor of epidural administration compared with intravenous administration [5]; however, further studies are needed to demonstrate the clinical safety of neuraxial oxycodone, and multiple models should be applied to assess neurotoxicity. Exposure of cell cultures may reveal potential toxicity, and can be useful for sorting out the possible mechanisms of toxicity of compounds. Exposing cell lines to naloxone would be interesting in evaluating whether the neurotoxicity is opioid receptor-related or not; however, data relating to morphine indicate that opioid receptor-related mechanisms may not be involved [19]. In addition, spinal cord or nerve root toxicity may also manifest itself as histologic, physiologic, or behavioral/clinical derangements. Experimental data in rats indicate that after intrathecal injection, opioid-induced hyperalgesia may last for 1–2 days [12, 20]. Ideally, a complete roster of histological, physiologic, and behavioral testing would be performed on spinal drugs in two or more animal species, followed by safety trials in humans prior to widespread clinical use [21].

In further studies, the safety of oxycodone local anesthetic combinations should also be evaluated. Intrathecal opioids are commonly used in combination with local anesthetics and vice versa to improve the analgesic effectiveness of neuraxial analgesia and also to reduce the requirement of single compounds. Contrary to opioids, local anesthetics are neurotoxic at subclinical concentrations, which has been demonstrated with neuronal cells [22, 23] and has also been found in clinical practice [24]. These studies are clinically paramount because epidural analgesia using local anesthetics with an opioid are highly effective and improve patient outcome compared with patients receiving systemic, standard-care analgesia [25].

5 Conclusions

Data indicate that the cytotoxic potential of oxycodone in human (SHSY5Y) and mouse (NSC-34) neuroblastoma spinal cord cells is similar or less than that of morphine, and occurs only at concentrations far above the peak clinical concentration in the cerebrospinal fluid after epidural administration of both compounds.

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