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Comparative Analysis of Opioids as Substrates and Inhibitors of the Human Organic Cation Transporter 1 (OCT1)

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Abbreviation	Full term
°C	temperature in degree Celsius
µg	microgramm (10^{-6})
µl	microliter (10^{-6})
µM or µMol/l	micromolar (10^{-6})
ASAM	American Society on Addiction Medicine
ASP^+	(4-(dimethylamino)steryl)-N-methylpyridinium iodine
bp	base pair
BSA	bovine serum albumin
CO_2	carbondioxide
CYP	cytochrome P450 monooxygenase family
ddH_2O	double distilled water
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DOP	delta opioid peptide receptor
EBM	evidence-based medicine
EDTA	ethylenediaminetetraacetic acid
EM	extensive metabolizer
ER	endoplasmatic reticulum
FCS	fetal calf serum
g	gramm
g	gravitational constant
h	hour(s)
$HBSS^+$	Hank's balanced salt solution
HEK293	human embryoic kidney cells 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
i.v.	intravenous (application of drug)
IC_{50}	half maximal inhibitory concentration
IS	internal standard
KOP	kappa opioid peptide receptor
l	liter
LC-MS	liquid tomography-mass spectrography
$logD_{(7.4)}$	distribution coefficient at pH 7.4
logP	partition coefficient
M	molar (mol/l)
MFS	major facilitator superfamily
mg	milligramm (10^{-3})
min	minute(s)
ml	milliliter (10^{-3})

mM or mMol/l	millimolar (10^{-3})
MOP	mu opioid peptide receptor
MPP+	1-methyl-4-phenylpyridinium
mRNA	messenger RNA
MS	mass spectrography
N.A.	not available
ng	nanogramm (10^{-9})
nl	nanoliter (10^{-9})
nM or nmol/l	nanomolar (10^{-9})
NMDA receptor	N-methyl-D-aspartate receptor
NOP	nociceptin opioid peptide receptor
NP-40	tergitol-type *NP-40*
OCT1	Organic Cation Transporter 1 (SLC22A1)
OIBD	opioid induced bowel dysfunction
ORL1	opioid receptor-like 1
p.o.	per os (oral intake of a drug)
PAMPA	parallel artificial membrane permeability assay
P_e	membrane permeability
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P_e	membrane permeability
pg	picogramm (10^{-12})
pK_a	dissociation constant
pM or pmol/l	picomolar (10^{-12})
PM	poor metabolizer
rfu	relative fluorescence unit
rOct1	rat organic cation transporter 1
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
s(ec)	second(s)
SEM	standard error of the mean
SNP	single nucleotide polymorphism
t.c.	transcutaneous (application of a drug)
t.m.	transmucosal (absorption of a drug)
TMH	transmembrane helix
UGT	uridine diphosphate-glucuronosyltransferase
WHO	World Health Organization
XTris-HCl	tris-buffered saline

1 Introduction

1.1 Pharmacogenomics

For most of its history, medicine has been considered an art. Medical practitioners' personal experience and their culture's prevailing belief systems dominated the way medicine was executed, spawning practices that were backed by little or no scientific evidence whatsoever. It is not possible to date the exact moment that medicine transgressed from being a healing art to a scientific discipline. In the Western European part of the world we can narrow it down to the nineteenth century, when empiricism relieved the practice of treating by anecdote and paved the way for what we consider modern medicine (Claridge and Fabian 2005). Though clinical experience remains an invaluable accessory in delivering good patient care, evidence-based medicine (EBM) –clinical observations supplemented and explained by scientific data- constitutes the mainstay of medicine these days.

However, despite the advances made in medical research over the past 150 years, we often still lack a comprehensive or even basic understanding of the mechanisms that govern our body in health and disease. As multi-morbidity and polypharmacy become the rule rather than the exception, a better understanding of pharmacodynamics (the effects a drug has on the body) and pharmacokinetics (the mechanisms by which the body processes and alters the drug) is necessary to avoid over- and underdosing the individual patient. Elucidating the correlations between clinically observed drug responses and specific quirks in our genetic makeup is the domain of a branch of pharmacology known as "pharmacogenetics".

Pharmacogenetics explores how genetic variations alter the function of those receptors, enzymes, transporters, and downstream signaling elements that ultimately affect drug response and metabolism. The term, coined by Friedrich Vogel in 1959, is often used interchangeably with the term "pharmacogenomics", which cropped up much later in the 1990s (Motulsky and Qi 2006). Strictly speaking, pharmacogenetics focuses on the exploration of single genes and their products, while pharmacogenomics takes a more holistic, i.e. *genome*-wide, approach. Budding in the 1950s, the field of pharmacogenetics only came into bloom at the turn of the millennium, when the advent of novel DNA sequencing methods accelerated genetic sequencing and capped its costs (Motulsky and Qi 2006). Since then, scanning patients for specific heritable genetic variants has become a viable option in clinical settings, adding a new dimension to the playing field of personalized medicine.

Generally, heritable genetic variants can affect gene products in two ways: the function of the gene product is either enhanced or impeded. The impact such altered functionality can have on metabolism is illustrated by the enzyme CYP2D6 of the cytochrome P450 family, which is involved in the metabolism of many opioids. Individuals with an impaired CYP2D6, so called "poor metabolizers" (PMs), are inefficient at metabolizing codeine into morphine. In consequence, they are exposed to codeine-related side effects without benefiting from morphine's analgesic effects (Eckhardt et al. 1998). At the other extreme, people with rapid variants of CYP2D6 may metabolize codeine to morphine at such high rates that the accumulating morphine reaches undesirably high levels, with potentially lethal outcome (Ciszkowski et al. 2009; Crews et al. 2012).

As DNA sequencing becomes faster and more readily accessible, pharmacogenetics can be an important tool in making personalized medicine more viable and efficient.

1.2 Opioids

1.2.1 General

Opioids are a class of drugs defined by their ability to bind to endogenous opioid receptors. They are best known for their analgesic properties, though they are capable of eliciting a wide array of responses, depending on their chemical properties and their receptor affinities. The term "opioid" comprises the opiates, their metabolites, and semi-synthetic derivatives, as well as the newer classes of structurally unrelated synthetic opioids, and opioid receptor antagonists. Opioids should not be confused with endorphins, endogenous peptides synthesized in the brain and the original ligands for opioid receptors (Davis and Pasternak 2009). Opiates morphine, codeine, and thebaine are plant alkaloids derived from *Papaver somniferum*, the opium poppy, and therefore considered "natural", as opposed to those substances artificially designed to bind to opioid receptors such as fentanyl, sufentanil, or pethidine (Freye and Levy 2008).

The analgesic properties of the juice won from the opium poppy have been exploited for thousands of years. The Ebers Papyrus, which dates back to 1500 BCE, documents the use of poppy seeds in sedative preparations, and the Sumerians are thought to have cultivated poppies as early as the third millennium BCE, supposedly to use them as euphoria-inducing stimulant in religious ceremonies- the Sumerian word for the poppy plant, "hul gil", translates to "plant of joy" (Brownstein 1993; Freye and Levy 2008). Although an opium tincture called "laudanum" was frequently employed by chemists from the 16th century

onwards (with the first reports of abuse and addiction cropping up at about the same time), the active compound of opium was only isolated and described in 1806 by Friedrich Sertürner, who named it morphium (morphine) after one of the Ancient Greek gods of dreams, Morpheus (Brownstein 1993). First marketed in 1817 by Sertürner and Company, the commercial production of morphine began in 1827 (Wikipedia 2018b). Bayer made heroin (diacetylmorphine) commercially available in 1898, and in 1939 pethidine (meperidine) was discovered, making it the first synthetic opioid with a structure unrelated to that of morphine (Brownstein 1993).

1.2.2 Pharmacodynamics

An opioid's effects depend on receptor affinity and intrinsic activity of the opioid, i.e. on how well it binds to a given receptor, and of what nature and magnitude the elicited response is, respectively.

To date, four classes of opioid receptors have been identified. They are known as MOP (μ or mu), DOP (δ or delta), KOP (κ or kappa), and NOP (nociceptin or nociceptin/orphanin FQ peptide), in which "-OP" is shorthand for "opioid peptide receptor" (Freye and Levy 2008; Dietis et al. 2011). Binding of an agonist to MOP is associated with analgesia, euphoria, respiratory depression, constipation, and itching, while KOP is thought to induce modest analgesia and feelings of dysphoria and displacement (Freye and Levy 2008; Dietis et al. 2011). Opioids receptors –DOR and KOR in particular– are also emerging as targets in the treatment of mood disorders (Lutz and Kieffer 2013). All four opioid receptor classes are 7-TM receptors that mediate their effects via G-class proteins, especially the Gi family, which inhibits the release of neurotransmitter from the presynaptic neuron in pain-transmitting fibers and thus accounts for much of the opioids' analgesic effect (Freye and Levy 2008).

A variety of MOP, DOP and KOP subtypes have been proposed. Originally, the existence of subtypes served to explain why the application of MOP agonists in combination with MOP antagonist selectively reversed some MOP-mediated effects while not affecting others (Dietis et al. 2011). The exploration of putative subtypes generated especial interest because it spurred hopes to tailor opioids that would bind to a receptor subset that induced analgesia but not to the ones implicated e.g. in respiratory depression (Dietis et al. 2011). However, knock-out experiments in mice yielded that the loss of the gene encoding for one opioid receptor caused the loss of all putative subtypes, leading to a reassessment of the traditional understanding of opioid receptor action (Dietis et al. 2011). Receptor heterodimerization, alternative splicing, and the interaction of downstream opioid receptor signaling pathways

with other signaling cascades have been proposed by more recent research to explain the observed differences in pharmacological responses (Corbett et al. 2006; Dietis et al. 2011).

Apart from their receptor profiles, opioids also differ in how strongly they bind to the individual receptors, a property termed "affinity". The stronger the affinity the opioids exhibit towards their respective receptors, the more potent is the response they elicit. Depending on their potency, opioids are roughly categorized as either weak or strong opioids. Codeine, dihydrocodeine, and tramadol are typical weak opioid analgesics, while morphine, oxycodone, levorphanol, methadone, hydromorphone, and fentanyl are considered strong opioids (WHO 1996; Brayfield 2014).

Additionally, and irrespective of their receptor affinity, opioids can also be classified depending on the effect they have on a receptor, i.e. whether they elicit a response or inhibit it. Opioids act either as agonists (e.g. morphine, fentanyl), antagonists (e.g. naltrexone, naloxone), or mixed agonist-antagonist (e.g. buprenorphine, pentazocine).

Due to their varied binding properties, opioids differ vastly in their pharmacological profile. While their ability to interact with opioid receptors classifies them as opioids, they do not bind to opioid receptors exclusively. The antitussive dextrorphan, for instance, binds little if at all to opioid receptors; it does, however, act as a NMDA-receptor antagonist. Morphine, the "classic" opioid, was shown to inhibit the release of the neurotransmitter acetylcholine and cause mast cell degranulation (Hermens et al. 1985; Davis 2009d).

Subsequently, each opioid has to be assessed individually with regard to pharmacologic and pharmacokinetic parameters.

1.2.3 Pharmacokinetics

The pharmacological response ultimately depends not only on the opioid, but also on its pharmacokinetics. The pharmacokinetics of any given drug –and thus its impact on an organism– are influenced by four factors: absorption, distribution, metabolism, and elimination (ADME).

Absorption of a drug depends on its chemical properties and the route of administration. While compounds such as fentanyl can be applied transdermally as patches, other opioids are best administered intravenously, either because they are absorbed poorly via the intestine (e.g. methylnaltrexone) or because they undergo extensive first-pass metabolism (e.g. morphine).

Distribution describes the compartments (vasculature, cells, extracellular space) a drug can access and is especially relevant in the context of the brain-blood barrier. Many opioids exert their desirable –and undesirable– effects by acting upon the central nervous system. Access to the brain is limited by the blood-brain barrier, and only highly lipophilic compounds are thought capable of penetrating the barrier. Distribution is, therefore, a matter of a compound's chemical properties. A highly lipophilic compound may diffuse into cells and accumulate in adipose tissue, whereas a highly polar substance will remain in the blood stream and be subjected to quick elimination through the kidneys. Thus, distribution affects half-life as much as the time and dose required to reach a steady state.

Metabolism determines to what extent a given substance will be present in either activated or inactivated form. Most metabolic transformations –so called biotransformations– take place in the liver, where enzymes located within the hepatocytes prime xenobiotics for elimination by introducing modifications that render the drugs more hydrophilic. CYP2D6 and CYP3A4 of the cytochrome P450 (CYP) family of enzymes are especially involved in opioid metabolism. For example, duplication of the CYP2D6 gene results in such accelerated metabolization of codeine that ultra-fast metabolizers have 50% higher plasma concentrations of morphine, a metabolite of codeine, than extensive metabolizers (Kirchheiner et al. 2007).

Elimination, like distribution, has a major impact on a drug's half-life. Since superfluous substances are excreted in bile and urine to a varying extent, especial care has to be taken in the application of drugs in patient suffering from liver or kidney disease. A number of commonly administered opioids, such as morphine, hydrocodone, or oxycodone, have higher bioavailability in patients with liver cirrhosis due to diminished metabolization. But while the effects of diminished CYP activity as well as that of other enzymes on opioid elimination and bioavailability have been extensively studied, the effects of diminished transporter activity have been less so. It is important to remember that non-membrane permeable substances rely on transporter proteins to shuttle them into the cell before they can be honed towards elimination. A study by Namisaki et al. showed that the expression of OCT1, among other transporters, was diminished in patients with hepatocellular carcinoma; however, chemotherapeutics employed in the treatment of this type of tumor depend on OCT1-mediated uptake into liver cells to reach their target (Namisaki et al. 2014). These findings indicate that not only phases 1 and 2, but also phases 0 and 3 of biotransformation –transporter-mediated uptake of drugs into cells and excretion from them, respectively– may be impaired in liver disease.

1.2.4 Effects of opioids and their role in clinical application

Opioids are mainly employed for their analgesic properties, though weaker opioids are also used as antitussives and anti-diarrheal agents. Despite a wide range of available non-opioid analgesics, opioids continue to be the mainstay of perioperative pain treatment (Brayfield 2014). Patients in chronic pain, such as cancer patients, also profit immensely from opioid therapy.

The importance of opioids is also reflected in the rise in global opioid consumption. Between 1989 and 2009 the consumption of morphine has risen sevenfold, that of oxycodone 26-fold, and that of fentanyl 100-fold (International Narcotics Control Board 2011). This trend is mainly observed for countries with high gross domestic income, but as the fields of pain medicine and palliative care advance and more people in so-called developing countries gain better access to adequate treatment of (chronic) pain, it can be expected that the global consumption of opioids will continue to rise.

Aside from the desired analgesic properties, opioids elicit a wide array of effects. Where opioids act upon the periphery, these effects take the shape of constipation, xerostomia (dry mouth), reduced libido, urinary retention, and pruritus (itching), while central nervous manifestations include sedation, respiratory depression, euphoria, dysphoria, and myoclonus.

In general, opioids are consumed in two manners: either sporadically, such as in a perioperative setting, or chronically, such as in protracted pain states. It is important to distinguish these two groups, since opioid-native patients will react more sensitively to even small doses of opioid, while patients accustomed to opioids will require higher doses and be more resilient towards certain side effects (e.g. respiratory depression).

When consumed on a regular basis, opioids cause gastrointestinal side effects. These side effects –often summarized as opioid-induced constipation, or opioid-induced bowel dysfunction (OIBD) – are the most pertinent to a patient's immediate well-being since they cause the greatest discomfort (Leppert 2015). Laxatives, such as lactulose or senna, are first-line therapies, but they can be supplemented with opioid receptor antagonists such as methylnaltrexone or alvimopan, both of whose effects are limited to the periphery since they cannot cross the brain-blood barrier (Davis 2009b). Nausea and vomiting are further common side effects when patients are started on opioids, but tolerance develops quickly, as it does in respiratory depression (Davis 2009b). Xerostomina (dry mouth) is caused by the anticholinergic effects of some opioids, especially morphine.

The most dreaded side effect of opioid therapy is respiratory depression. Depression of the respiratory center is dose-dependent, which is why opioids with high potency, such as sufentanil, are only titrated under closely monitored conditions, e.g. during general anesthesia. Accidental opioid overdosing that leads to respiratory depression can also happen when the opioid in question is insufficiently cleared from the bloodstream, such as in acute kidney injury (Conway et al. 2006). Although nociceptive stimuli counteract respiratory depression by increasing respiratory rate, respiratory depression continues to be a serious problem in patients who are either very old or very young, opioid-naïve, or already suffering from respiratory impairment (Davis 2009b). In this context, knowledge of genetic variations in the metabolic pathway of opioids can be helpful in averting a potentially life-threatening accumulation of opioids.

Addiction is another major adverse effect to bear in mind when administering opioids. The term "addiction" describes a chronic disease with a strong psychological component and detrimental socioeconomic consequences for affected individuals (in contrast to "tolerance" and "dependence", which describe physical adaptation to drug consumption) (ASAM 2001; Rosenblum et al. 2008). In 2013, 37% of the 44,000 drug-related deaths in the US were attributed to prescription opioids, an additional 19% to heroin (Volkow and McLellan 2016). While substance abuse in cancer patients under chronic opioid medication with no prior history of drug abuse is rare, and addiction is not known to develop in patients who receive opioids for a short time as part of their peri-operative analgesic treatment, the prevalence of opioid addiction and dependence among patients on (recklessly prescribed) long-term opioid therapy is high (Kirsh et al. 2009; Volkow and McLellan 2016).

1.2.5 Role of OCT1 in opioid metabolism

All the effects mentioned above –the desirable as well as the undesirable– depend on the bioavailability of opioids. Pharmacists and physicians have begun to recognize that bioavailability is influenced not only by route of application and co-medication, but also by genetics. The ideal dose of medication may differ significantly between individuals, or even within an individual, depending on whether they receive additional medication or supplements that interfere with signaling cascades and drug metabolism.

Since the vast majority of opioids are weak bases, which are protonated and carry a charge at physiological pH, the OCT family of organic cation transporters have come to attention as likely mediators of phase 0 biotransformation in opioid metabolism. For instance, Tzvetkov et al. demonstrated that the uptake of morphine is affected by polymorphisms in

the OCT1 gene. Loss-of-function phenotypes resulted in elevated plasma concentrations of morphine in affected patients, which improved analgesia but also increased the risk of accidental overdosing (Fukuda et al. 2013; Tzvetkov et al. 2013).

Another opioid whose plasma concentration is elevated in the presence of loss-of-function OCT1 genotypes is O-desmethyltramadol, the active metabolite of tramadol (Tzvetkov et al. 2011). The effect is so marked that, in a clinical study, patients with either one or two loss-of-function OCT1 alleles consumed less tramadol after surgery than patients with two wild types OCT1 alleles (Stamer et al. 2016).

Thus, OCT1 has been shown to affect plasma concentrations of both a classic and a synthetic opioid to a clinically significant degree.

1.3 Human organic cation transporter 1 (OCT1)

Everything we ingest –food, fluid, pharmaceuticals– has to be transported across the barrier of the gastrointestinal tract into our bloodstream. Even when administered intravenously or epidurally, many compounds will only be effective once they arrive inside our cells.

These transitions –from outside the body to within, and from extracellular to intracellular– are mediated by two mechanisms: diffusion and transport. While for a long time drug-metabolizing enzymes were the sole focus of pharmacogenomic research, attention has shifted over the past decade to membrane transporters (Yee et al. 2010). The role of membrane transporters on tissue and plasma concentrations of certain drugs is now widely recognized, and current and future research is expected to provide a better understanding of interactions between membrane transporters, drug-metabolizing enzymes, and their genes (Giacomini et al. 2010; Yee et al. 2010). The human organic cation transporter 1 (OCT1) is only one of the transporter in the spotlight of current research.

1.3.1 OCT1 gene (*SLC22A1*)

The human organic cation transporter 1 (OCT1, occasionally hOCT1 to better distinguish it from rat and mouse Oct1) is a transporter primarily expressed in the sinusoidal membranes of the liver, where it facilitates diffusion of polyspecific cations from the bloodstream into the liver cells (Grundemann et al. 1994; Zhang et al. 1997). It is a member of the solute carrier family of transport proteins, which belong to the major facilitator superfamily (Koepsell 2011; Pelis and Wright 2014). The gene encoding for OCT1, *SLC22A1*, is located on the long arm of chromosome 6 (6q25.3), and its corresponding mRNA comprises an

open reading frame of 1662bp, encoding a protein 554 amino acids in length (Gene: *SLC22A1* ; Zhang et al. 1997). Apart from in the liver, OCT1 is weakly expressed in the kidneys and intestine, and OCT1 mRNA has been detected in the adrenal gland and lung (Nies et al. 2009; Tzvetkov et al. 2009). There is also some evidence that OCT1 expressed in the endothelial cells of brain vessels mediates the passage of drugs across the blood-brain barrier (Dickens et al. 2012).

1.3.2 Structure of OCT1

OCT1 is believed to be made up of twelve transmembrane helices (TMH) connected by intracellular and extracellular loops (Grundemann et al. 1994; Zhang et al. 1997; Keller et al. 2011). As the crystal structure of OCT1 has yet to be obtained, considerations as to OCT1's structure rely mostly on models fashioned after LacY (lactose permease), a bacterial transporter that also belongs to the major facilitator superfamily (Pelis and Wright 2014). Site-directed mutagenesis is another tool that has helped to elucidate the structure of the binding regions (Popp et al. 2005; Gorbunov et al. 2008). According to these models, OCT1 contains two large loops: one located between TMH 1 and 2 on the extracellular side, and one between TMH 6 and 7 on the intracellular side (Keller et al. 2011). The extracellular loop does not directly participate in substrate translocation, but mutations within the loop of rOct1 lead to a decrease in plasma membrane translocation of rOct1 (Keller et al. 2011). This observation is supported by findings from Seitz et al, who showed that six polymorphisms of OCT1, two of which were located in the extracellular loop, led to a retention of transporter within the endoplasmic reticulum (Seitz et al. 2015). The intracellular loop contains four potential phosphorylation sites; a fifth is at the C-terminal domain, which, like the N-terminus, is located on the intracellular side (Zhang et al. 1997). A study by Ciarimboli et al proposes that OCT1 activity is inhibited by PKA-mediated phosphorylation and stimulated by Ca2+/Calmodulin, Ca2+/Calmodulin-dependent CamKII, and p56lck tyrosine kinase (Ciarimboli et al. 2004).

Binding of a substrate to OCT1 induces a conformational change that displaces the bound substrate from extracellular to intracellular (or vice versa). rOct1 has three putative binding sites: one with a high, the other two with a low substrate affinity (Koepsell 2011). It has been hypothesized that substrate binding to the high affinity site may allosterically impact the low affinity sites and alter their transport capacities (Gorbunov et al. 2008). The proposed mechanism is one of many that would explain why some molecules act as inhibitors of OCT1

without being transported, and why IC_{50} values vary depending on the concentration of the inhibited agent (Gorbunov et al. 2008).

In order to shuttle a substrate from one side of the cell to the other, be it by passive or active transport, the transport protein has to undergo a number of conformational changes: open outward-facing, occluded, and open inward-facing (Gouaux 2009). This model applies to OCT1, where conformational changes are induced by substrate binding, in contrast e.g. to voltage-gated Na-channels, where conformation is determined by membrane potential (Egenberger et al. 2012). Due to its penchant for (positively) charged molecules, the transport processes mediated by OCT1 are electrogenic, and it has been proposed that membrane potential influences the equilibrium between the open outward-facing and the open inward-facing conformation (Busch et al. 1996b; Koepsell 2011).

1.3.3 Substrates and inhibitors of OCT1

To date, a number of compounds with diverse chemical structures have been identified as substrates of OCT1 (see Figure 1), among them morphine, O-desmethyltramadol, odansetron, sumatriptan, amisulpride, tiatropium, and amiloride (Tzvetkov et al. 2011; Tzvetkov et al. 2012; Hendrickx et al. 2013; Matthaei et al. 2016; Chen et al. 2017). Putative endogenous substrates include monoamines, such as the neurotransmitters serotonin, norepinephrine, dopamine, acetylcholine, and histamine, as well as thiamine (vitamin B1) (Busch et al. 1996a; Boxberger et al. 2014; Chen et al. 2014). MPP^+ (1-methyl-4-phenylpyridinium), TEA (tetraethylammonium), and ASP^+ (4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide) are established model substrates for OCT1 in research (Busch et al. 1996b; Gorboulev et al. 1997; Schlatter et al. 2002; Ahlin et al. 2008).

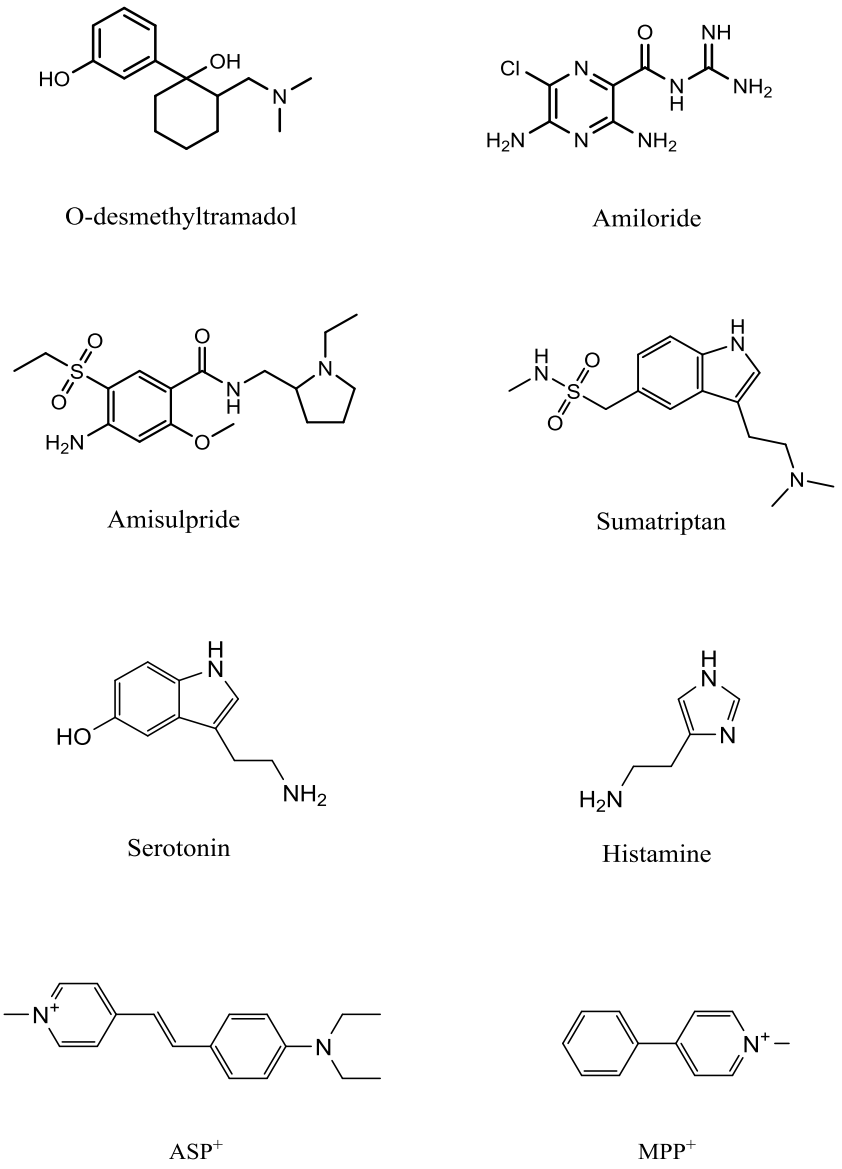


Figure 1: Chemical structures of selected substrates of OCT1

Despite their diversity, the substances intersect in at least four respects, as demonstrated by the example of morphine (see Figure 2). The first is charge. Although OCT1 does not exclusively transport molecules that are positively charged, most of its substrates do have a basic nature. The second is that they contain nitrogen, which may be quaternary at physiological pH; methylation can further stabilize the resulting positive charge at the nitrogen atom (as in N-methylated tilidine and unmethylated nortilidine). A third property is a cyclic structure or aromatic ring, which is integral to the substrate's structure. Finally, OCT1 substrates tend to exhibit low lipophilicity. Low lipophilicity in a compound, e.g. in the form

of hydroxyl groups, increase the likelihood that it cannot diffuse through cell membranes, thus having to rely on transporter proteins to ferry it across. However, data suggest that a certain degree of lipophilicity is necessary to enable substrate-transporter interaction, which is enhanced in the absence of hydrogen-bond donors (Ahlin et al. 2008). In addition, a study by Hendrickx et al. concluded that molecular volume was an important characteristic of OCT1 substrates, with increased volumes diminishing the likelihood that a given substances is a substrate of OCT1 (Hendrickx et al. 2013).

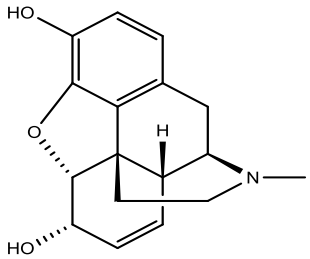


Figure 2: Chemical structure of morphine.

Morphine illustrates some of the features typical of an OCT1 substrate: a methylated nitrogen atom, which, at physiological pH, will carry a positive charge; five cyclic rings that enhance lipophilicity, one of which is aromatic; and two polar hydroxyl groups.

On the other hand, various compounds inhibit OCT1 without being transported by it. Ahlin et al. concluded that the four most important features for an OCT1 inhibitor are hydrophobicity, lipophilicity, positive charge, and an absence of hydrogen –bonding moieties (Ahlin et al. 2008). While it is not entirely possible to predict what substance will act upon OCT1 as substrate or inhibitor –or at all– there is some evidence that lipophilic substances tend to be inhibitors rather than substrates (Ahlin et al. 2008).

1.3.4 Genetic variability of OCT1

The OCT1 gene is highly genetically polymorphic. Seitz et al. reported 85 variants within the 2770bp long *SLC22A1* gene locus; of those, 44 lay within the coding region, and 29 caused amino acid substitutions (Seitz et al. 2015). OCT1 polymorphisms leading to incorrect compartmentalization of the transporter resulted in a global loss of activity, indiscriminate of substrate, whereas loss-of-function variants inserted into the plasma membrane displayed substrate specific loss-of-function (Seitz et al. 2015).

Loss-of-function OCT1 polymorphisms in the general population vary globally among regions and ethnic groups. While the frequency of reduced or loss-of-function alleles is

below 2% in Chinese and Japanese populations, 40% of Caucasians carry one allele and 9% carry two alleles with the most common loss-of-function polymorphisms (Chen et al. 2010; Seitz et al. 2015).

1.3.5 OCT1 and pharmacogenetics research

Given the plethora of transporters so far identified (more than 15,000 transport protein sequences in over 1,200 families as of December 2017 (Transporter Classification Database)), what distinguishes OCT1 and makes it an especially interesting target for medical research?

First of all, OCT1 is mainly located in the sinusoidal membrane of hepatocytes (Gorboulev et al. 1997; Zhang et al. 1997). The liver is the body's powerhouse of metabolism; detoxification and elimination of both endogenous and exogenous compounds occur primarily here. This process, dubbed biotransformation, ultimately leads to the deactivation and elimination of xenobiotics. As gatekeeper to the intracellular metabolic machinery of the hepatocytes, OCT1 is part of phase 0 biotransformation. However, in some cases, instead of rendering xenobiotics ineffective, CYP-mediated modifications can also lead to their activation, as is the case with prodrugs tilidine and codeine, whose metabolites nortilidine and morphine, respectively, elicit the clinical effects associated with their parent compounds. Occasionally, activation also results in toxification, as with pethidine or acetaminophen, causing a potentially harmful accumulation of toxic metabolites in vulnerable patient groups, such as patients suffering from impaired renal clearance (Ramirez et al. 2004; Laine et al. 2009). Slightly overstated, it can be said that transport of many drugs into the liver cells, where they are deactivated (their effects on the body thus being terminated) or activated (thus becoming effective) would be diminished without OCT1.

Secondly, OCT1's high genetic variability has been shown to impact OCT1's transport capacities to a degree that is clinically significant. Diminished uptake in loss-of-function OCT1 polymorphisms has been shown for morphine, tramadol, and sumatriptan, effectively causing an increase in their bioavailability and half-life (Tzvetkov et al. 2012; Tzvetkov et al. 2013; Matthaei et al. 2016). Considering that a great number of drugs fulfill the formal criteria for OCT1 substrates and inhibitors, it would be of both use and interest for clinicians to know which drugs interact with OCT1, and whether their pharmacokinetic properties are altered in other-than wild type OCT1 variants.

Thirdly, the current list of substrates and inhibitors of OCT1 comprises substances that are staple drugs in clinical practice. Xenobiotics transported by OCT1 include morphine, O-

desmethyltramadol, odansetron, tropisertron, and sumatriptan, to name only a few (Tzvetkov et al. 2011; Tzvetkov et al. 2012). Endogenous substrates of OCT1, on the other hand, remain elusive; so far only monoamines and vitamin B1 have been identified (Busch et al. 1996a; Chen et al. 2014). It seems, therefore, as if OCT1's primary role is to shuttle exogenous substrates into the liver for deactivation and detoxification.

For these reasons, OCT1 is a variable in drug metabolism worthy of attention. Knowing which opioids are impacted by OCT1 polymorphisms –and which opioids in turn impact its transport capacities– could help clinicians make better informed choices concerning the administration of opioids, their dosage, and co-medication.

1.4 Aim

The aim of this work was to perform a systematic screening of opioids in order to identify substrates and inhibitors of OCT1 beyond the previously identified morphine and O-desmethyltramadol. Screening for individual compounds as substrates and inhibitors of OCT1 was performed in four steps:

- *in silicio* predictions of the compounds' basic properties and lipophilicity

- experimental determination of their passive membrane permeability using parallel artificial membrane permeability assays (PAMPAs)

- determination of their inhibitory potential on OCT1

- determination of their uptake by OCT1

The *in silicio* predictions helped us in the initial selection process by identifying opioids with low or even negative $logD_{(7.4)}$ values, which indicate limited lipophilicity. The premise was that substances with low membrane permeability are more likely to require transporters to penetrate biological membranes than lipophilic substances. The PAMPAs that followed experimentally validated the substances' membrane permeability in an artificial, well-defined but cell-free model. The degree to which substances did or did not interact with OCT1 was evaluated with the help of the inhibition experiments, while the direct transport measurements identified those substances that interacted with OCT1 as substrates (as opposed to mere inhibitors).

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals	Manufacturer
ASP^+	Invitrogen, Darmstadt, Germany
Bicinchoninic Acid Solution	Sigma-Aldrich, Steinheim, Germany
Copper sulfate solution	Sigma-Aldrich, Steinheim, Germany
EDTA 0.5 M solution	AppliChem, Darmstadt, Germany
Fecal Calf Serum (FCS)	Gibco Life Technologies, Darmstadt, Germany
Formic acid	Merck-Schuchard, Hohenbrunn, Germany
$HBSS^+$	Gibco Life Technologies, Darmstadt, Germany
Helipur® H plus N	B.Braun Medical AG, Sempach, Switzerland
Medium	Gibco Life Technologies, Darmstadt, Germany
Methanol LCMS grade	LCG Standards GmbH, Wesel, Germany
MPP^+	Sigma-Aldrich, Steinheim, Germany
NaCl	Merck, Darmstadt, Germany
Na-deoxycholate	Sigma-Aldrich, Steinheim, Germany
Nonidet®P40 Substitute (Nonylphenylethylenglycol)	Fluka (Sigma), Germany
PBS buffer	AppliChem, Darmstadt, Germany
Penicillin and Streptomycin (P/S)	Gibco Life Technologies, Darmstadt, Germany
Poly-D-lysine bromide	Sigma-Aldrich, Steinheim, Germany
PromoChem Acetonitrile Optigrade	LCG Standards GmbH, Wesel, Germany
Sodium dodecyl sulfate (SDS)	BioRad, Hercules, USA
Tris-HCl	Roth, Karlsruhe, Germany
TrypLE	Gibco Life Technologies, Darmstadt, Germany
Tryptane Blue	Sigma-Aldrich, Steinheim, Germany
Water LCMS grade	LCG Standards GmbH, Wesel, Germany

2.1.2 Drugs

Drugs	Manufacturer
3-Methoxymorphinan hydrochloride	Sigma-Aldrich, Steinheim, Germany
3-Hydroxymorphinan hydrobromide	Sigma-Aldrich, Steinheim, Germany
Codeine	Sigma-Aldrich, Steinheim, Germany
Dextromethorphan solution	Sigma-Aldrich, Steinheim, Germany
Dextrorphan tartrate	Sigma-Aldrich, Steinheim, Germany
Fentanyl solution	Sigma-Aldrich, Steinheim, Germany
Hydrocodone bitartrate	Sigma-Aldrich, Steinheim, Germany
Hydromorphone	Sigma-Aldrich, Steinheim, Germany
Meptazinol hydrochloride	Sigma-Aldrich, Steinheim, Germany
Methylnaltrexone bromide	Sigma-Aldrich, Steinheim, Germany

Morphine	Sigma-Aldrich, Steinheim, Germany
Naltrexone solution	Sigma-Aldrich, Steinheim, Germany
N-Desmethyl-cis-tramadol hydrochloride solution	Sigma-Aldrich, Steinheim, Germany
Norfentanyl oxalate solution	Sigma-Aldrich, Steinheim, Germany
Noroxycodone hydrochloride solution	Sigma-Aldrich, Steinheim, Germany
Nortilidine hydrochloride solution	Sigma-Aldrich, Steinheim, Germany
Oxycodone	Sigma-Aldrich, Steinheim, Germany
Oxymorphone (solution)	Sigma-Aldrich, Steinheim, Germany
Pethidine (Meperidine) hydrochloride	Sigma-Aldrich, Steinheim, Germany
Piritramide	Janssen-Cilag GmbH, Neuss, Germany
Sufentanil citrate	LGS Standards, Wesel, Germany
Tapentadol	Sigma-Aldrich, Steinheim, Germany
Tilidine hydrochloride	Sigma-Aldrich, Steinheim, Germany

2.1.3 Cell lines

Cell lines	Generated by
HEK 293 hOCT1	Ali Reeza Saadatmand
HEK 293 pcDNA5	Ali Reeza Saadatmand

2.1.4 Consumables

Equipment	Manufacturer
96-well plate, clear	Sigma-Aldrich, Nümbrecht, Germany
96-well PCR plate	Thermo Scientific, Loughborough, UK
Adhesive Sealing Sheets	Thermo Fisher Scientific, Darmstadt, Germany
Cakes and cookies	Tina, Marleen, Mladen, Karo, Ellen, Helen, Jiayin
Cell culture flasks, 75 cm^2	Sarstedt, Nümbrecht, Germany
Centrifuge tubes, 15 ml	Sarstedt, Nümbrecht, Germany
Centrifuge tubes, 50 ml	Sarstedt, Nümbrecht, Germany
Discardit II™ 20 ml syringe	BD Biosciences, Heidelberg, Germany
Disposable gloves	Rösner-Mautby Meditrade Holding GmbH, Kiefersfelden, Germany
Flat cap strips, 8	Thermo Fisher Scientific, Darmstadt, Germany
Ethanol 99 % (methylated), diluted with ddH2O to 70 %	Walter CMP GmbH, Ratingen, Germany
Filtered tips, 200 µl	Kisker, Steinfurt, Germany
Glass pasteur pipet, 230 mM	Brand, Wertheim, Germany
Glass Vial Micro-Insert 0.05 ml	Th. Geyer, Renningen, Germany
Minisart® plus Syringe Filters	Sartorius, Göttingen, Germany
Narrow tips, 100 µl	Sarstedt, Nümbrecht, Germany
NunclonTM Delta Surface, 12-well plates	ThermoScientific, Roskilde, Denmark
Parafilm®	Brand, Wertheim, Germany
Pre-coated PAMPA Plate System	Corning, Bedford, MA, USA

Septum 8 mM PTFE virginal	VWR International GmbH, Darmstadt, Germany
Serological pipet, 5 ml	Sarstedt, Nümbrecht, Germany
Serological pipet, 10 ml	Sarstedt, Nümbrecht, Germany
Serological pipet, 25 ml	Sarstedt, Nümbrecht, Germany
Test tubes, 1.5 ml	Sarstedt, Nümbrecht, Germany
Test tubes, 2.0 ml	Sarstedt, Nümbrecht, Germany
Test tubes, 5.0 ml	Sarstedt, Nümbrecht, Germany
Tips, 10 µl	Sarstedt, Nümbrecht, Germany
Tips, 1000 µl	Sarstedt, Nümbrecht, Germany
Tips, 200 µl	Sarstedt, Nümbrecht, Germany

2.1.5 Reusable equipment

Equipment	Manufacturer
96-well plate, black with clear bottom	Corning Incorporated, Corning, USA
Cover slip	Schütt, Göttingen, Germany
Neubauer Cell Counting Chamber	Schütt, Göttingen, Germany
Screw neck vials for autosampler	VWR International GmbH, Darmstadt, Germany
Screw tops without septa for vials	VWR International GmbH, Darmstadt, Germany
Spring for Micro Insert 0.05 ml	VWR International GmbH, Darmstadt, Germany

2.1.6 Instruments

Instruments	Manufacturer
Analytical balance	Sartorius, Göttingen, Germany
API4000 LC-MS/MS system	AB Sciex, Darmstadt, Germany
Arium® pro VF Ultrapure Water System	Sartorius, Göttingen, Germany
Axiovert 40 CFL microscope	Carl Zeiss Microscopy GmbH, Göttingen, Germany
Centrifuge 5810R	Eppendorf, Hamburg, Germany
CO_2Incubator BBD 6220	ThermoFischer Scientific, Osterode, Germany
Ecovac safety vacuum system	Schuett-biotec GmbH, Göttingen, Germany
Fridges and freezers	Liebherr, Biberach an der Riß, Germany
Heraeus Biofuge Pico	ThermoFischer Scientific, Osterode, Germany
Heraeus Multifuge X3R	ThermoFischer Scientific, Osterode, Germany
Magnetic stirrer	2mag, Munich, Germany
MS2 Minishaker	W.Krannich GmbH, Göttingen, Germany
Polymax 1040 Platform Shaker	Heidolph Instruments GmbH, Schwabach, Germany
Sample Concentrator	Techne, Stone, UK
Stretching Table OTS40, 150 x 400 x 80 mm (W/D/H)	Medite, Burgdorf, Germany
Tecan Ultra microplate reader	Tecan, Crailsheim, Germany
Thermomixer 5436	Eppendorf, Hamburg, Germany
Waterbath GFL 1083	Schuett-biotec GmbH, Göttingen, Germany

2.1.7 Software

Software	Manufacturer
ADMET Predictor	Simulations Plus Inc., Lancaster, USA
Analyst® Version 1.4.2	Applied Biosystems, Darmstadt, Germany
ChemBioDraw Ultra 14.0.0.117	PerkinElmer Inc.
EndNote X7.3.1	Thomson Reuters, Philadelphia PA, USA
GIMP Image Editor 2.8 (GNU Image Manipulation Program)	Spencer Kimball, Peter Mattis and the GIMP Development Team
GraphPad Prism 5	GraphPad Software Inc.
Microsoft Excel 2013	Microsoft Corporation, Redmond, WA, USA
Microsoft Word 2013	Microsoft Corporation, Redmond, WA, USA
SigmaPlot 12.0	Systat Software Inc., Erkrath, Germany
XFluor4 Software	Tecan, Crailsheim, Germnay

2.2 Methods

2.2.1 Cell culture

The cells were kept in 75 cm^2 cell culture flasks with Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 10 % fecal calf serum (FCS) and 1 % penicillin/streptomycin mix (P/S) (hereafter referred to as "medium"). The flasks were cultivated in an incubator at 37 °C and 5% CO_2. Depending on cell growth and plating frequency, the cells were split every 2-5 days. Passages between 4 and 31 were used for plating.

2.2.2 Plating

The cells were grown on 12-well plates. To enhance cell adhesion, the wells were coated with poly-D-lysine by adding 0.5 µl of poly-D-lysine solution to each well and incubating the plate in a cell culture incubator at 37 °C for 15 min. The superfluous poly-D-lysine was then removed from the wells and stored at 4 °C for reuse.

Poly-D-lysine solution was prepared by mixing 100 mg of D-lysine with 50 ml of ddH_2O. This solution was filtered through a syringe with a filter tip before use.

For plating, the medium was removed from the cell culture flask and 3.5 ml of trypsine added to the flask. The flask was then incubated in the cell culture incubator at 37 °C for 4 min. during which time the trypsine dissolved the extracellular matrix that bonded the cells together into a biofilm. As soon as the time was up, the flask was removed from the incubator and 10 ml of medium were added to terminate the trypsine reaction. The cells were then ablated from the bottom of the flask, transferred into a falcon tube, and the tube centrifuged for 3min. at 700 x *g*. The medium was then removed from the tube and the cell pellet at the bottom of the tube resuspended in 10 ml of fresh medium. Of this cell suspension, 20 µl was added to a test tube and mixed with 20 µl of trypthane blue. A sample of the dyed cell solution was pipetted onto a cell counting chamber and the cells counted under a light microscope. The sum of the cells from the four fields of the counting chamber was divided by two and multiplied with 10^4 to yield the total number of cells per milliliter. The cell solution was then diluted with medium to meet the concentration of cells required, i.e. $6x10^5$ cells per well. Of this final cell solution, 1ml was added to each well.

For the inhibition experiments, three wells of a 12-well plate were plated with pcDNA5 cells and nine wells with OCT1-overexpressing cells. For the transport experiment four wells of

a 12-well plate were plated with pcDNA5 cells and eight wells with OCT1-overexpressing cells.

2.2.3 *In silicio* predictions

Initially, we compiled a list of 60 opioids and opioid congeners by reviewing pharmacological and medical textbooks, and by browsing PubMed and Wikipedia. Especially those opioids and their congeners were considered of interest that are either well established in clinical practice and widely used, or else belong to newer, emerging classes of opioids, whose pharmacological interactions have yet to be elucidated.

On the basis of the substances' chemical structure, which were obtained from PubChem (The PubChem Project), three values were predicted with ADMET Predictor Software: the dissociation constant pKa; the partition coefficient logP (the log of the ratio of unionized compounds in a lipophilic and a hydrophilic phase); and the distribution coefficient $logD_{(7.4)}$ (log of the ration of the sum of both unionized and ionized compound in a lipophilic and a hydrophilic phase). The $logD_{(7.4)}$ values were of greatest interest to us as they best reflect lipophilicity under physiological conditions, which were also the conditions emulated in the inhibition and transport experiments.

2.2.4 Parallel artificial membrane permeability assays (PAMPAs)

Drug solutions of the 23 substances selected to undergo this experiment were prepared at three different concentrations each. They were prepared in phosphate buffered solution (PBS) to yield aliquots of 400 µl with concentrations of 20 µM, 100 µM, and 500 µM, except for codeine, fentanyl, and morphine, which were prepared at 10 µM, 20 µM, and 50 µM.

Of these drug solutions, 300 µl was added to the donor wells (lower plate) of the PAMPA plate. The remaining 100 µl was set aside as C0 samples and their exact concentration determined via mass spectrography (MS) in order to adjust for inaccuracies that might have occurred during pipetting.

Once the donor wells had been prepared, 200 µl of PBS was added to each well of the acceptor wells (upper plate). The upper plate was then carefully placed into the lower, trapping as little air between the membranes as possible, since bubbles impede diffusion between the two plates. The PAMPA plates were then set aside at room temperature (21°C) and not disturbed for 5 h.

After incubating for 5 h, the PAMPA plates were separated and the lower plate set aside. Starting with the acceptor plate, 180 µl of solution from each well was transferred into a

corresponding well on a standard 96-well PCR plate. Analogously, 180 µl of solution from the donor wells was transferred into corresponding wells on a second standard 96-well PCR plate. The samples from the acceptor plate were designated C5A (concentration after 5 h, acceptor plate), those of the donor plate C5D (concentration after 5 h, donor plate).

Before MS measurements, the samples from C0 and C5D were diluted 1:10,000 in 0.1 % formic acid. The samples from C5A were initially measured undiluted, but as their concentrations exceeded the detection capacities of the mass spectrometer, the samples were likewise diluted in 0.1% formic acid, either 1:100 or 1:10,000 depending on how strong the initial signal detected by the mass spectrometer was. The different dilution ratios were later taken into account when the results of the MS measurements for C0, C5A, and C5D were converted into mM (millimolar) for calculation.

Permeability and mass retention were calculated according to the formula provided by the producer of the PAMPA plates.

Recommended formula for data analysis provided by the producer

$$Pe = \frac{-\ln\left(1 - \frac{CA(t)}{C\ equilibrium}\right)}{A \times \left(\frac{1}{VD} + \frac{1}{VA}\right) \times t}$$

C_0 = initial compound concentration in donor well in [mM]

$C_D(t)$ = compound concentration in donor well at time t in [mM]

$C_A(t)$ = compound concentration in acceptor well at time t in [mM]

V_D = donor well volume

V_A = acceptor well volume

$C_{equilibrium}$ = $\frac{(CD(t) \times VD + CA(t) \times VA)}{(VD + VA)}$

A = filter area (0.3 cm^2)

t = incubation time in s (5 h= 18,000 s)

2.2.5 Inhibition experiments

The IC_{50}s of the substrates were determined with 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodine (ASP^+). A fluorescent molecule that can be easily quantified by photometer measurements, ASP^+ is also an established substrate of OCT1. As such, its uptake depends on OCT1's transport capacities, which will be diminished in the presence of an inhibitor or that of a competing substrate. The competing substances (hereafter referred to as "test drug") were the 23 opioids selected for testing.

ASP^+ uptake was measured at eight different concentrations of test drug, including a concentration of zero, which served as the baseline of ASP^+ uptake by OCT1 and pcDNA5 cells. Drug concentrations of 1, 5, 10, 50, 100, 500, and 1000 µl were obtained through serial dilution of a stock solution with high molar mass. Oxycodone was measured at higher concentrations of 0, 10, 50, 100, 500, 1000, 2000, 5000 µl after initial measurements at lower concentrations yielded inconclusive results. All samples were diluted with Hank's Balanced Salt Solution ($HBSS^+$) and prepared at twice the target concentration, so that when the test drug solution was mixed with an equal amount of ASP^+ solution shortly before the experiment the final solution yielded the concentrations desired for testing.

ASP^+ solution was prepared from a 10 mM stock solution with $HBSS^+$ as diluent. As with the inhibitor solutions, the solution was prepared at a concentration of 1 µM and the target concentration of 0.5 µM obtained when equal volumes of 1 µM ASP^+ and test drug solution were mixed prior to the experiment.

The uptake measurements were performed under (human) physiological conditions. These conditions were met by adjusting the $HBSS^+$ buffer to fall within a pH range of 7.3- 7.5 and by warming it up to 37 °C in a water bath prior to the experiments. Eppendorf tubes containing the ASP^+-test drug solutions were also kept on a heating block at 37 °C until the solutions were added to the cells.

The cells were prepared for the experiment by discarding the old medium within the wells and washing them once with 2ml of pre-warmed $HBSS^+$ per well. This, too, was completely removed.

The experiment began with the addition of the ASP^+-test drug solution to the wells. 400 µl of ASP^+-test drug solutions were added to the OCT1-containing wells, with ASP^+ at a concentration of 0.5 µM and test drug at the aforementioned concentrations. Additionally, pure 0.5 µM ASP^+ solution without test drug was added to two of the OCT1 wells and both of the pcDNA5 wells (see pipetting scheme, Figure 3).

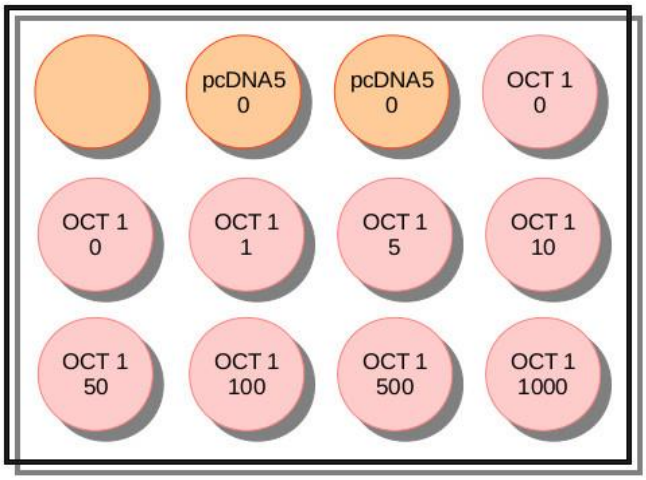


Figure 3: Plating and pipetting scheme for inhibition experiments, concentrations in µM

The plates were kept at 37 °C on a hot plate during the entire course of the experiment to allow for optimal uptake of ASP^+ and test drug under physiological conditions. After an incubation period of 2 min, the reaction was stopped by adding 2 ml of ice-cold $HBSS^+$ to each well. This was discarded, and the cells were washed two more times with ice-cold $HBSS^+$.

The cell lysate required for fluorescence measurement was won by removing all vestiges of $HBSS^+$ buffer and lysing the cells with 0.5 µl of RIPA buffer per well.

RIPA buffer	
Tris-HCl	50 mM
SDS	0.1%
NP-40	1%
Na-deoxycholate	0.25%
NaCl	150 mM
EDTA	1 mM

The plates were then placed on the Shaker for 10 min. to ensure complete lysis had taken place, followed by 10 min. of centrifugation at 700 x *g*. Centrifugation was not mandatory, but performed nonetheless because it helped to isolate the DNA and thereby facilitated pipetting. Cell lysate from each well, 200 µl and 10 µl, were transferred to a 96-well black bottom for fluorescence measurements and to a standard 96-well plate for protein measurements, respectively. A standard of bovine serum albumin (BSA) of 0, 1, 3, 5, 10, and 15 µl was added in duplicate to the standard 96-well plate. 200 µl of a bicinchoninic acid mastermix prepared from 200 µl of bicinchoninic acid and 4 µl of cupric sulfate solution was then added to each of the protein samples and the plate incubated at 37 °C for 30 min.

Fluorescence of the cell lysate in the 96-well black bottom plate was measured by a Tecan Ultra Plate Photometer using XFluor4 Software.

Measurement Parameters

Mode	Fluorescence
Excitation wavelength	486nm
Emission wavelength	615nm
Gain (Manual)	50
Number of flashes	10
Lag time	0
Integration time	40
Mirror selection	Dichronic 2
Z-position	Adjust manually

Protein measurements were performed analogously, with measurement mode "Absorbance" and an absorption wavelength of 570 nm.

The relative fluorescent measurement unit (rfu) per milligram served as a relative scale that helped to quantify the amount of ASP^+ taken up in the presence of inhibitor at different concentrations. It was determined by calculating the average rfu from the two measurements performed for each sample and extrapolated to 500 µl, the initial amount of cell lysate. The amount of protein per well was calculated from the degree of absorption of the samples within the 96-well plate. These values were corrected through the BSA standard and converted to milligrams. The quotient of rfu and protein in milligram (rfu/mg) represented ASP^+ uptake and yielded absolute values, which were subsequently converted into relative values. The IC_{50} was calculated by equating the average amount of ASP^+ uptake by OCT1-overexpressing cells in the wells without any inhibitor (i.e. non-inhibited ASP^+ uptake) minus the uptake by the pcDNA5 cells (i.e. the baseline uptake) with 1,0. The amount of ASP^+ uptake at all other concentrations was set in relation to this baseline. Their values were expressed in percent of non-inhibited ASP^+ uptake and the percentages plotted as functions of their respective inhibitor concentration in a graph with SigmaPlot. The IC_{50}s of the tested substances were also calculated with SigmaPlot by plotting the percentages as functions of their respective inhibitor concentrations and fitting a nonlinear regression curve through the graph.

The ASP^+ uptake inhibition experiments were conducted at least thrice in independent experiments for each of the test drugs.

2.2.6 Transport experiments

The transport experiments were performed with OCT1-overexpressing HEK293 cell and two negative controls: pcDNA5 cells and OCT1-overexpressing cells supplemented with MPP^+, an inhibitor of OCT1.

Drug uptake was measured at three concentrations: 0.05, 0.1, and 0.5 μM. This was done once for pcDNA5 and twice for OCT1, one batch of which was supplemented with 2 mM MPP^+, a potent inhibitor of OCT1, and served as an additional negative control. The three remaining wells –one with pcDNA5 and two with OCT1— were reserved for protein measurements (see Figure 4).

Drug solutions were prepared at twice the desired concentration (i.e. 0.1, 0.2, and 1.0 μM) and the final concentrations obtained by adding an equal amount of either pure $HBSS^+$ or $HBSS^+$ spiked with 4 mM of MPP^+ (which would be diluted to the desired concentration of 2 mM, analogously to the ASP^+ in the inhibition experiments). For a single experiment, 0.75 ml of drug solution at double concentration was prepared for each drug. The stock solutions of 0.1, 0.2, and 1.0 μM were each split into 0.5 and 0.25 ml aliquots. 0.5 ml of pure $HBSS^+$ was added to the 0.5ml aliquots, yielding 1ml of 0.05, 0.1, and 0.5 μM drug solutions, the volume sufficient for testing one pcDNA5 and one OCT1 well. 0.25 ml of $HBSS^+$ containing 4 mM of MPP^+ was added to the 0.25 ml aliquots, tallying up to 0.5 ml of 0.05, 0.1, and 0.5 μM drug solutions with 2 mM MPP^+ each that were added to the negative control OCT1 wells.

In addition to the tested concentrations, a standard curve was prepared for each drug at concentrations of 0.1, 1, 10, and 100 nM. It was prepared in 80 % LC/MS-grade acetonitrile containing 10 ng/ml of an internal standard (IS), which varied depending on the substances tested (see 2.2.7, LC-MS). They underwent the same treatment as the samples did subsequent to cell lysis. The 80 % acetonitrile/IS solution also served as lysis buffer for the cells.

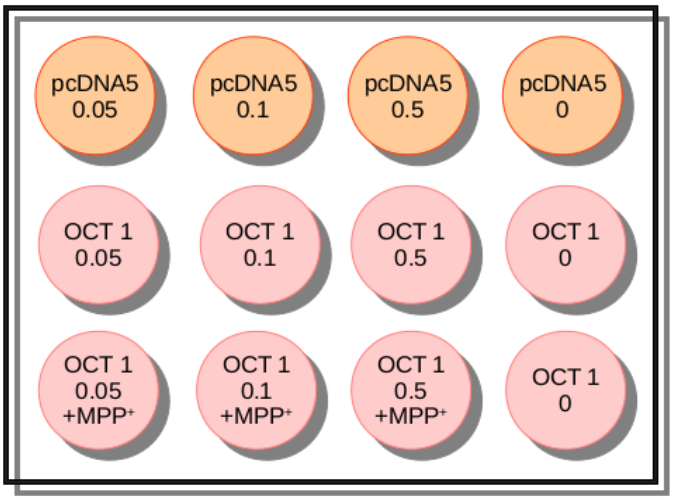


Figure 4: Plating and pipetting scheme for transport experiments, concentrations in µM

Treatment before, during, and after drug uptake was the same as in the inhibition experiments. The solutions were warmed up to 37°C and 400 µl added to the wells every ten seconds, while the wells for protein measurement (the zero concentration wells, see Figure 4) remained in pure $HBSS^+$. After incubating for two minutes, the cells were doused in 2 ml of ice-cold $HBSS^+$ per well and all transport processes thus terminated. The wells were then washed two more times before the buffers were added to the cells. Analogously to the inhibition experiments, 500 µl of RIPA buffer was added to the zero concentration wells destined for protein measurement, whereas the cells incubated in drug solution were treated with 500 µl of acetonitrile/IS buffer.

After placing the plates on the shaker for ten minutes, approx. 450 µl of the lysate (more if possible) were transferred from the wells on the plate and into test tubes. The samples, as well as the standards, were then centrifuged in a tabletop centrifuge at 13 x *g* for 10 min. 350 µl of the supernatant was subsequently transferred into new, carefully labeled (!) test tubes. These tubes were either stored at -20 °C until testing or immediately processed further.

For further processing, the tubes containing the samples were placed in a heating block and the samples evaporated with molecular nitrogen. Once the fluid had completely evaporated, the samples were resuspended in 350 µl of formic acid, vortexed, and centrifuged at 10 x *g* for 5 min. Finally, 100 µl of the centrifuged samples were transferred to LC-MS bottles and stored at 8 °C until measurement in the mass spectrometer

Protein measurements were performed analogously to those in the inhibition experiments (see 2.2.5).

The results of the LC-MS/MS measurement were given by the program in ng of detected drug per ml of eluent. These concentrations were converted into pmol/µl and extrapolated via the total volume of lysis buffer per well (500 µl/well) to pmol/well. The concentration of pmol/well was then divided by the amount of protein in mg per well and the incubation time of 2min, resulting in the uptake of measured drug in pmol/min/mg protein.

2.2.7 LC-MS/MS

Direct transport measurements were performed via mass spectrometry using an API4000 LC-MS/MS system with the Analyst® Version 1.4.2 software. The samples were processed as described above and then subjected to measurements following the measurement protocols listed in Table 1. The mass-to-charge ratio of precursor and product ions were mostly obtained from the literature, but frequently supplemented with data from our own tuning process since we preferred to measure the mass-to-ratio values of at least two product ions when possible. The remaining parameters –retention time, mobile phase, flow– are also the results of priming and testing conducted during the course of the experiments.

Table 1: Parameters for LC-MS/MS

Analyte	Qualifier Precursor Ion to Product Ion (m/z)	Quantifier Precursor Ion to Product Ion (m/z)	Retention time (min)	Internal Standard	Internal Standard Precursor Ion to Product Ion (m/z)	Retention time Internal Standard (min)	Mobile phase (% aceto-nitrile)	Flow (µl/min)
3-Hydroxymorphinan	244.1/157.0	244.1/132.9	7.3'	Nortilidine	260.3/155.1	8.3'	15	300
3-Methoxymorphinan	258.4/215.1	258.4/171.2	8.4'	Nortilidine	260.3/155.1	5.0'	20	500
Codeine	300.3/215.1	300.3/165.1	4.8'	Morphine	286.2/201.1	3.2'	8	300
Dextromethorphan	272.2/171.1	272.2/215.2	8.1'	Nortilidine	260.3/155.1	5.0'	20	500
Dextrorphan	258.4/157.1	258.4/199.1	4.7'	Nortilidine	260.3/155.1	5.0'	20	500
Fentanyl	337.4/104.9	337.35/188.2	5.0'	3-Methoxymorphinan	258.4/215	5.4'	20	700
Hydrocodone	300.5/199.1	300.5/128.1	6.8'	Codeine	300.3/215.1	5.0'	8	300
Hydromorphone	286.2/185.2	286.2/157.1	4.0'	Codeine	300.3/215.1	5.0'	8	300
Levorphanol	258.1/157.1	258.1/133.0	7.2'	Nortilidine	260.3/155.1	8.3'	15	300
Meptazinol	234.5/107.0	234.5/76.9	6.3'	Nortilidine	260.3/155.1	7.9'	15	300
Methylnaltrexone	356.2/338.2	356.2/284.1	5.6'	Morphine	286.2/201.1	3.2'	8	300
	356.2/338.2	356.2/284.1	3.6'	Nortilidine	260.3/155.1	8.1'	15	300
Morphine	286.2/201.1	286.2/165.1	3.2'	Codeine	300.3/215.1	4.8'	8	300
Naltrexone	342.3/324.2	342.3/270.1	6.3'	Codeine	300.3/215.1	4.8'	8	300
N-Desmethyltramadol	250.2/232.3	250.2/44.1	6.5'	Norfentanyl	233.2/84.1	4.8'	15	300
Norfentanyl	233.2/84.1	233.2/150.1	4.7'	N-Desmethyltramadol	250.2/232.3	6.4'	15	300
Noroxycodone	302.4/284.1	302.4/187.0	5.8'	Codeine	300.3/215.1	4.9'	8	300

Analyte	Qualifier Precursor Ion Analyte to Product Ion (m/z)	Quantifier Precursor Ion to Product Ion (m/z)	Retention time (min)	Internal Standard	Internal Standard Precursor Ion to Product Ion (m/z)	Retention time Internal Standard (min)	Mobile phase (% aceto-nitrile)	Flow (µl/min)
Nortilidine	260.3/155.1	260.3/229.1	8.0'	N-Desmethyltramadol	250.2/232.3	6.5'	15	300
Oxycodone	316.3/298.2	316.3/256.2	5.7'	Codeine	300.3/215.1	4.8'	8	300
Oxymorphone	302.3/284.1	302.3/277.1	3.7'	Codeine	300.3/215.1	4.8'	8	300
Pethidine (Meperidine)	248.4/174.1	248.4/91.0	8.0'	Nortilidine	260.3/155.1	7.9'	15	300
Sufentanil	387.2/238.1	387.15/110.9	10.5'	3-Methoxymorphinan	258.4/215	5.4'	20	700
Tapentadol	222.1/107.1	222.1/-	7.5'	Nortilidine	260.3/155.1	7.9'	15	300
Tilidine	274.1/154.9	274.1/91.1	8.3'	Nortilidine	260.3/155.1	8.0'	15	300

m/z: mass-to-charge ratio. The mass-to-charge ratio for qualifier and quantifier precursor ion were obtained from Vengurlekar et al. (Vengurlekar et al. 2002) for 3-hydroxymorphinan and 3-methoxymorphinan; from Eichhold et al. (Eichhold et al. 1997) for dextrorphan and dextromethorphan; from Fernandez et al. (Fernandez Mdel et al. 2013) for codeine, fentanyl, hydrocodone, hydromorphone, morphine, norfentanyl, oxycodone, and oxymorphone; from Bonn et al. (Bonn et al. 2010) for levorphanol; from Qiao et al. (Qiao et al. 2009) for meptazinol; from Oswald et al. (Oswald et al. 2011) for methylnaltrexone and naltrexone; from Godoy et al. (Godoy et al. 2011) for N-desmethyltramadol; from Fang et al. (Fang et al. 2013) for noroxycodone; from Köhler et al. (Kohler et al. 2011) for nortilidine and tilidine; from Agilent Technologies (Gulyas and Payne 2011) for pethidine; from Cooreman et al. (Cooreman et al. 2010) for sufentanil; and from Coulter et al. (Coulter et al. 2010) for tapentadol.

3 Results

3.1 *In silicio* predictions

We utilized *in silico* methods to narrow down the spectrum of possible OCT1 substrates by evaluating the pK_a, logP, and $logD_{(7.4)}$ values of 60 opioids and excluding drugs with very high hydrophobicity. The average pK_a, logP, and $logD_{(7.4)}$ of the complete set of opioids were 8.52, 2.55, and 1.51, respectively (Table 2). The predicted pK_a values lay between 6.71 and 10.02, and only three substances (alfentanil, remifentanil, and naloxone) had pK_a values below 7.4, indicating that overall the analyzed substances are moderate to strong bases and that, at a physiological pH in the range of 7.3-7.5, the equilibrium of 57 of the 60 substrates will favor an ionized state. LogP values ranged from -1.34 to 5.71, and $logD_{(7.4)}$ values from -1.57 to 4.66.

Based on this data, we initially selected compounds with low $logD_{(7.4)}$ values for further analyses and then expanded the list of candidate substances to include commonly used opioids (e.g. fentanyl, $logD_{(7.4)}$ value of 3.47), opioid/metabolite pairs (e.g. oxycodone/noroxycodone), and opioids that, despite yielding high $logD_{(7.4)}$ values, bore a promising structural resemblance to established OCT1 substrates (e.g. similarity of meptazinol to O-desmethyltramadol).

In this way, we selected a total of 23 opioid to undergo further experiments(Table 2). Their average pK_a, logP, and $logD_{(7.4)}$ values were 8.68, 2.25, and 1.00, respectively. The average pK_a and logP values of the complete set containing the original 60 substances and those of the experiment subset barely differ (+1.8 and -1.6%, respectively). In contrast, the average $logD_{(7.4)}$ value of the experiment subset was 33.8% lower than the average of the complete set (Table 2). This was in keeping with our strategy of testing substances with probable low lipophilicity.

Table 2: Opioids and their predicted pK_a, logP and $logD_{(7.4)}$ values, in alphabetical order

Opioid	pKa	logP	logD(7.4)	Included in further analysis	Reasons for inclusion
3-Hydroxymorphinan	**9.39**	**3**	**1.03**	Yes	Metabolite of dextromethorphan
3-Methoxymorphinan	**9.72**	**3.65**	**1.36**	Yes	Metabolite of dextromethorphan
3-Monoacetylmorphine	8.23	1.13	0.21		
6-Monoacetylcodeine	8.24	1.84	0.94		
6-Monoacetylmorphine	8.1	1.39	0.62		
Alfentanil	6.71	2.26	2.18		
alpha-L-Acetylmethadol	9.01	4.83	3.21		
Alvimopan	7.9	0.73	0.61		
Buprenorphine	7.94	4.72	4.07		
Butorphanol	7.87	3.71	3.11		
Codeine	**8.43**	**1.5**	**0.44**	Yes	Opioid used in clinical practice (cough syrups, analgesia), precursor of morphine
Dextromethorphan	**8.92**	**4.04**	**2.51**	Yes	Opioid used in clinical practice (cough syrups)
Dextrorphan	**8.81**	**3.34**	**1.92**	Yes	Metabolite of dextromethorphan
Dezocine	8.99	3.36	1.77		
Dihydrocodeine	8.69	1.78	0.47		
Dihydromorphine	8.54	1.14	-0.02		
Dipipanone	9.08	4.78	3.1		
Diprenorphine	7.97	3.33	2.66		
D-Propoxyphene	8.77	4.74	3.35		
EDDP	8.77	4.98	3.59		
Ethorphine	8.04	3.09	2.36		
Fentanyl	**8.23**	**4.36**	**3.47**	Yes	Opioid used in clinical practice (analgesia)
Heronie (Diamorphine)	8.07	1.55	0.79		
Hydrocodone	**8.59**	**1.54**	**0.32**	Yes	Opioid used in clinical practice (analgesia)
Hydromorphone	**8.43**	**1.09**	**0.04**	Yes	Opioid used in clinical practice (analgesia)
Levorphanol	**8.81**	**3.34**	**1.92**	Yes	Opioid used in clinical practice (analgesia), enantiomer of dextrorphan
L-Methadone	9.1	3.92	2.21		
Lofentanil	7.64	4.46	4.02		
Loperamide	7.74	5.17	4.66		

Opioid	pKa	logP	logD(7.4)	Included in further analysis	Reasons for inclusion
Meptazinol	**8.9**	**3.36**	**1.85**	Yes	Opioid used in clinical practice (analgesia), chemical structure
Methylnaltrexone	**9.15**	**-1.27**	**-1.59**	Yes	Opioid used in clinical practice (OIBD), negative $logD_{(7.4)}$ (lowest of all)
Morphine	**8.3**	**0.9**	**-0.05**	Yes	Opioid used in clinical practice (analgesia), negative $logD_{(7.4)}$
Morphine-3-Glucuronide	8.41	-1.23	-1.27		
Morphine-6-Glucuronide	8.19	-1.34	-1.4		
Nalbuphine	7.63	1.83	1.4		
Nalmefene	7.72	2.6	2.11		
Nalorphine	7.78	1.65	1.12		
Naloxone	7.26	1.09	0.85		
Naltrexone	**7.63**	**1.24**	**0.81**	Yes	Opioid used in clinical practice (antidote to opioid overdose)
N-Desmethyltramadol	**10.02**	**2.5**	**0.08**	Yes	Metabolite of tramadol
Norbuprenorphine	9.1	3.34	1.64		
Norfentanyl	**9.62**	**1.86**	**-0.33**	Yes	Metabolite of fentanyl, negative $logD_{(7.4)}$
Noroxycodone	**8.82**	**0.41**	**-0.93**	Yes	Metabolite of oxycodone, negative $logD_{(7.4)}$
Norpethidine	9.69	2.16	0.16		
Norpropoxyphene	9.73	4.26	2.01		
Nortilidine	**8.43**	**2.76**	**1.71**	Yes	Metabolite of tilidine (and active compound)
O-Desmethyltramadol	8.98	2.19	0.6		
Oripavine	7.79	2.01	1.47		
Oxycodone	**8**	**0.81**	**0.11**	Yes	Opioid used in clinical practice (analgesia)
Oxymorphone	**7.89**	**0.33**	**-0.28**	Yes	Opioid used in clinical practice (analgesia), negative $logD_{(7.4)}$
Pentazocine	8.23	4.25	3.36		
Pethidine (Meperidine)	**8.91**	**2.45**	**0.93**	Yes	Opioid used in clinical practice (analgesia, to counter shivering), chemical structure
Phenazocin	8.33	4.7	3.73		
Piritramide	7.57	3.48	3.08		
Remifentanil	7.25	1.96	1.73		
Sufentanil	**7.78**	**3.91**	**3.38**	Yes	Opioid used in clinical practice (analgesia, esp. during general anesthesia)
Tapentadol	**9.29**	**3.27**	**1.39**	Yes	Opioid used in clinical practice (analgesia)

Thebaine	7.92	2.63	1.99		
Opioid	pKa	logP	logD(7.4)	Included in further analysis	Reasons for inclusion
Tilidine	**7.64**	**3.33**	**2.89**	Yes	Opioid used in clinical practice (analgesia, prodrug)
Tramadol	9.04	2.83	1.18		

AVERAGE (n=60)	8.43	2.55	1.51
SEM	± 0.09	± 0.20	± 0.19
AVERAGE (n=23)	**8.68**	**2.51**	**1.00**
SEM	± **0.14**	± **0.30**	± **0.28**
Difference AVERAGES in %	+ 1.8	- 1.6	- 33.8

SEM: standard error of the mean. The 23 opioids that were selected to undergo further experiments are marked in bold. Averages and SEMs of all 60 analyzed opioids are at the bottom of the table, along with the averages of the 23 opioids of the experiment subset. The difference between the average pK_a, logP, and $logD_{(7.4)}$ of the 23 opioid subset as compared to all 60 opioids is given in percent.

3.2 Parallel artificial membrane assays (PAMPAs)

We conducted the PAMPAs in order to establish in how far the *in silicio* $logD_{(7.4)}$ values corresponded to actual membrane permeability in an *in vitro* cell membrane model.

The membrane permeabilities (P_e) of the 23 substances tested in the PAMPAs ranged across three potencies, from $0.002x10^{-5}$ (methylnaltrexone) to $6.4x10^{-5}$ (dextromethorphan). Despite the wide distribution, only six substances had values greater than $2x10^{-5}$. Seven substances had values smaller $0.5x10^{-5}$ (Table 3).

All substances were tested in duplicate at three concentrations, except for morphine and codeine, which were only tested once at three concentrations. However, since their P_e's correspond to those published previously by Tzvetkov et al. ($5 \pm 0.5\ x10^{-7}$ versus our $1.58 \pm 0.1\ x10^{-7}$ for morphine, and $8.2 \pm 0.4\ x10^{-6}$ versus our $8.01 \pm 1.3\ x10^{-6}$ for codeine (Tzvetkov et al. 2013)), we can assume that three measurements are sufficient to provide reliable P_e values.

Determining the exact P_e values of the substances with the highest membrane permeability caused some difficulty. Because their P_e's lie close to equilibrium, even slight imprecisions in MS measurements led to C_A becoming nominally larger than C_{equil}. In those cases, the formula used for calculating the P_e became invalid, so that the amount of data for the affected substances is not as large as we would have liked it to be. However, there was enough data to ensure that at least three individual P_e values contributed to the mean P_e of each substance presented. In addition, the high P_e's determined for dextromethorphan, fentanyl, pethidine, sufentanil, tilidine, and nortilidine are in line with established knowledge regarding their lipophilicity.

Overall, the P_e values correspond to the predicted $logD_{(7.4)}$; however in the cases of 3-hydroxymorphinan, dextrorphan, and levorphanol the experimentally determined P_e values are lower than the $logD_{(7.4)}$ values would originally have led us to expect. Conversely, oxycodone and pethidine yielded relatively high P_e values considering their $logD_{(7.4)}$ values. It follows that, while the $logD_{(7.4)}$ values are indicators of a substance's lipophilicity, actual membrane permeability should be assessed with cell-free membrane models such as PAMPA.

Table 3: P_e(mean) and SEM(mean) in comparison to $logD_{(7.4)}$ values

Opioid	P_e(mean) 10^{-5}	SEM 10^{-5}	$logD_{(7.4)}$
Methylnaltrexone	0.002	0.001	-1.59
Morphine	0.016	0.001	-0.05
3-Hydroxymorphinan	0.073	0.009	1.03
Noroxycodone	0.094	0.016	-0.93
Hydromorphone	0.17	0.04	0.04
Norfentanyl	0.26	0.07	-0.33
Oxymorphone	0.32	0.16	-0.28
Dextrorphan	0.54	0.16	1.92
Codeine	0.80	0.13	0.44
Hydrocodone	0.91	0.21	0.32
Levorphanol	0.92	0.18	1.92
N-Desmethyltramadol	1.3	0.32	0.08
Tapentadol	1.4	0.26	1.39
Meptazinol	1.4	0.35	1.85
Naltrexone	1.5	0.22	0.81
3-Methoxymorphinan	1.7	0.29	1.36
Oxycodone	1.8	0.42	0.11
Nortilidine	2.7	0.88	1.71
Tilidine	3.2	1.2	2.89
Sufentanil	3.8	0.59	3.38
Pethidine (Meperidine)	4.0	0.72	0.93
Fentanyl	5.7	0.10	3.47
Dextromethorphan	6.4	0.71	2.51

The PAMP assay simulated diffusion across a cell membrane under nearly physiological conditions (pH 7.4, ambient temperature of approx. 21°C). The substances are ranked from the substance with the smallest P_e down to the one with the largest. Opioids that emerged as substrates of OCT1 are printed in bold, opioids that emerged as inhibitors of OCT1 are highlighted in pale red. The smaller the partition coefficient P_e, the lower membrane permeability. The $logD_{(7.4)}$ values are the same as those discussed in section 3.1 and are listed here to facilitate the direct comparison of the values.

3.3 Inhibition experiments

Twenty-three opioids were tested for their potency to inhibit OCT1. To this end, OCT1-overexpressing cells were incubated with the model substrate ASP^+ in the presence of increasing concentrations of opioids. An inhibitor was defined as a substance with an IC_{50} of 100 µM or less, i.e. ASP^+ uptake had to be reduced by 50 % or more at inhibitor concentrations of 100 µM or less. Fourteen inhibitors of OCT1 were identified in this manner (Table 4).

Table 4: Tested substances sorted by IC_{50} from lowest to highest

Opioids	IC_{50} [µM]	SEM [µM]
Dextrorphan	6.40	1.63
Levorphanol	8.48	0.60
3-Methoxymorphinan	10.29	2.14
Dextromethorphan	15.15	2.52
Meptazinol	18.96	3.06
Sufentanil	19.36	0.19
Tapentadol	21.89	1.24
Pethidine (Meperidine)	22.25	3.66
3-Hydroxymorphinan	24.93	5.13
Tilidine	38.71	13.95
Fentanyl	46.17	6.81
N-Desmethyltramadol	55.75	9.41
Morphine	71.79	6.74
Nortilidine	88.76	18.81
Norfentanyl	117.74	16.56
Hydromorphone	137.97	19.22
Naltrexone	157.51	7.54
Noroxycodone	199.82	22.27
Methylnaltrexone	234.39	29.97
Codeine	235.50	29.42
Oxymorphone	250.33	35.24
Hydrocodone	536.96	87.58
Oxycodone	2003.78	172.90

OCT1 substrates are marked in bold. The dashed line indicates the cut-off between IC_{50}s lower and larger than 100 µM.

The inhibition curves of the tested substances are shown in Figure 5. The previously reported OCT1 substrate morphine had an IC_{50} of 71.8 ± 6.7 μM. Dextromethorphan and its derivatives were more potent OCT1-inhibitors than morphine and other morphinans with a more "morphine-like" structure (Fig. 8A and B). The group of synthetic opioids is mixed (Figs. C and D), but overall their IC_{50} values tend to lie below that of morphine, as do the IC_{50} values of the opioid receptor antagonists naltrexone and methylnaltrexone (Fig. E).

The nine most potent inhibitors had IC_{50} values below 25 μM, ranging from dextrorphan (IC_{50} of 6.4 μM) to 3-hydroyxmorphinan (IC_{50} of 34.9 μM). Tilidine, fentanyl, N-desmethyltramadol, morphine, and nortilidine also qualified as inhibitors.

In contrast, the substances with the highest IC_{50} values -methylnaltrexone, codeine, oxymorphone, hydromorphone and especially oxycodone- spanned a range from 234 μM to above 2 mM.

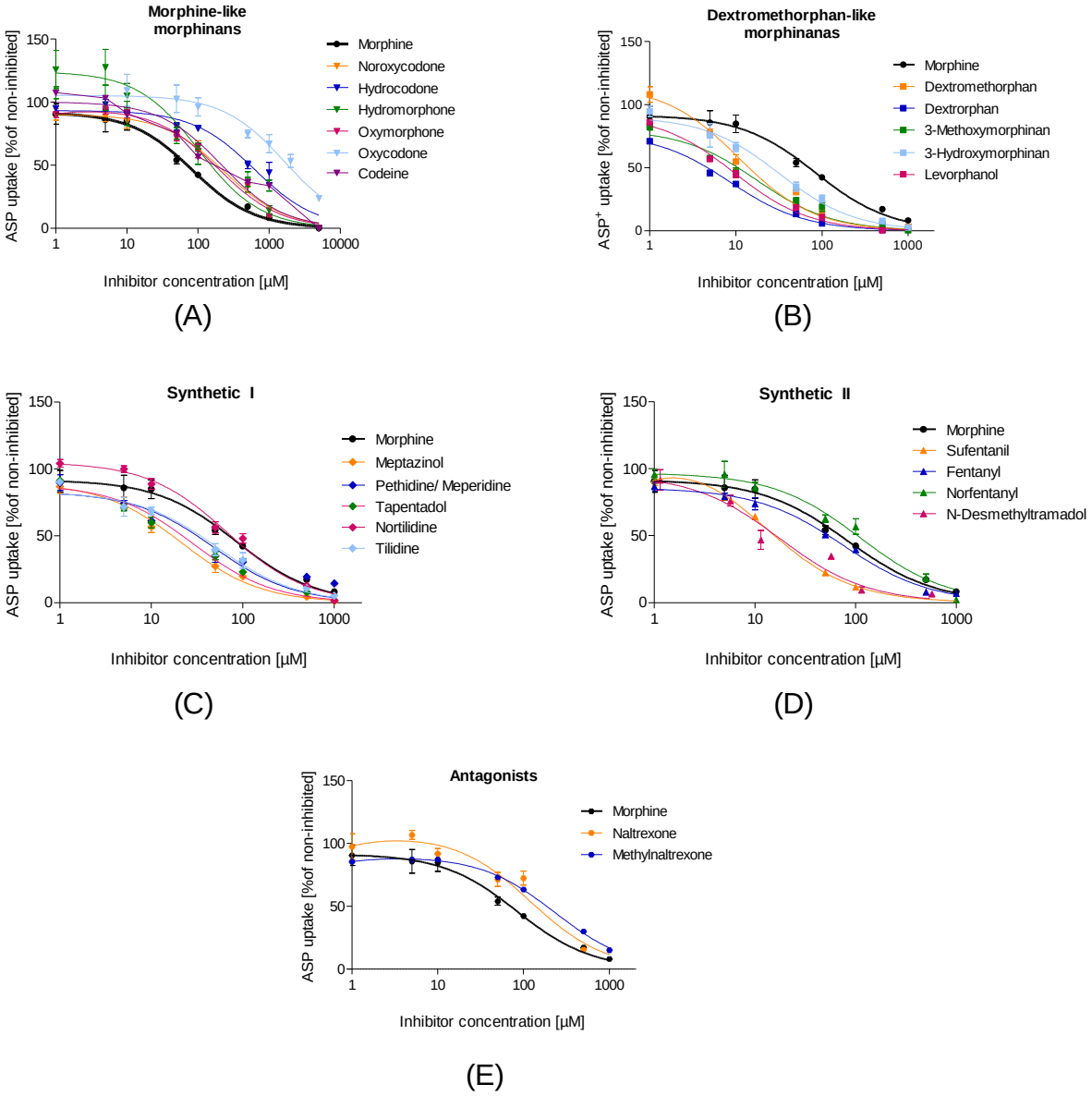


Figure 5: Comparative analyses of opioids as inhibitors of OCT1

For better comparability, the substances were assigned to one of five different groups based on their structure and inhibitory potency. The first group (A) comprises those morphinans with a structure similar to morphine. The second group (B) is made up of dextromethorphan and its metabolites, plus levorphanol, the enantiomer of dextromethorphan-metabolite dextrorphan. Although they, too, are morphinans, they differ from morphine and the antagonists structurally in that they contain only four rings instead of five. The synthetic opioids (C and D) are structurally more heterogeneous than the morphinans and were distributed across two graphs to provide a better overview over the curves. The final group (E), the antagonists, are also morphine-like morphinans, but in contrast to the other opioids they act antagonistically upon opioid receptors. The morphine curve was added in black to all graphs to allow for better comparability of the curves between graphs.

3.4 Transport experiments

3.4.1 Relative transport

The main objective of this work was to determine which opioids may profit from OCT1 for their cellular uptake, i.e. which opioids are OCT1 substrates. To this end, we performed direct uptake measurements of the different opioids into OCT1-overexpressing cells via mass spectrometry. A substance was arbitrarily defined as a substrate if uptake of the substance into OCT1-overexpressing cells was at least 2.0 times as high as that into pcDNA5 cells at two or more of the three concentrations tested. In addition to the negative control pcDNA5 cells, we also measured uptake in MPP^+-inhibited OCT1-overexpressing cells to confirm that the values obtained from the pcDNA5 cells were the result of diffusion and not of some fault in the cell line.

Overall, we assigned the tested substances to one of three groups: strong transport, moderate transport, and no transport. Out of 23 tested substances the transport experiment yielded - next to the previously known morphine- seven further OCT1 substrates; three with strong transport and four with moderate transport.

Strong transport

Strong transport was defined as an at least five-fold increase of uptake of tested substance into OCT1-overexpressing cells compared to the negative control. In total, three substances strongly transported by OCT1 were identified: methylnaltrexone, 3-hydroxymorphinan, and norfentanyl (Table 5). The strongest increase in uptake was seen in methylnaltrexone, with a 32-, 67-, and 85-fold increase in uptake at concentrations of 0.05, 0.1, and 0.5 μM, respectively. 3-Hydroxymorphinan experienced a 14 to 17-fold increase in uptake across the concentration range, while norfentanyl showed an approximately 7-fold increase in uptake at all three concentrations.

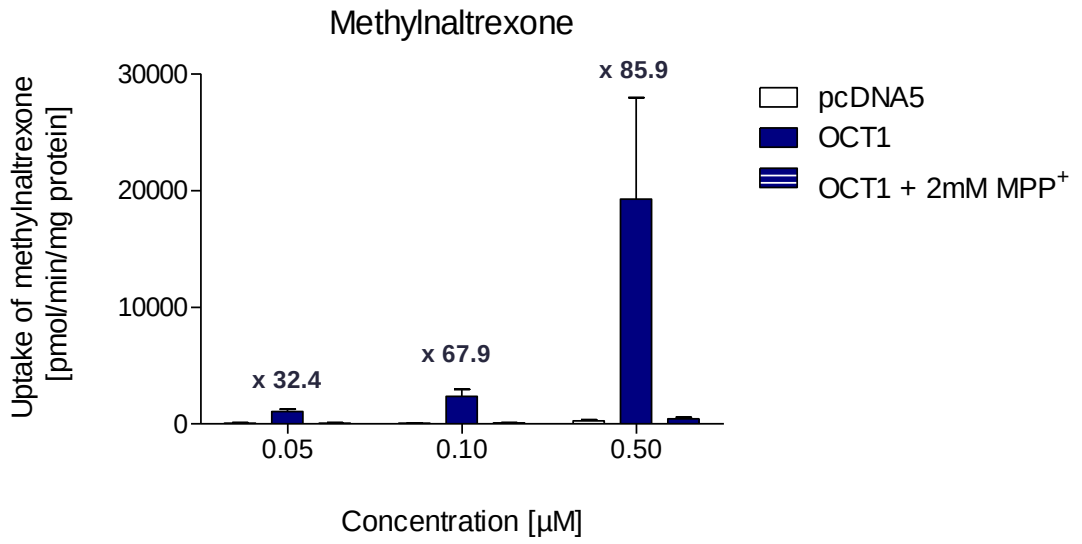


Figure 6: Relative increase in uptake of methylnaltrexone

Negative control pcDNA5 cells, OCT1 cells, and OCT1 cells inhibited with 1 mM MPP^+ were incubated with opioid solutions at concentrations of 0.05 µM, 0.1 µM, and 0.5 µM for two minutes and uptake of the opioid into the cells determined via LC-MS/MS. On the scale presented, the bars of the pcDNA5 cells and the OCT1 + 2 mM MPP^+ cells are barely visible compared to the bars of the OCT1 cells.

Moderate transport

Moderate transport was defined as an at least two-fold, but less than five-fold increase in uptake of tested substance into OCT1-overexpressing cells compared to the negative control cells. Next to morphine, which has already been established as a substrate, four other substances were identified to be transported by OCT1 (Table 5). In descending order of uptake, these substances were morphine (maximally 5.1-fold increase), noroxycodone (maximally 4-fold increase), 3-methoxymorphinan (maximally 2.5-fold increase), meptazinol (maximally 2.3-fold increase, and hydromorphone (maximally 2-fold increase).

No transport

Transport activity was deemed non-existent when the uptake of substance into OCT1-overexpressing cell was less than two times high as that into the negative controls. Codeine, dextromethorphan, dextrorphan, fentanyl, hydrocodone, levorphanol, naltrexone, oxycodone, oxymorphone, pethidine, sufentanil, tapentadol, and tilidine were allocated to this group.

Data on N-desmethyltramadol (the inactive M2 metabolite of tramadol) and nortilidine was inconclusive; whether or not and to what extent N-desmethyltramadol and nortilidine are transported by OCT1 could therefore not be determined.

Table 5: Relative increase in uptake of tested substances

	0.05 µM		0.1 µM		0.5 µM	
Substance	**Mean**	SEM	**Mean**	SEM	**Mean**	SEM
3-Hydroxymorphinan	14.0	±1.7	14.9	±1.2	17.4	±4.8
3-Methoxymorphinan	2.5	±0.7	2.2	±0.4	2.0	0.1
Codeine	0.7	±0.2	1.0	±0.3	0.8	±0.0
Dextromethorphan	1.1	±0.2	1.3	±0.4	0.8	±0.2
Dextrorphan	2.1	±0.2	1.9	±0.8	1.5	±0.1
Fentanyl	1.0	±0.1	1.0	±0.1	1.0	±0.1
Hydrocodone	0.9	±0.1	1.6	±0.3	1.2	±0.1
Hydromorphone	2.0	±0.1	1.9	±0.1	2.0	±0.1
Levorphanol	1.5	±0.2	1.7	±0.1	1.3	±0.0
Meptazinol	0.8	±0.2	2.2	±0.5	3.2	±1.4
Methylnaltrexone	32.4	±20.4	67.9	±39.8	85.9	±24.7
Morphine	4.6	±0.5	5.1	±0.7	4.3	±0.7
Naltrexone	0.8	±0.2	0.8	±0.2	1.0	±0.2
N-desmethyltramadol	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Norfentanyl	7.0	±0.5	6.6	±0.6	7.2	±0.6
Noroxycodone	4.0	±1.4	3.9	±0.3	3.6	±0.1
Nortilidine	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Oxycodone	0.9	±0.0	0.8	±0.1	0.9	±0.0
Oxymorphone	2.4	±1.6	1.3	±0.4	0.9	±0.2
Pethidine (Meperidine)	1.1	±0.1	1.2	±0.1	1.2	±0.1
Sufentanil	1.1	±0.0	1.0	±0.1	1.1	±0.1
Tapentadol	0.9	±0.2	1.6	±0.3	1.5	±0.2
Tilidine	1.3	±0.2	1.3	±0.3	1.3	±0.1

The relative increase of substrate into OCT1-overexpressing cells was calculated in relation to the negative control cells at concentrations of 0.05, 0.1, and 0.5µM. Substances identified as substrates of OCT1 are highlighted in pale green.

3.4.2 Absolute transport

The absolute transport of the different opioids into the different cells lines is of less interest than the relative uptake; nevertheless, the data are included in Table 6 in order to supplement the relative values and help with their interpretation.

Table 6: Absolute uptake of the tested substance

	Uptake in pmol/min/mg protein								
	pcDNA 0.05	pcDNA 0.1	pcDNA 0.5	OCT 1 0.05	OCT 1 0.1	OCT1 0.5	OCT1 0.05 + MPP^+	OCT1 0.1 + MPP^+	OCT1 0.5 + MPP^+
3-Hydroxymorphinan	168.5 ±16.5	333.7 ±50.8	1852.3 ±168.1	2324.2 ±198.2	4857.0 ±376.6	30594.4 ±5098.1	209.1 ±14.4	418.0 ±21.3	2601.9 ±78.8
3-Methoxymorphinan	3950.1 ±2169.5	2706.4 ±746.1	18262.3 ±4635.4	6903.9 ±1343.8	6246.3 ±1942.0	37657.1 ±10295.7	3849.1 ±1057.1	4581.9 ±1358.3	30503.0 ±11154.5
Codeine	434.0 ±82.3	909.5 ±270.8	4281.8 ±1027.8	322.7 ±131.5	749.9 ±100.5	3499.0 ±774.9	567.9 ±318.2	734.7 ±114.3	4389.3 ±1227.4
Dextromethorphan	2417.9 ±622.4	6314.6 ±2575.9	30502.3 ±9044.0	2692.5 ±1055.3	9233.4 ±5900.6	21134.0 ±4605.1	2539.8 ±940.2	4375.0 ±1382.9	24182.7 ±6976.0
Dextrorphan	897.5 ±151.7	2613.0 ±919.7	11808.0 ±4056.5	2357.3 ±479.4	5897.5 ±1663.0	17326.3 ±5610.3	1284.3 ±179.8	2037.2 ±588.0	11235.1 ±2397.8
Fentanyl	591.1 ±177.6	1153.6 ±199.3	5488.4 ±727.0	604.3 ±155.5	1127.8 ±198.5	5402.9 ±746.6	497.4 ±145.8	872.1 ±165.5	5009.9 ±760.2
Hydrocodone	80.8 ±18.8	176.4 ±25.7	1751.0 ±153.0	77.9 ±27.9	265.0 ±40.9	2175.8 ±308.7	85.0 ±18.9	223.9 ±32.6	1761.6 ±271.6
Hydromorphone	95.5 ±2.2	196.5 ±9.2	1089.8 ±86.6	193.3 ±13.6	371.6 ±22.3	2158.1 ±102.2	95.0 ±1.2	175.3 ±2.3	991.6 ±83.4
Levorphanol	1215.3 ±503.5	1457.6 ±137.9	8104.7 ±777.7	1339.6 ±190.7	2427.6 ±213.1	10654.0 ±1297.4	795.7 ±85.5	2146.2 ±728.8	8098.2 ±587.1
Meptazinol	599.0 ±170.0	806.1 ±148.1	3930.5 ±874.2	415.2 ±50.7	1645.5 ±373.9	13038.5 ±5564.5	356.6 ±98.0	884.2 ±167.1	3353.4 ±355.3
Methylnaltrexone	71.2 ±42.6	54.3 ±21.5	261.0 ±106.7	1061.2 ±200.7	2374.6 ±588.8	19269.2 ±8701.0	61.6 ±40.5	86.6 ±34.6	427.1 ±144.4

Morphine	141.8 ±7.9	257.5 ±8.7	1367.0 ±146.0	654.0 ±52.4	1324.8 ±180.1	5803.9 ±620.1	102.4 ±3.2	440.9 ±249.9	872.0 ±104.4
Naltrexone	380.5 ±113.7	788.9 ±31.6	3949.2 ±87.8	274.9 ±11.4	596.1 ±107.6	4133.9 ±908.1	301.1 ±70.1	720.9 ±130.5	4452.6 ±1093.3
N-desmethyltramadol	N.A. N.A.	N.A. N.A.	N.A. N.A.	N.A. N.A.	N.A. N.A.	N.A. N.A.	N.A. N.A.	N.A. N.A.	N.A. N.A.
Norfentanyl	109.9 ±12.8	232.1 ±17.8	1189.6 ±56.3	757.4 ±50.9	1503.8 ±21.8	8554.4 ±621.9	105.0 ±16.5	185.8 ±7.3	875.3 ±21.3
Noroxycodone	94.0 ±20.1	163.8 ±24.4	979.2 ±29.5	320.5 ±58.5	623.9 ±39.7	3496.0 ±45.5	45.4 ±21.8	120.1 ±20.9	755.1 ±31.6
Nortilidine	N.A. N.A.	N.A. N.A.	N.A. N.A.	N.A. N.A.	N.A. N.A.	N.A. N.A.	N.A. N.A.	N.A. N.A.	N.A. N.A.
Oxycodone	227.0 ±28.3	463.1 ±25.5	2402.4 ±247.6	212.1 ±22.3	370.3 ±40.7	2212.5 ±272.1	56.6 ±28.5	183.9 ±41.6	1083.6 ±158.2
Oxymorphone	114.2 ±98.1	857.0 ±408.9	1849.4 ±87.1	189.0 ±17.6	761.2 ±295.1	1568.0 ±366.4	470.9 ±272.3	605.7 ±365.3	2021.8 ±283.5
Pethidine	458.1 ±154.5	806.5 ±231.3	5177.4 ±1777.5	503.5 ±149.8	959.0 ±188.9	5728.5 ±1162.8	579.7 ±181.5	1120.8 ±289.3	5502.8 ±1299.8
Sufenanil	849.2 ±77.2	2040.3 ±99.3	11525.7 ±776.0	931.8 ±107.9	2105.2 ±217.6	12482.4 ±2033.8	718.7 ±138.1	1895.6 ±303.0	11161.1 ±1853.8
Tapentadol	1070.0 ±89.1	1886.5 ±572.3	10534.1 ±1581.6	944.1 ±244.2	2921.7 ±854.4	15453.2 ±2753.6	963.3 ±276.1	1553.6 ±343.7	8182.0 ±1888.1
Tilidine	171.0 ±19.3	312.7 ±30.5	1704.4 ±319.9	229.8 ±56.8	437.2 ±132.4	2158.1 ±496.7	215.5 ±47.9	350.3 ±118.4	2041.6 ±687.1

Printed above in each cell is the average uptake of substrate in pmol/min/mg; below are the respective SEMs. OCT1 substrates are highlighted in green. N.A. (not available): the measurements performed with these substances were inconclusive and not further pursued due to unavailability of the required amounts of test substance.

4 Discussion

We will discuss the results of our experiments under four aspects: pharmaco-clinical properties, structural aspects, aspects of metabolism, and clinical aspects.

4.1 Pharmaco-chemical properties

In order to better evaluate our findings regarding pK_a, $logD_{(7.4)}$, and P_e, we are going to explore these parameters under the aspects of OCT1 transport (OCT1 substrates vs. non-substrates), OCT1 inhibition (OCT inhibitors vs. non-inhibitors), and pharmacologic class (morphinans vs. synthetic opioids).

Substrates vs. non-substrates

A histogram of the pK_a values of OCT1 substrates compared to those of non-substrates yielded no marked difference between the two groups (Figure 7), though the substrates' average pK_a lay slightly above that of the non-substrates, with 9.04 and 8.49, respectively (Table 7).

With respect to $logD_{(7.4)}$, substrates of OCT1 displayed lower values than non-substrates, with substrates yielding an average $logD_{(7.4)}$ of 0.17 and non-substrates of 1.44 ($p=0.026$, Table 7). This was to be expected since only substances with low lipophilicity and presumed low membrane permeability would require a transporter to enable their uptake into cells. This finding was further bolstered by the results of the substrates' and non-substrates' P_e values. With a value of $0.47x10^{-5}$, the average P_e's of the substrates is considerably lower than that of the non-substrates, which lies at $2.35x10^{-5}$ ($p=0.013$, Table 7). Of the seven substances with P_e values below $5x10^{-6}$, six are substrates of OCT1. The two substrates of OCT1 with larger P_e values, meptazinol and 3-methoxymorphinan, lay between 1.4 and $1.7x10^{-5}$ (see Table 3, section 3.2). The P_e values of non-substrates were evenly spread across the spectrum (Figure 9).

These results nicely illustrate that substances with low membrane permeability are more likely to be substrates of OCT1 than those with high $logD_{(7.4)}$ and P_e values.

Inhibitors vs. non-inhibitors

At 8.80 and 8.51, the average pK_a's of inhibitors and non-inhibitors happen to be very similar. Their average $logD_{(7.4)}$ and P_e values, however, differ markedly. While the average $logD_{(7.4)}$

and P_e values of the inhibitors were 3.16 and 1.74, respectively, those of the non-inhibitors lay far below that, with a $logD_{(7.4)}$ of 0.83 and a P_e value of -0.16 ($p=0.00014$ and $p=0.023$, respectively, Table 7). The histogram in Figure 9 especially illustrates that non-inhibitors have low P_e values, whereas inhibitors span the entire spectrum.

It appears that, while low $logD_{(7.4)}$ and P_e values are common in substrates of OCT1, they also correlate with a low probability for inhibitory potential.

Morphinans vs. synthetic opioids

Following fourteen substances are morphinans: morphine, codeine, oxycodone, oxymorphone, hydrocodone, hydromorphone, noroxycodone, naltrexone, and methlynaltrexone as well as dextromethorphan, dextrorphan, levorphanol, 3-methoxymorphinan, and 3-hydroxymorphinan. Opposed to the morphinans are the synthetic opioids and their metabolites, whose chemical structure bears no resemblance to the polycyclic structure of the morphinans. The group of synthetic opioids was made up by nine substances: fentanyl, meptazinol, N-desmethyltramadol, norfentanyl, nortilidine, pethidine, sufentanil, tapentadol, and tilidine.

While there is little difference in the pK_a values of morphinans and synthetic opioids (8.64 and 8.74, respectively; $p=0.675$), the average $logD_{(7.4)}$ of the synthetic opioids lay above that of the morphinans, with values of 1.94 and 0.54, respectively ($p=0.038$, Table 7, Figure 7, and Figure 8). These values indicate that synthetic opioids are more lipophilic than the classic morphinans, which is indeed their purpose. Synthetic opioids were designed to be more potent than classic opioid, and higher lipophilicity means these opioids can cross the blood-brain barrier more easily and have a more powerful effect on the central nervous system. In comparison, the difference between the P_e values of morphinans and synthetic opioids seems less marked (1.09 and 2.64, respectively; $p=0.042$); however, the discrepancy in the distribution of their P_e values becomes visible in Figure 9, with the morphinans clearly clustered around low P_e values. Dextromethorphan, on the far right of the x-achsis, is the only morphinan with a P_e value larger $2x10^{-5}$ (Figure 9). In contrast, the P_e values of the synthetic opioids did not fall below $1x10^{-5}$.

Methylnaltrexone yielded the lowest P_e ($0.002 \pm 0.001x10^{-5}$) by far, indicating very poor membrane permeability. It was followed by morphine, 3-hydroxymorphinan, noroxycodone, hydromorphone, and norfentanyl, all of which are substrates of OCT1, and all of which, except for norfentanyl, are morphinans.

Overall, OCT1 substrates, non-inhibitors, and morphinans have significantly lower $logD_{(7.4)}$ and P_e values than their respective counterparts. Opioids with low lipophilicity are therefore more likely to be a substrate of OCT1, a non-inhibitor, or a morphinan (or a combination thereof) while more lipophilic opioids are more likely to be non-substrates, inhibitors, and synthetic opioids.

Table 7: pKa, $logD_{(7.4)}$, and P_e values and their significance

Opioid	pKa	$logD_{(7.4)}$	P_e x10^{-5}
3-Hydroxymorphinan	9.39	1.03	0.073
3-Methoxymorphinan	9.72	1.36	1.7
Codeine	8.43	0.44	0.80
Dextromethorphan	8.92	2.51	6.4
Dextrorphan	8.81	1.92	0.54
Fentanyl	8.23	3.47	5.7
Hydrocodone	8.59	0.32	0.91
Hydromorphone	8.43	0.04	0.17
Levorphanol	8.81	1.92	0.92
Meptazinol	8.90	1.85	1.4
Methylnaltrexone	9.15	-1.59	0.002
Morphine	8.30	-0.05	0.016
Naltrexone	7.63	0.81	1.5
N-Desmethyltramadol	10.02	0.08	1.3
Norfentanyl	9.62	-0.33	0.26
Noroxycodone	8.82	-0.93	0.094
Nortilidine	8.43	1.71	2.7
Oxycodone	8.00	0.11	1.8
Oxymorphone	7.89	-0.28	0.32
Pethidine (Meperidine)	8.91	0.93	4.0
Sufentanil	7.78	3.38	3.8
Tapentadol	9.29	1.39	1.4
Tilidine	7.64	2.89	3.2
Average Total	**8.68**	**1.00**	**1.70**
Average substrates	**9.04**	**0.17**	**0.47**
Average non-substrates	**8.49**	**1.44**	**2.35**
Significance (p-value)	**0.057**	**0.026**	**0.013**
Average inhibitors	**8.80**	**1.74**	**2.37**
Average non-inhibitors	**8.51**	**-0.16**	**0.65**
Significance (p-value)	**0,318**	**0,00014**	**0,023**
Average morphinans	**8.64**	**0.54**	**1.09**
Average synthetic opioids	**8.76**	**1.71**	**2.64**
Significance (p-value)	**0.675**	**0.038**	**0.042**

The averages are given in bold, along with the significance of the differences between the averages for substrates vs. non-substrates, inhibitors vs. non-inhibitors, and morphinans vs. synthetic opioids.

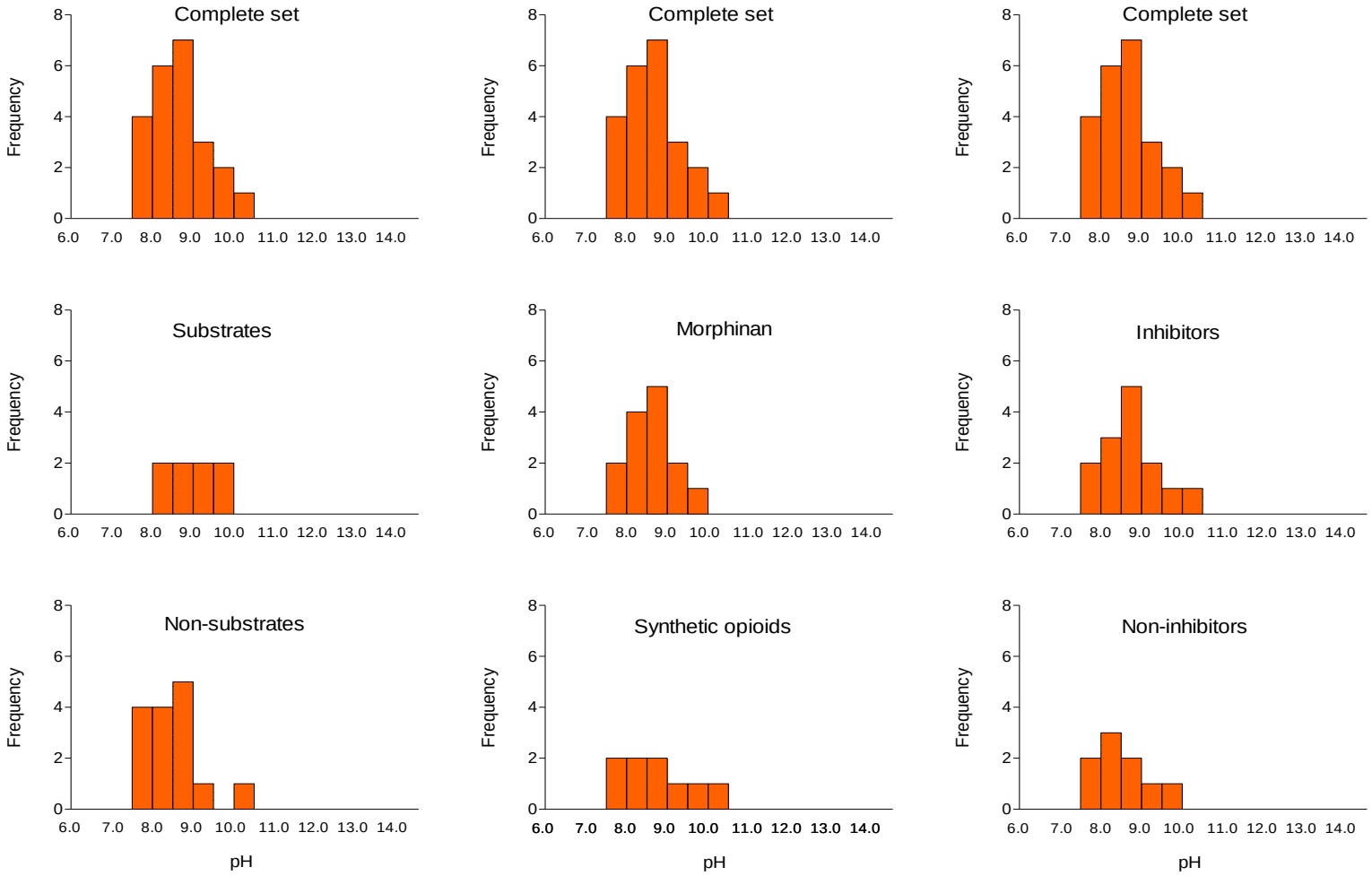


Figure 7: Histograms of pKa values for OCT1 substrates and non-substrates, morphinans and synthetic opioids, and OCT1 inhibitors and non-inhibitors

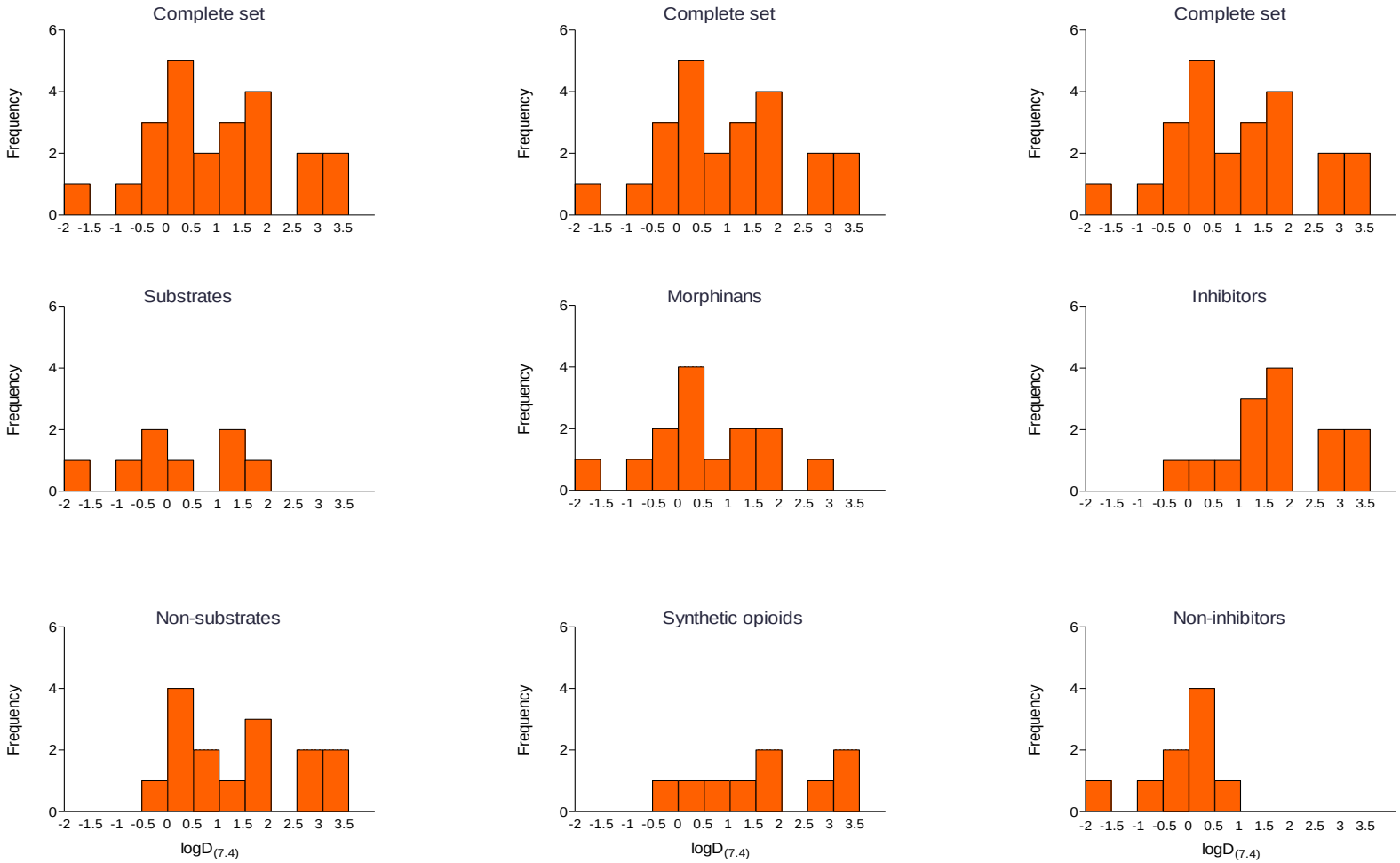


Figure 8: Histograms of $logD_{(7.4)}$ values for OCT1 substrates and non-substrates, morphinans and synthetic opioids, and OCT1 inhibitors and non-inhibitors

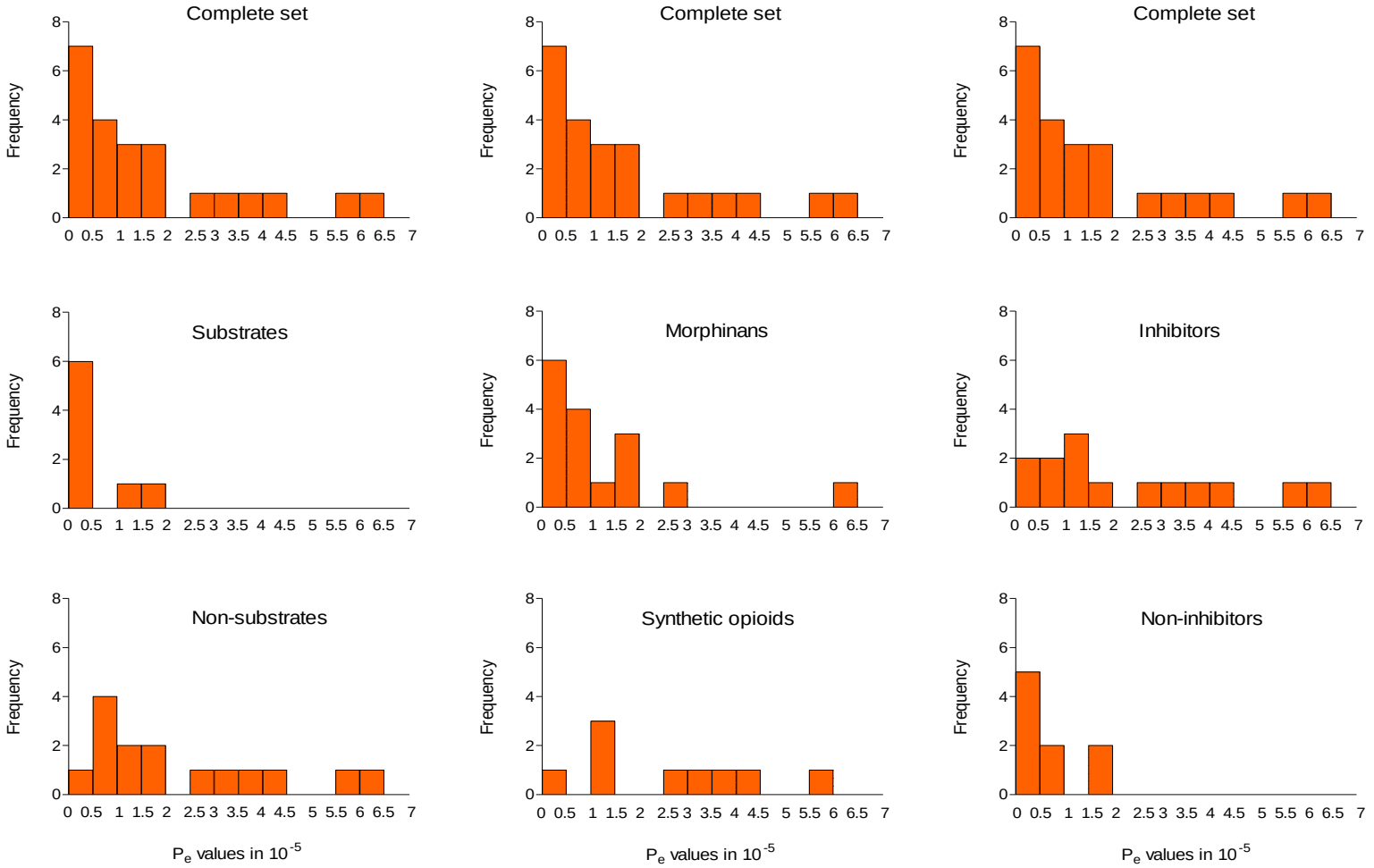


Figure 9: Histograms of P_e values for OCT1 substrates and non-substrates, morphinans and synthetic opioids, and OCT1 inhibitors and non-inhibitors

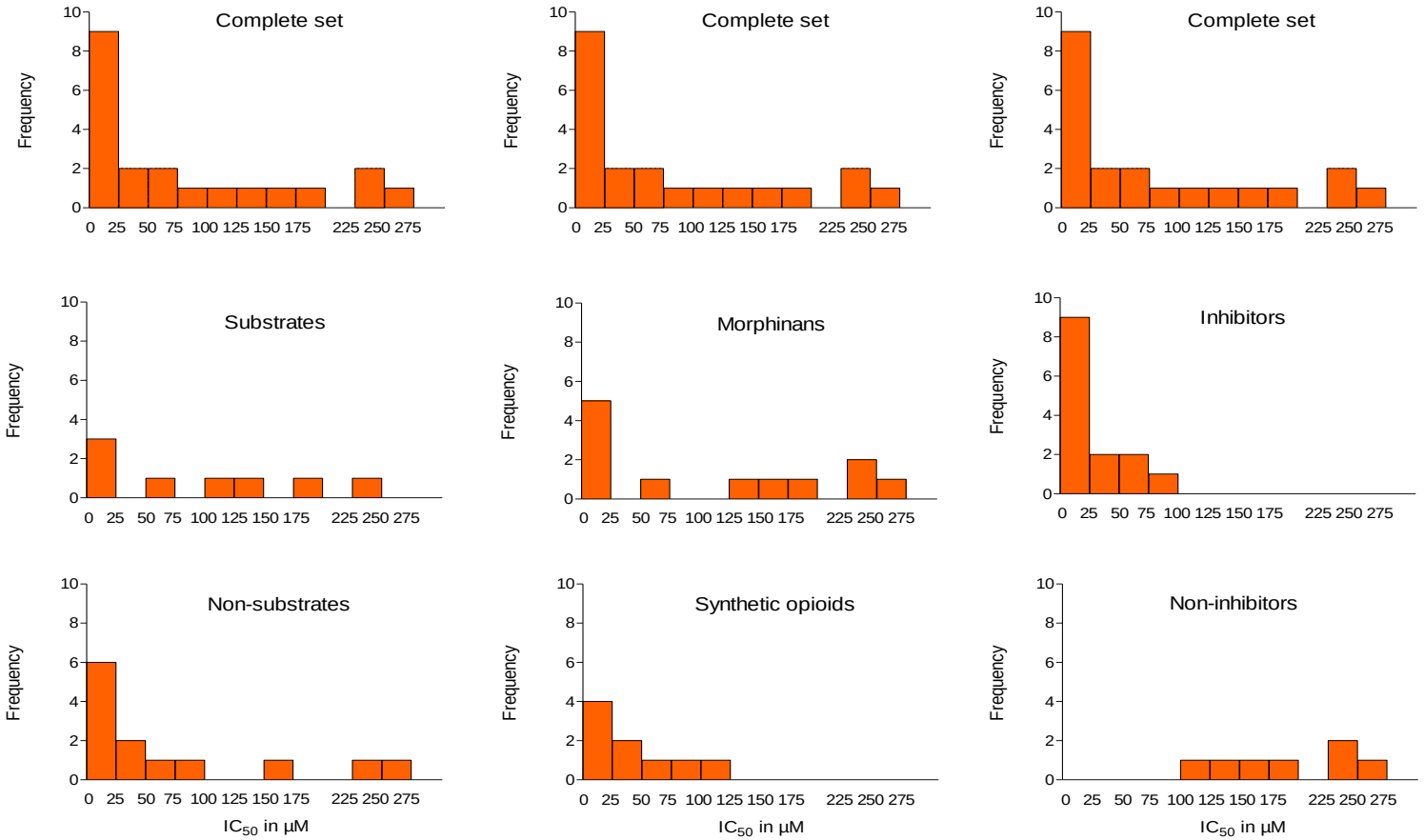


Figure 10: Histograms of IC_{50} values for OCT1 substrates and non-substrates, morphinans and synthetic opioids, and OCT1 inhibitors and non-inhibitors In order to provide a better overview, outliers hydrocodone (IC_{50}=537 µM) and oxycodone (IC_{50}=2004 µM) have been omitted.

4.2 Structural aspects

In the following we shall discuss which structural aspects might predispose a substance to be either a substrate of OCT1 or an inhibitor of OCT1, or to have no observed interaction with OCT1.

Substrates

Six of eight substrates are opioids with a morphinan structure. The two exceptions are meptazinol, a synthetic opioid, and norfentanyl, metabolite of the synthetic opioid fentanyl. Norfentanyl, despite being a synthetic opioid, resembles the morphinans in its chemical properties more than it does the synthetic opioids (see Table 7, section 4.1).

Despite obvious shared characteristics (polycyclic structure, aromatic ring), a common denominator that singles out these opioids as substrates in contrast to, for example, dextrorphan or oxycodone, remains elusive.

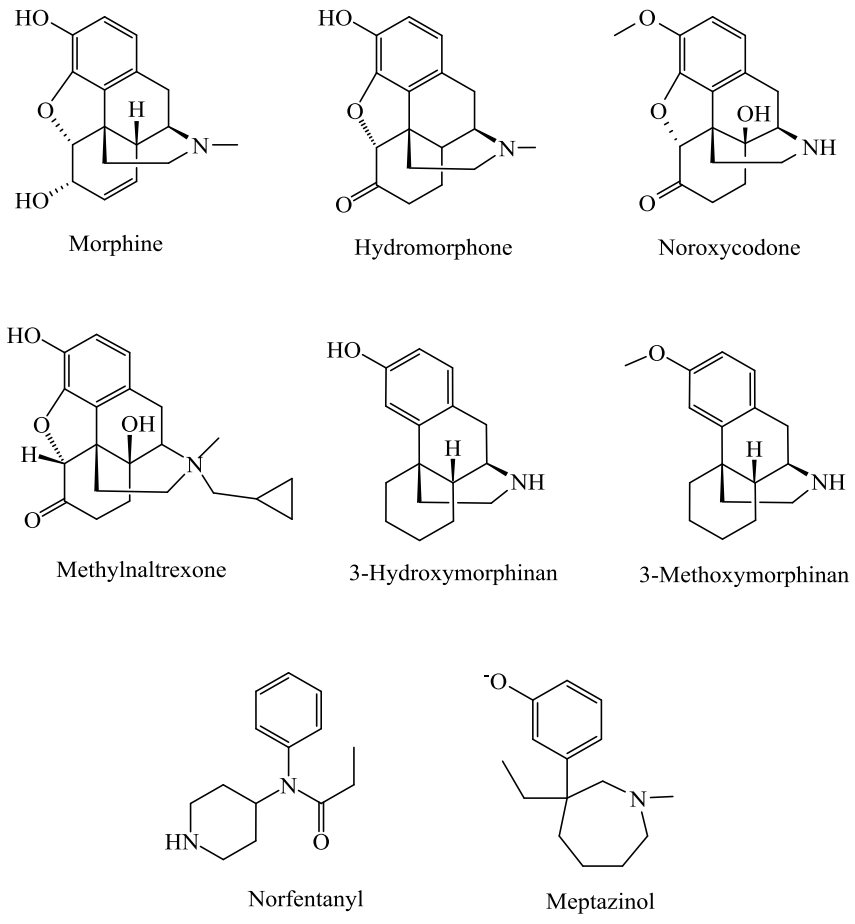


Figure 11: Identified substrates of OCT1

Inhibitors

Synthetic opioids and their metabolites make up more than half the roster of inhibitors; of the fourteen inhibitors, eight are synthetic. Of the six inhibitors with a morphinan structure, morphine is the only "classic" morphinan; the other five inhibitors are dextromethorphan and its metabolites.

As mentioned in the introduction, substances with an inhibitory effect on OCT1 have different qualities in common. One of them is lipophilicity. The results from our PAMP assays confirm that the opioids identified by us as inhibitors are indeed more lipophilic than those that did not inhibit OCT1 (see Table 7). Additionally, all our inhibitors are at least partially charged at a physiological pH and have a relative dearth of hydrogen-bonding moieties (Figure 12). The properties intrinsic to our set of inhibitors are therefore in keeping with the model developed by Ahlin et al. (Ahlin et al. 2008).

As can be seen in Figure 13 and Figure 14, synthetic opioids and dextromethorphan-like morphinans interact strongly with OCT1. The dextromethrophans interact even more strongly than the synthetic opioids; of the five inhibitors with IC_{50} values below 20 μM, four are dextromethorphan-like morphinans. But again, we cannot name a specific moiety responsible for making an opioid an inhibitor of OCT1.

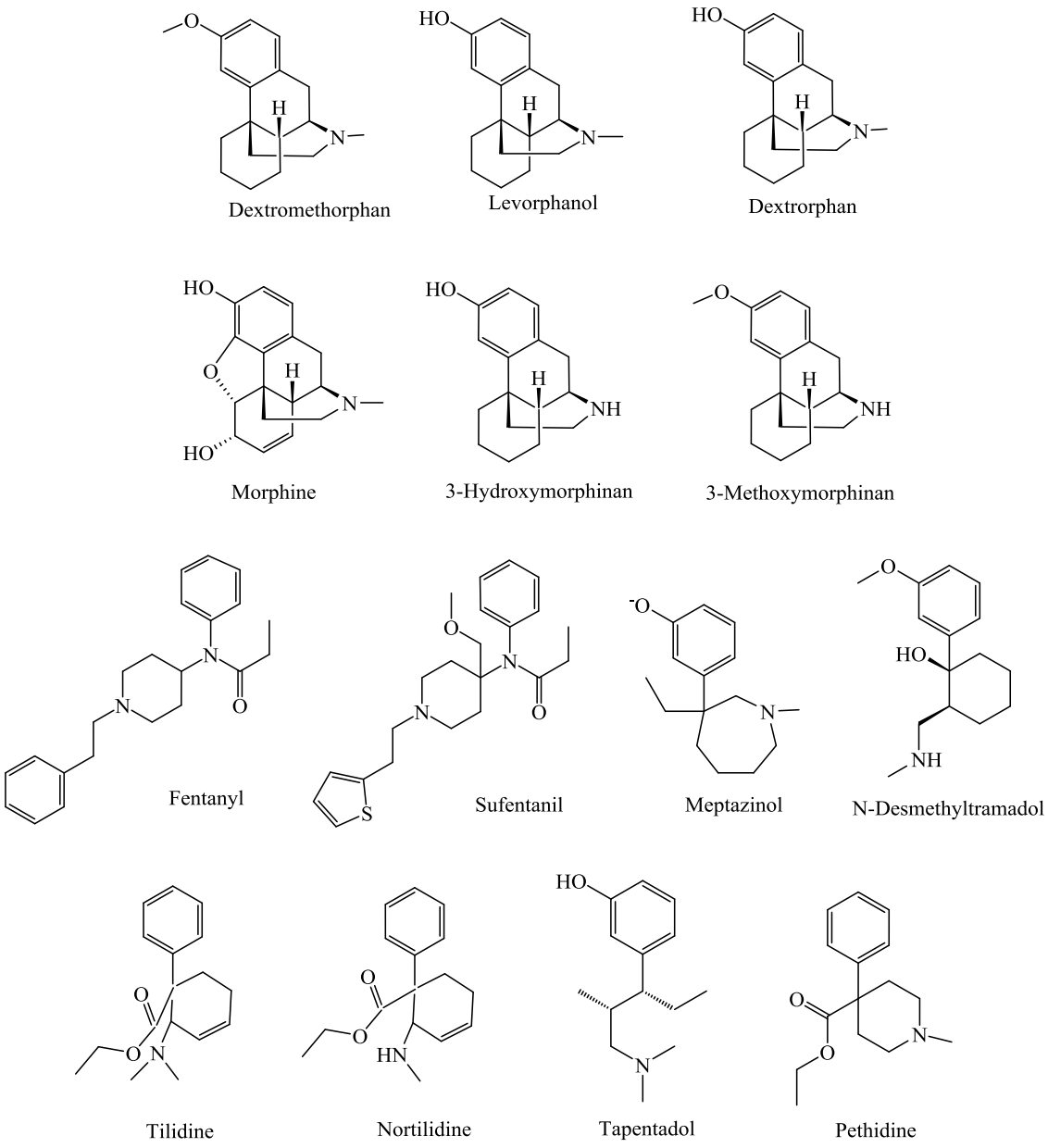


Figure 12: Identified inhibitors of OCT1

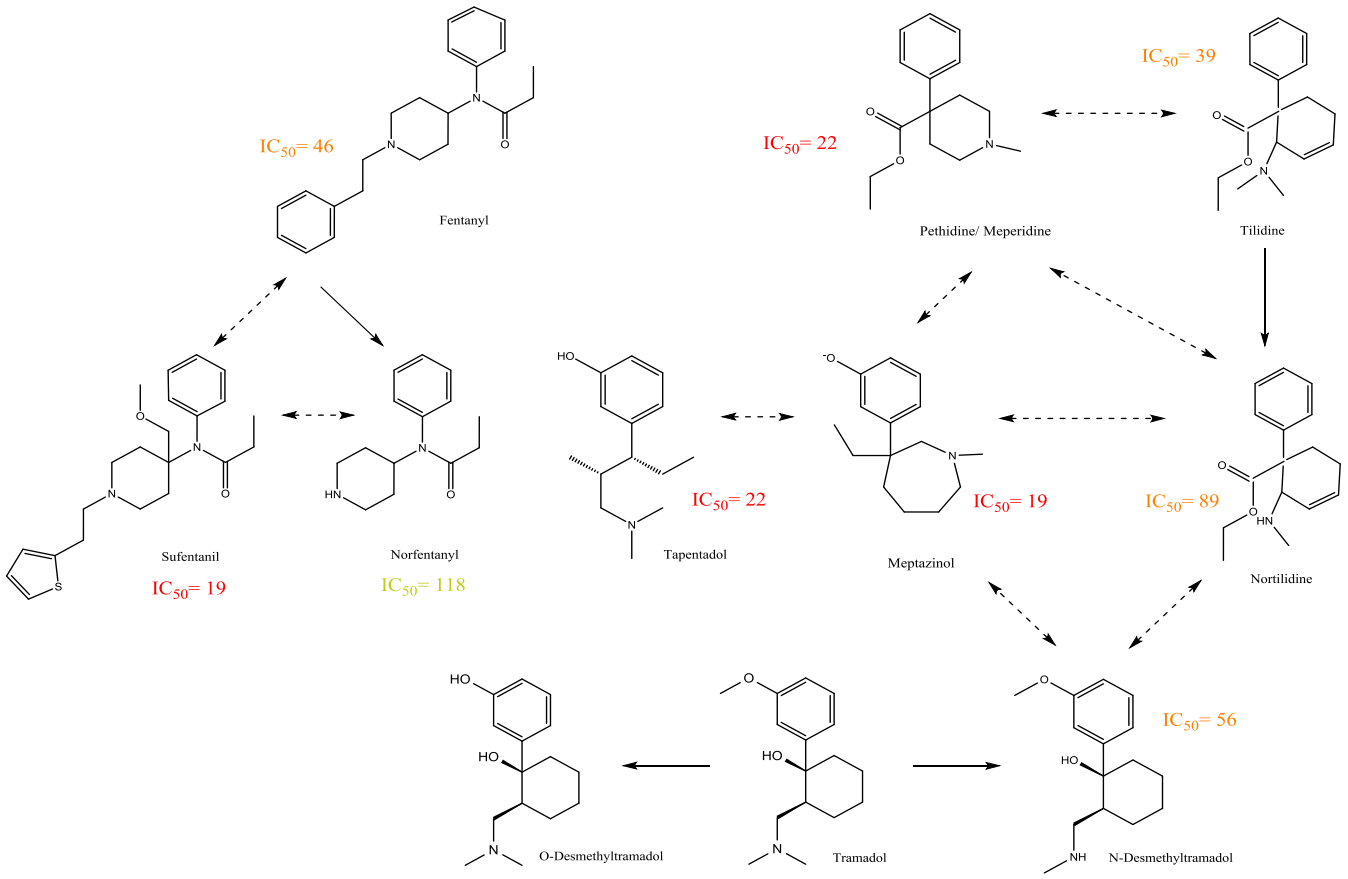


Figure 13: Synthetic opioids and their respective IC_{50} values

Normal arrows indicate a metabolic, dashed arrows a structural relationship. Tramadol und O-desmethyltramadol have been discussed elsewhere (Tzvetkov et al. 2011) and are merely included to provide a better overview. IC_{50} values are given in µM.

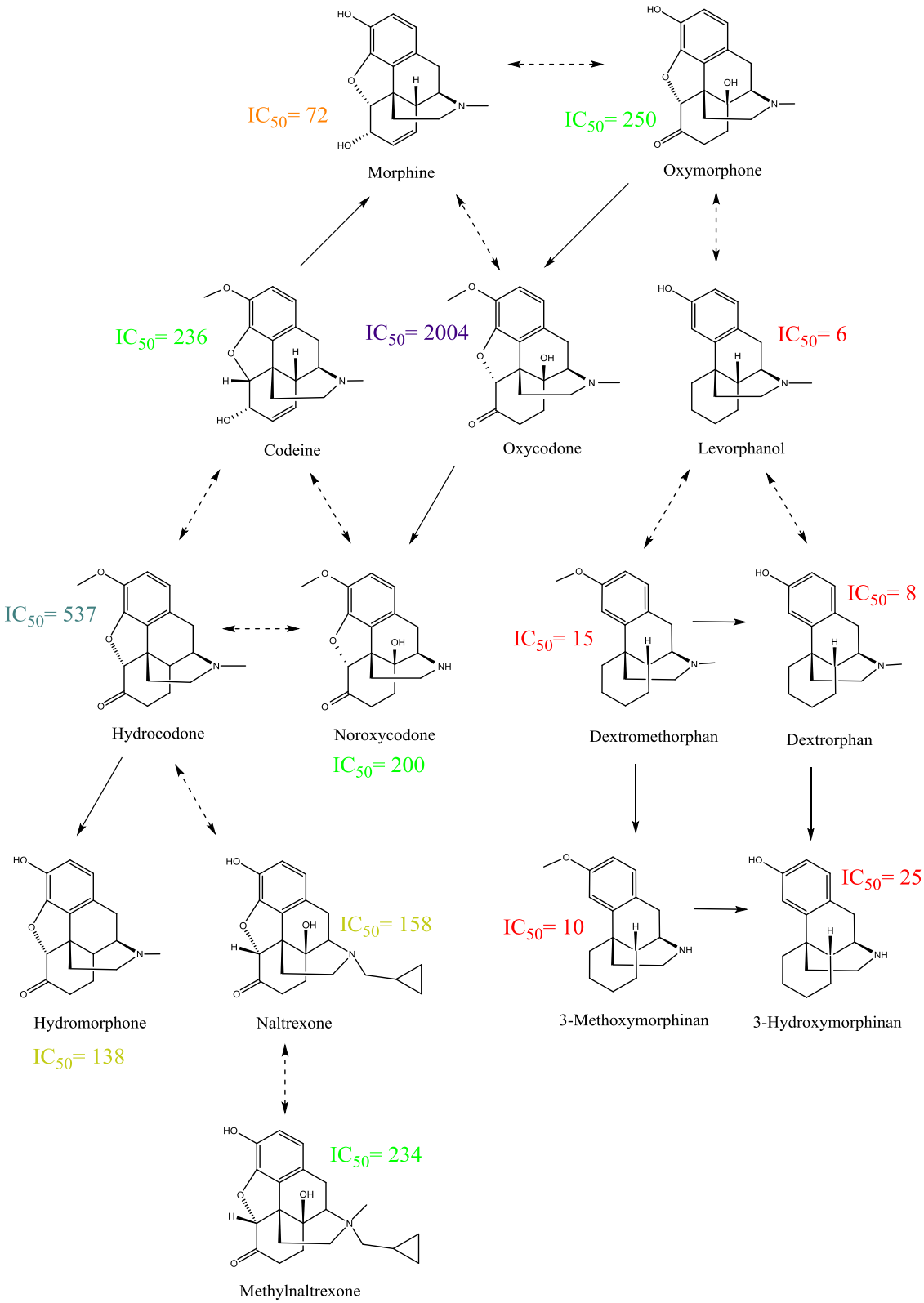


Figure 14: Morphinans and their respective IC_{50} values

Normal arrows indicate a metabolic, dashed arrows a close structural relationship. IC_{50}s are given in μM.

No observed interaction

Five substances did not observably interact with OCT1 either in the way of substrates or in that of inhibitors: codeine, hydrocodone, naltrexone, oxycodone, and oxymorphone (Figure 15).

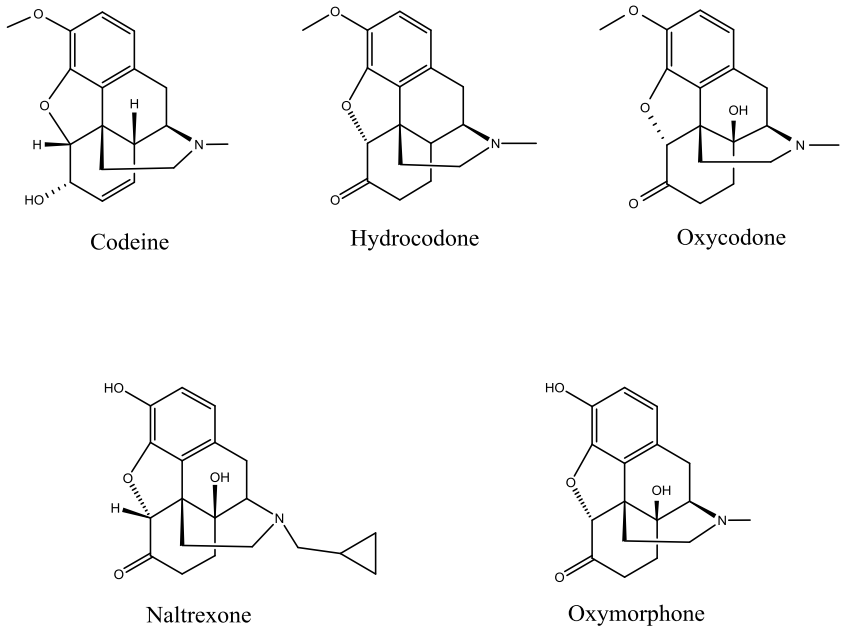


Figure 15: Opioids with no observed interaction with OCT1

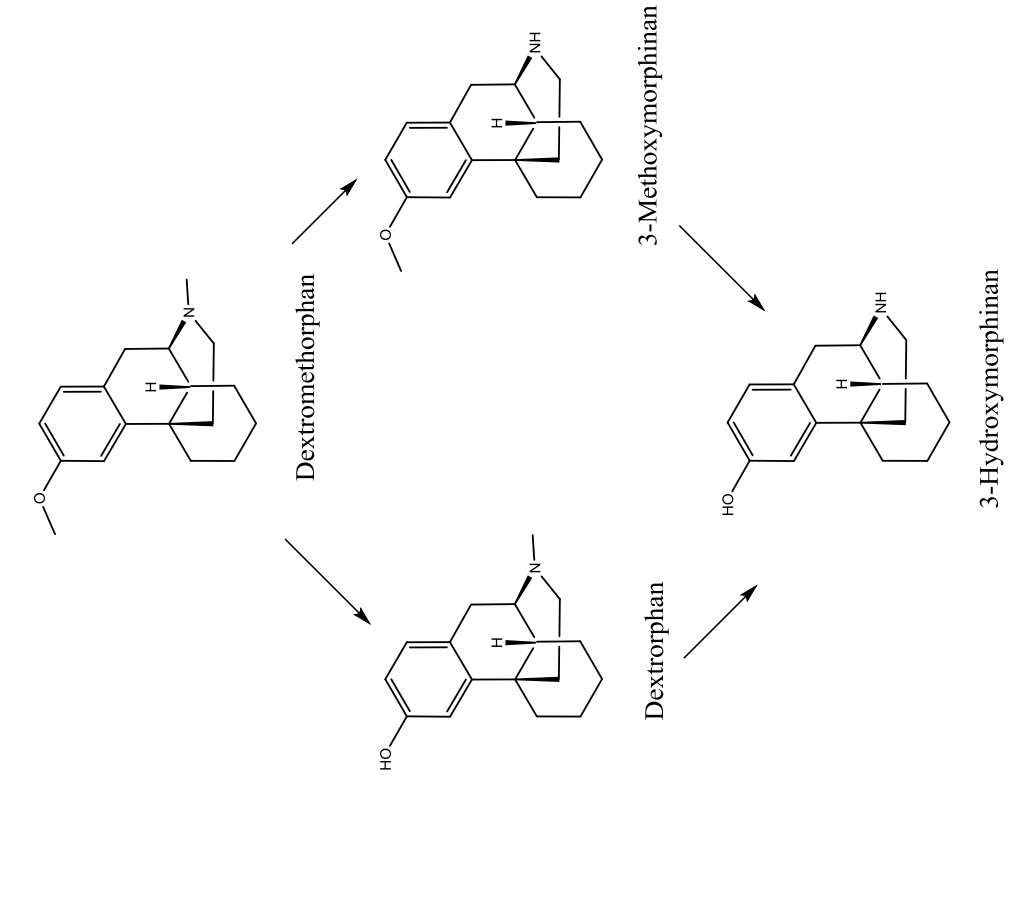
The observation that naltrexone does not interact with OCT1 is surprising because only a methyl group at the nitrogen atom distinguishes it structurally from methylnaltrexone, which proved to depend heavily on OCT1-mediated transport. However, with a P_e of $1.5x10^{-5}$ naltrexone is a lot more lipophilic than methylnaltrexone (P_e of $0.002x10^{-5}$). It remains similarly mystifying why hydromorphone should be a substrate of OCT1 while oxymorphone is not, or why noroxycodone relies on OCT1-mediated transport while oxycodone, quite overwhelmingly, does not interact with OCT1 at all.

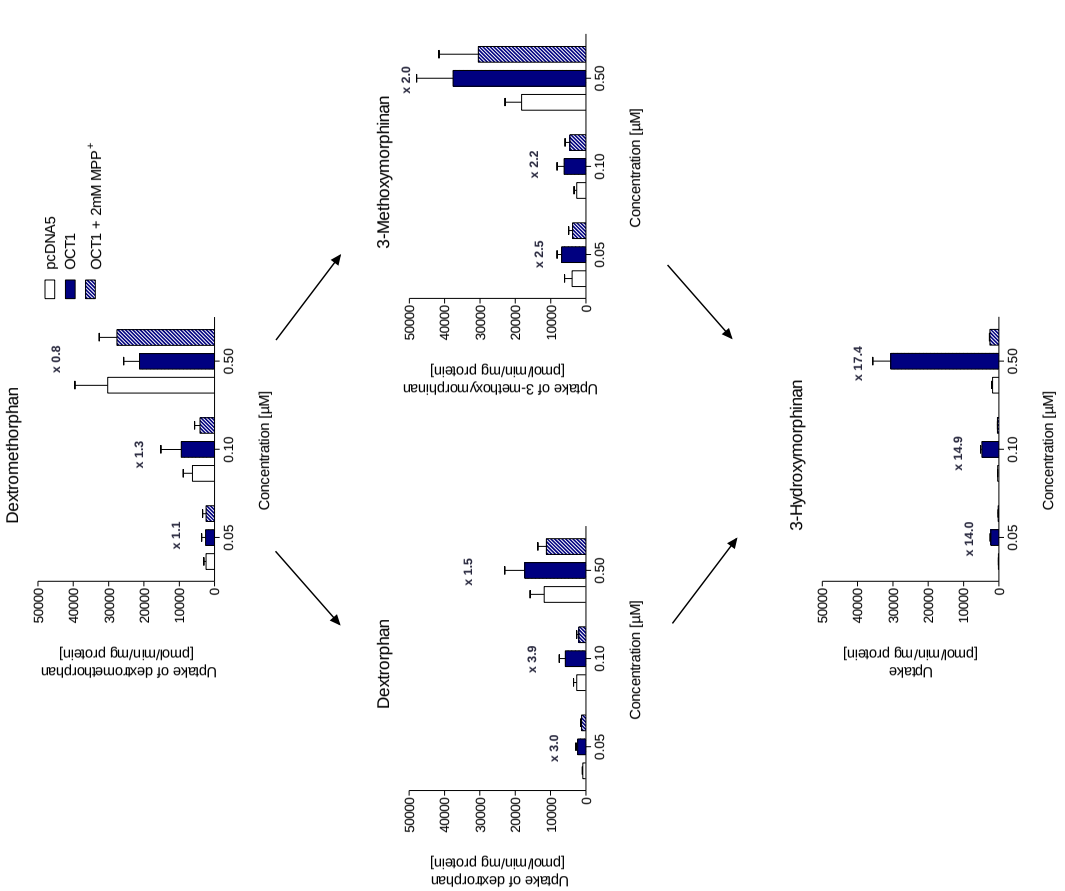
4.3 Aspects of metabolism

Six of the eight substances we identified as OCT1 substrates are metabolites of other opioids: 3-methoxymorphinan and 3-hydroxymorphinan are metabolites of dextromethorphan; hydromorphone is a metabolite of hydrocodone, morphine of codeine, noroxycodone of oxycodone, and norfentanyl of fentanyl. At the same time, four of the substances –3-methoxymorphinan, morphine, meptazinol and hydromorphone– are amenable to further metabolization, i.e. they are parent compounds themselves. These four compounds are active until biotransformation, mainly hepatocytic glucuronidation, leads to their inactivation (see Appendix for more detail).

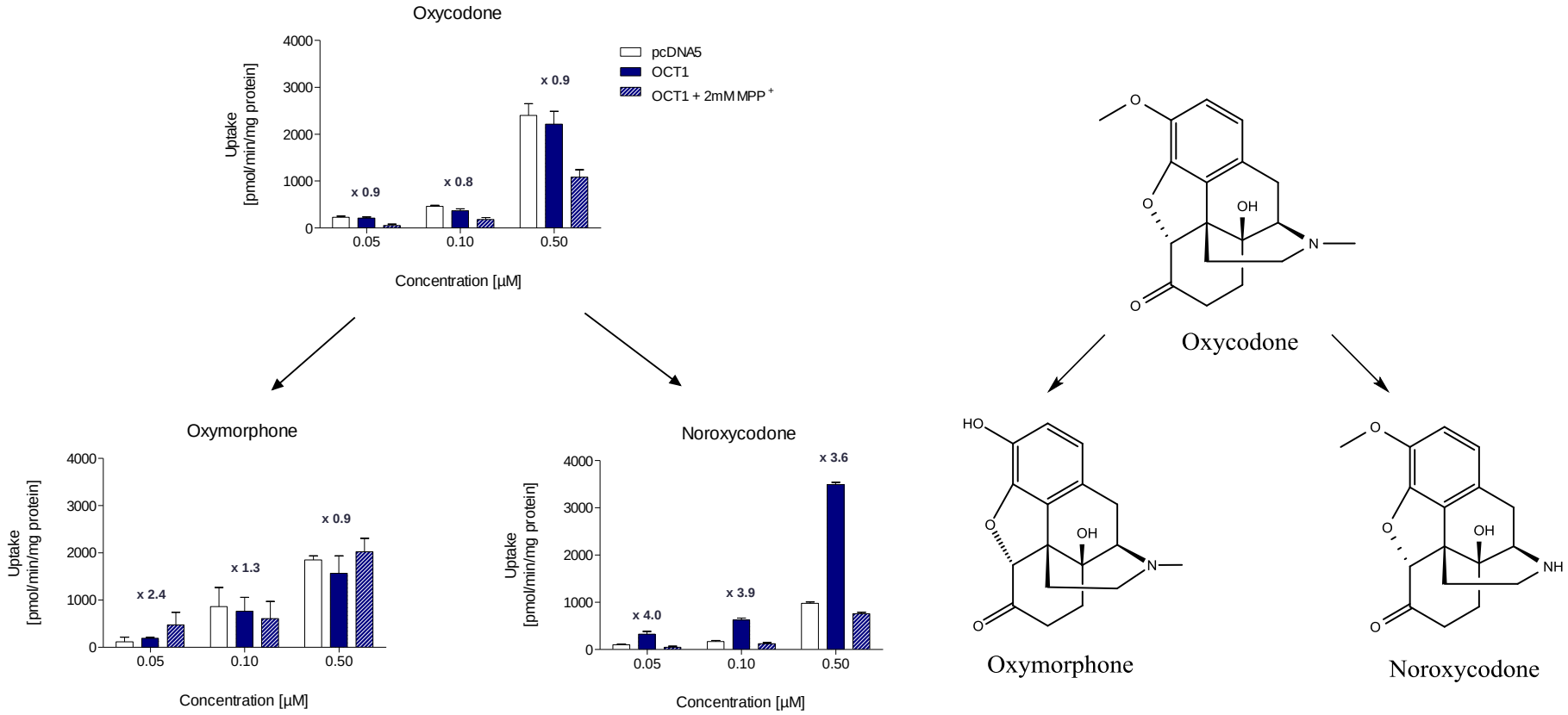
We observed that uptake of metabolite opioids by OCT1-overexpressing cells was elevated compared to that of their parent compounds, which were often not transported by OCT1 at all (Figure 16). A possible explanation for this observation is that biotransformation renders opioids more hydrophilic and thus more dependent on transporter-mediated uptake and release from hepatocytes, whereas the parent compounds are more lipophilic and thus capable of crossing cell membranes independently of OCT1 via diffusion.

The metabolic progression of dextromethorphan and its metabolites in Figure 16 nicely illustrates how OCT1-mediated transport progresses with each modification introduced by biotransformation. Unfortunately, we lack data on OCT1-mediated transport -if present- of the glucuronidated metabolites of hydromorphone, meptazinol, and morphine. It would be interesting to see if OCT1-mediated transport of their metabolites increases in the same fashion as observed with dextromethorphan.

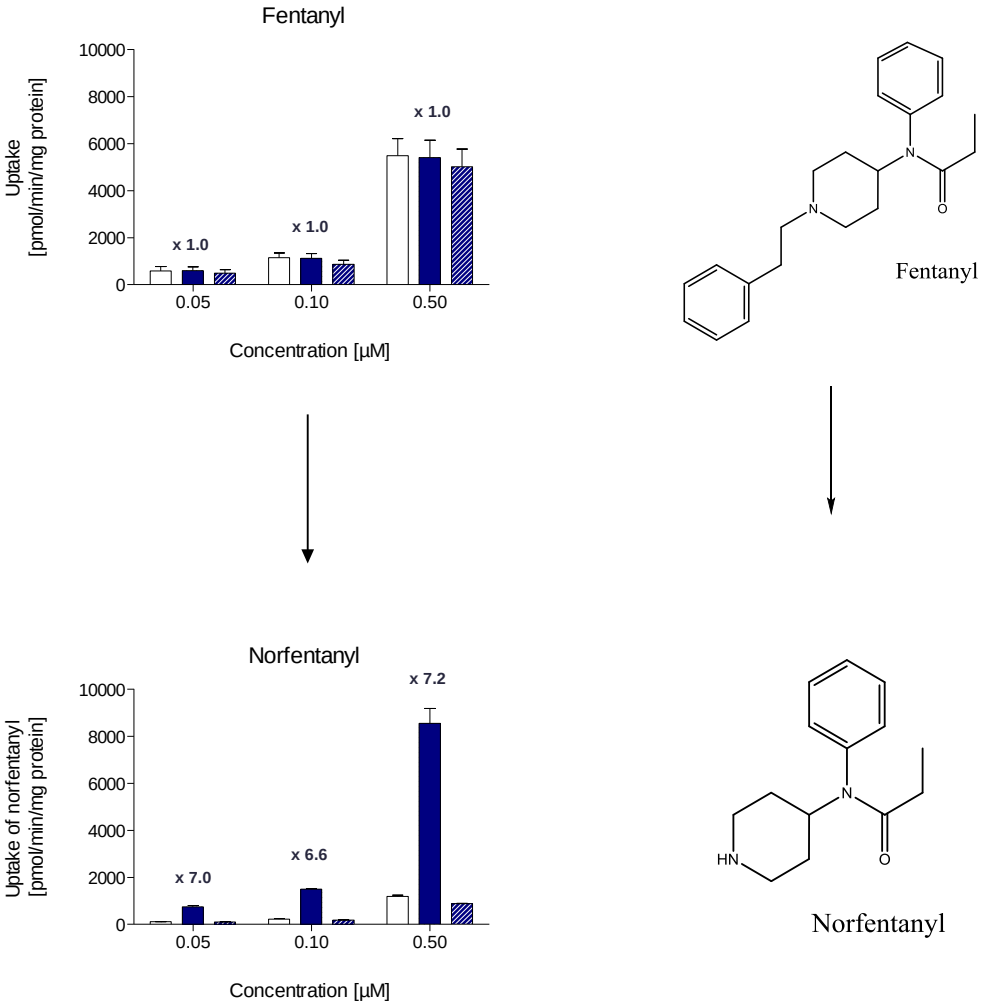

Dextromethorphan
Dextrorphan
3-Methoxymorphinan
3-Hydroxymorphinan
HO
O
NH
N
H


pcDNA5
OCT1
OCT1 + 2mM MPP+
Dextromethorphan
Uptake of dextromethorphan [pmol/min/mg protein]
x 1.1
x 1.3
x 0.8
Dextrorphan
Uptake of dextrorphan [pmol/min/mg protein]
x 3.0
x 3.9
x 1.5
3-Methoxymorphinan
Uptake of 3-methoxymorphinan [pmol/min/mg protein]
x 2.5
x 2.2
x 2.0
3-Hydroxymorphinan
Uptake [pmol/min/mg protein]
x 14.0
x 14.9
x 17.4
Concentration [µM]
0.05
0.10
0.50
0
10000
20000
30000
40000
50000

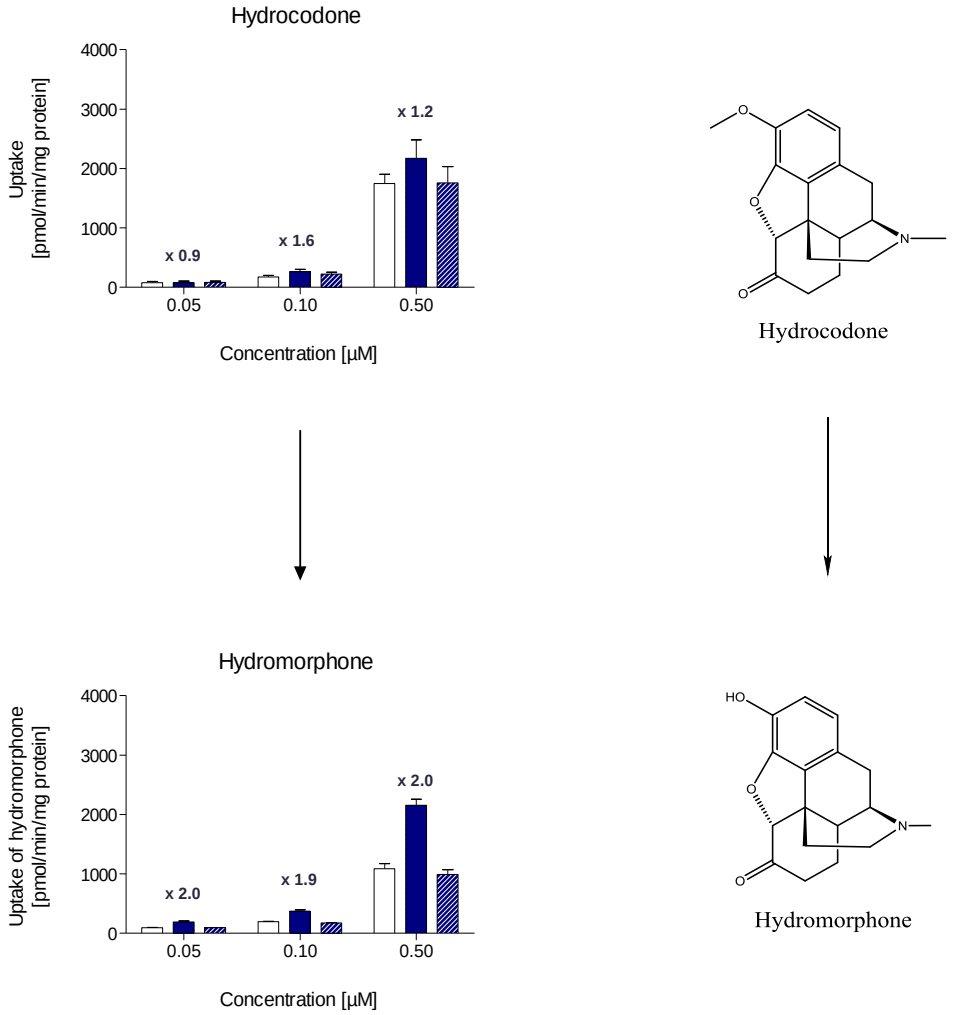
(A)


Oxycodone
Uptake [pmol/min/mg protein]
4000
3000
2000
1000
0
pcDNA5
OCT1
OCT1 + 2mM MPP+
x 0.9
x 0.8
x 0.9
0.05
0.10
0.50
Concentration [µM]
Oxymorphone
x 2.4
x 1.3
x 0.9
Noroxycodone
x 4.0
x 3.9
x 3.6
HO
O
OH
N
NH

(B)


Fentanyl
Uptake
[pmol/min/mg protein]
10000
8000
6000
4000
2000
0
x 1.0
x 1.0
x 1.0
0.05
0.10
0.50
Concentration [µM]
N
N
O
Fentanyl
Norfentanyl
Uptake of norfentanyl
[pmol/min/mg protein]
x 7.0
x 6.6
x 7.2
Concentration [µM]
HN
N
O
Norfentanyl

(C)



(D)

Figure 16: Uptake of parent compounds and their metabolites

(A) Progressive increase in OCT1-dependent uptake of dextromethorphan and its metabolites. (B) Uptake is only increased for oxycodone metabolite noroxycodone, not for its alternative metabolite oxymorphone. (C) Uptake of fentanyl is not altered in the presence of OCT1 but that of its metabolite norfentanyl is. (D) OCT1-dependent transport of hydromorphone is increased compared to that of its parent compound hydrocodone.

4.4 Clinical aspects

Identifying opioids as either substrates or inhibitors of OCT1 is not merely an exercise born from academic interest; it is also an attempt to understand –and possibly predict– how opioids administered in a clinical setting may affect patients. We shall therefore now discuss the ramifications of our findings and attempt to put them into a practical context.

Substrates

Morphine aside, we identified seven novel substrates, three of which act upon opioid receptors: hydromorphone, meptazinol, and methylnaltrexone. The other four –3-hydroxymorphinan, 3-methoxymorphinan, norfentanyl, and noroxycodone– are considered inactive with respect to opioid receptor activity.

The relative increase in the uptake of hydromorphone was the lowest of all substrates. Taking into account that the absolute uptake of oxycodone –not a substrate of OCT1– into OCT1-overexpressing cells was equal to that of hydromorphone, it seems highly improbable that OCT1 polymorphisms would have a clinically noticeable impact on hydromorphone plasma concentrations. However, hydromorphone is a widely employed analgesic, and the impact of OCT1 polymorphisms on its metabolization, however marginal, should not be easily dismissed.

Meptazinol is the most lipophilic of the substrates, and the only active member of the class of synthetic opioids that qualifies as a substrate. It is also extensively metabolized by the liver, so that OCT1 might indeed play a role in its phase 0 biotransformation and elimination (Franklin et al. 1975). It would be worth investigating if and in how far plasma concentrations of meptazinol are affected by diminished OCT1 transport capacities.

Methylnaltrexone turned out to rely heavily on OCT1-mediated transport. Though methylnaltrexone interacted poorly with OCT1 in the presence of ASP^+ and failed to qualify as an inhibitor by a wide margin (IC_{50} of 234.39 μM), its uptake via OCT1 skyrocketed in the absence of competing substrates. Assumedly, when in direct competition for the binding site that mediates transportation, ASP^+ binds more efficiently to OCT1 than methylnaltrexone. Otherwise the fact that methylnaltrexone is transported by OCT1 came not entirely surprising, considering that methylnaltrexone's quaternary nitrogen atom and low membrane permeability essentially render it unsuitable for diffusion. The relative increase in uptake (32x, 68x, and 86x) was impressive nevertheless (see Figure 6, section 3.4.1).

It also remains uncertain in how far OCT1-mediated transport of methylnaltrexone into the liver is clinically relevant, considering that 60% of methylnaltrexone are excreted unchanged and thus independently of hepatic biotransformation (see Appendix for more detail). Instead, methylnaltrexone might play a greater role in research as a new, highly sensitive model substrate for OCT1.

The other substrates do not currently have any confirmed clinical effects. Even if their plasma concentrations were affected by diminished OCT1 transport capacities, the consequences of such diminished transport would bear little or no clinical relevancy.

Inhibitors

Though our main interest initially lay with the substrates of OCT1, our results gradually shifted our focus to the inhibitors. Since OCT1 is one of the transporters most involved in the metabolism of a wide range of drugs, the opioids identified by us as inhibitors of OCT1 have at least the potential to affect the transport of such drugs into OCT1-expressing cells.

In order to assess which of the inhibitors might, at least in theory, affect OCT1-mediated uptake *in vivo*, we calculated the ratio of the opioids' maximal unbound plasma concentration in the portal vein and our experimentally determined IC_{50} values as described elsewhere (Ito et al. 2002; Ahlin et al. 2011). These calculations are of interest in so far as they attempt to translate our findings from an *in vitro* set up into an *in vivo* environment. However, they cannot level out the experimental nature of our findings entirely.

The IC_{50}s were determined by measuring how effectively they inhibited the uptake of a test substrate. In our case, this test substrate was ASP^+, which yielded an IC_{50} of 72 μM for morphine. This value is more than fifteen-times as high as the 4.2 μM reported by Tzvetkov et al.; however, latter determined the IC_{50} of morphine using the model substrate MPP^+ and liquid scintillating counting (Tzvetkov et al. 2013). So while the calculations presented in Table 8 nudge us into promising directions in terms of future research, the opioids' K_m and V_{max} are needed in order to fully appreciate their effects on OCT1.

Of the fourteen in vitro inhibitors, dextromethorphan, levorphanol, and tapentadol emerged as candidates that may be potent enough at clinically realistic plasma concentrations to inhibit OCT1 *in vivo*. Tapentadol yielded especially high ratios in both the immediate and extended release formulation (0.86 and 0.88, respectively) as did levorphanol (0.88) when administered repeatedly. Tapentadol and levorphanol are both opioids employed in chronic pain management, which means that they are likely to reach steady-state concentrations and that, due to the respective patient population, they are likely to be co-administered along with

other drugs, which may well rely on OCT1-mediated uptake for metabolization (e.g. the anti-emetic odansetron). Further studies would be needed to confirm or invalidate such interactions in actual patients, but given the broad spectrum of available opioids, our experimental data may invite clinicians to defer from administering tapentadol and levorphanol in favor of opioids such as oxycodone or hydrocodone (always provided the patients tolerate them).

Dextromethorphan -somewhat confusingly on first glance- only seems to have an effect in extensive metabolizers of CYP2D6. The CYP2D6 genotype should not affect the portal venous concentrations of dextromethorphan, since the drug has only just been absorbed from the gut and not yet been metabolized by the liver (and even if we assumed we had reached steady-state plasma concentrations after repeated dosing, we would expect the concentration of dextromethorphan to be higher in poor than in extensive metabolizer). However, in the studies from which we took the plasma concentrations for dextromethorphan, test subjects with a known PM CYP2D6 genotype were given lower doses of dextromethorphan than those with an EM CYP2D6 genotype (Thummel et al. 2005). It is fair to assume that, had the PMs been given the same dose of dextromethorphan as the EMs rather than just half of it, that their portal venous concentration of dextromethorphan would have equalled that of the EMs. We can only speculate that patients with a PM CYP2D6 genotype, since less effective at metabolizing dextromethorphan, will suffer prolonged inhibition of OCT1. But since patients have unchecked access to cough syrups with dextromethorphan and the PM CYP2D6 is relatively common in the Caucasian population, it would be well worth it to further explore the inhibitory effects of dextromethorphan on OCT1 in clinical studies.

Table 8: Inhibition of OCT1-mediated uptake by opioids identified as inhibitors of OCT1 in vitro

Enteral administration

Inhibitor	IC_{50}, µM	Dose p.o., mg	Plasma conc. max, µg/l	Plasma conc. max, µM	Cmax portal unb., µM	Cmax portal unb/IC_{50}
Dextromethorphan EM	15.1	60	5.2	0.019	4.813	0.318
Dextromethorphan PM	15.1	30	33	0.122	2.446	0.161
Levorphanol, repeated dose	8.5	50	10	0.039	7.473	0.881
Levorphanol, single dose	8.5	2	100	0.389	0.313	0.037
Morphine IR, single dose	71.8	10	10	0.035	1.438	0.020
Morphine ER, single dose	71.8	50	7.4	0.026	7.091	0.099
Tapentadol IR	21.9	86	22.5	0.102	19.539	0.892
Tapentadol ER	21.9	86	64.2	0.290	19.388	0.886
Tilidine	38.7	50	30	0.110	8.029	0.207

Parenteral administration

Inhibitor	IC_{50}, µM	Dose, mg	Plasma conc. max, µg/l	Plasma conc. max, µM	Cmax portal unb., µM	Cmax portal unb/IC_{50}
Fentanyl	46.2	0,4mg t.m.	0.8	0.002	< 0.000	< 0.000
Meptazinol	19.0	25mg i.v.	90	0.386	0.104	0.005
Morphine	71.8	10mg i.v.	400	1.402	0.911	0.013
Pethidine (Meperidine)	22.3	24mg/hr i.v. to steady state	670	2.709	1.138	0.051
Sufentanil	19.4	i.v.	10	0.026	0.002	0.000
Tilidine	38.7	50mg i.v.	906	3.314	2.320	0.060

In order to assess the inhibitory potential of the opioids, we calculated the ratio of the opioids' maximal unbound concentration in the portal vein and the experimentally determined IC_{50} values. Ratios greater 0.25 indicate inhibitory potential. In parenteral administration, the peripheral plasma concentrations are equivalent to portal venous concentrations; calculating the portal venous concentration was therefore not necessary. Data on 3-hydroxymorphinan, 3-methoxymorphinan, dextrorphan, N-desmethyltramadol, and nortilidine was not sufficiently available to calculate their ratios.

Cmax plasma unb., maximal concentrations of the unbound drug in plasma; Cmax portal unb., maximal concentrations of the unbound drug in portal vein; EM, extensive metabolizer (regarding CYP2D6); PM, poor metabolizer (regarding CYP2D6); IR, immediate release; ER, extended (sustained) release; N.A., not available

[a]The maximal plasma concentration of the unbound drug (Cmax plasma unb.) was calculated by multiplying the Cmax by the fraction unbound in plasma. The maximal plasma concentrations and the fraction unbound to plasma proteins were obtained from Gilman and Goodman's The Pharmacological Basis of Therapeutics (Thummel et al. 2005) for fentanyl, morphine, and pethidine; from Gilman and Goodman's The Pharmacological Basis of Therapeutics (Thummel et al. 2005) and Taylor (Taylor et al. 2016) for dextromethorphan; from Hajda et al (Hajda et al. 2002) for tilidine; from the FDA's Professional Drug Information (FDA) and Dixon et al (Dixon et al. 1983) for levorphanol; from Gohler et al (Göhler et al. 2013) for tapentadol; and from Opioids in Cancer Pain (Hall and Hardy 2009) and Schmerztherapie (Standle et al. 2010) for sufentanil.

[b]Cmax portal unb. values were estimated according to the method described before by Ito et al (2002) and Ahlin et al (2011).

4.5 Conclusion

We were able to identify seven novel substrates of OCT1, three of which could be relevant in a clinical context should they be affected by diminished OCT1 transport capacities, and fourteen inhibitors of OCT1, again three of which could affect OCT1-mediated transport *in vivo*.

From the point of view of OCT1 and its polymorphisms, we were able to identify five opioids –namely codeine, hydrocodone, naltrexone, oxycodone, and oxymorphone– as clinically "safe" opioids, i.e. as opioids that interact neither as substrates nor inhibitors with OCT1. Since they have no touching points with OCT1, we can expect plasma concentrations of these opioids to go unaffected by variegations in OCT1 transport capacities, whether brought about by either genetic variations or by pharmacologic interactions. Nor would we expect them to affect the plasma concentrations of drugs dependent on OCT1-mediated transport. However, we only dealt with wild type OCT1 in our experiments and cannot exclude that these opioids do not interact with certain variations of OCT1. Further testing would be needed in order to elucidate if these opioids are indeed "genetically indifferent" as far as variations in *SLC22A1* are concerned.

From a chemical point of view, our results yielded that low lipophilicity –in the semblance of low $logD_{(7.4)}$ and P_e values– increase the probability that a given opioid will be an OCT1 substrate, a non-inhibitor, and a morphinan, while high lipophilicity can be associated with non-substrates, inhibitors, and synthetic opioids. Additionally, the class of synthetic opioids and the one of dextromethorphan-like morphinans –which are mostly lipophilic substances– turned out to be especially potent inhibitors of OCT1 *in vitro*, whereas morphine-like morphinans –generally less lipophilic opioids– inhibit OCT1 transport capabilities only to a moderate degree. However, we are as yet unable to pinpoint a specific moiety or structural quirk that flags a drug as either substrate or inhibitor.

All in all, further experiments will be needed to elucidate the interactions between inhibitors and substrates with OCT1 and each other. Inhibitor-substrate interactions should be evaluated in OCT1 non-overexpressing cells (e.g. human hepatocytes) and at therapeutic concentrations (e.g. OCT1 inhibitor tapentadol in combination with OCT1 substrate rocuronium) to elucidate if and to what extent the opioids identified as potent inhibitors of OCT1 affect OCT1-mediated uptake of other substances. The most common OCT1 variants ought also to be assessed with respect to their uptake of the identified substrates.

5 Abstract

The organic cation transporter 1 (OCT1) is primarily located in the sinusoidal membranes of hepatocytes and mediates phase 0 biotransformation of xenobiotics, including weak basic and cationic drugs. Variations in *SLC22A1*, the gene encoding OCT1, are common throughout the Caucasian population. Close to 9% of Caucasians are homozygotic for OCT1 alleles that encode insufficient transporters, resulting in a partial or complete loss of OCT1 transport activity in affected individuals. Clinical studies have demonstrated that diminished OCT1 transport activity leads to elevated plasma levels of opioids morphine and O-desmethyltramadol (the active metabolite of tramadol). Patients with diminished-activity OCT1 alleles require 20% less tramadol for efficient postoperative pain management and suffer more adverse effects after morphine consumption. These findings are all the more relevant when considering the high prevalence of diminished-activity OCT1 variants and the extensive use of opioids in clinical practice.

The aim of this study was to perform a systematic screening of opioids *in vitro* in order to identify substrates and inhibitors of OCT1 beyond morphine and tramadol that might be affected by reduced OCT1 activity. The screening was performed in a four-step process that involved *in silicio* predictions, membrane permeability testing in an artificial membrane model (so-called parallel artificial membrane permeability assays), inhibition experiments, and transport experiments. For the inhibition experiments, we photometrically determined to what extent our test drugs inhibited the uptake of the fluorescent OCT1 substrate ASP^+, while for the transport experiments we measured the uptake of test drugs into OCT1-overexpressing HEK293 cells directly via LC-MS/MS. Sixty opioids underwent the initial *in silicio* predictions; of those, we chose 23 opioids to run the entire course of experiments.

Seven novel substrates and fourteen inhibitors of OCT1 were identified. Three of the substrates –methylnaltrexone, meptazinol, and hydromorphone– are active compounds used in clinical practice. Uptake of methylnaltrexone into OCT1-overexpressing cells was 32 to 85 times higher than that into negative control cells, making it one of the substances most dependent on OCT1-mediated transport so far reported. Inhibitors were defined as substances with IC_{50} values of 100 μM or below; of the fourteen inhibitors identified, nine were especially potent with IC_{50} values of less than 25 μM. Notably, dextromethorphan and its metabolites were among the substances with the lowest IC_{50} values. Three of the inhibitors –namely dextromethorphan, levorphanol, and tapentadol– could, theoretically, reach portal vein concentrations high enough to inhibit OCT1 *in vivo*.

We also identified five opioids –codeine, hydrocodone, naltrexone, oxycodone, and oxymorphone– that can be considered pharmacokinetically "safe" with respect to variations in *SLC22A1* since they do not seem to interact with OCT1 in any way. Their uptake into OCT1-overexpressing cells was less than two times the uptake into negative control cells, indicating a lack of OCT1-mediated transport. They also failed to inhibit the uptake of ASP^+ into OCT1-overexpressing cells by 50 % at concentrations of 100 μM.

Analysis of the *in silicio* data showed that opioids with low $logD_{(7.4)}$ and P_e values are more likely to be morphinans, and substrates or non-inhibitors of OCT1 than opioids with high $logD_{(7.4)}$ and P_e values. It also appears that the structures of synthetic opioids and dextromethorphan-like opioids lend themselves to more potent inhibition of OCT1 than the "classic" morphinan structure of morphine and its congeners.

Overall, this study identified at least three substrates and three inhibitors that warrant further research in humans in order to gauge their pharmacological and pharmacokinetic impact on patients with diminished OCT1 transport activity. In addition, our *in vitro* data suggests five opioids that may be safer to use in patients with diminished OCT1 transport activity.

6 Appendix

6.1 An overview of the tested drugs

6.1.1 3-Hydroxymorphinan

Colloquially, 3-hydroxymorphinan refers to (+)-3-hydroxymorphinan; its levo isomer, (—)-3-hydroxymorphinan, is better known as norlevorphanol. While lacking intrinsic activity with regard to opioid receptors, (+)-3-hydroxymorphinan has emerged as a potent neuroprotective agent in animal models and may be of use in the therapy of Parkinson's disease (Zhang et al. 2005). Ultimately a metabolite of dextromethorphan, (+)-3-hydroxymorphinan is metabolized from dextrorphan via CYP3A4 or from 3-methoxymorphinan via CYP2D6 (Vengurlekar et al. 2002).

6.1.2 3-Methoxymorphinan

A metabolite of dextromethorphan, 3-methoxymorphinan is generated via CYP3A4 mediated N-demethylation in the liver (Vengurlekar et al. 2002) .It is currently thought to be inactive, though further processing by CYP2D6 yields 3-hydroxymorphinan.

6.1.3 Codeine

Codeine is a prodrug and precursor of the opioid morphine. It is considered a weak opioid that unfolds its analgesic effects after CYP2D6-mediated O-demethylation of the phenylic hydroxyl group transforms it into morphine (Hardy and Jackson 2009). Codeine is administered as an antitussive and as an antidiarrheal agent. Although codeine is part of many antitussive preparations, it is probably no more effective than a placebo at battling coughs (Smith et al. 2014). Loperamide –another opioid commonly employed against diarrhea- causes fewer side effects, but codeine is cheaper and therefore more likely to be used in countries where access to superior –and more expensive- opioids is limited (Hardy and Jackson 2009; International Narcotics Control Board 2011)

6.1.4 Dextromethorphan

Dextromethorphan is the prodrug of dextrorphan; all its metabolites are dextrorotatory. Though displaying only weak affinity for opioid receptors, it acts agonistically on the sigma 1 receptor and inhibits the uptake of norepinephrine and serotonin (Pioro 2014). In 2010, the FDA approved of a formulation of dextromethorphan in combination with quinidine –

a CYP2D6 inhibitor that delays metabolization of dextromethorphan- for the treatment of pseudobulbar affect (Pioro 2014). Additionally, dextromethorphan may act as a weak serotonin reuptake inhibitor, though supra-therapeutic doses (as may be encountered in abuse) are required in combination with MAOIs to produce serotonin toxicity (Gillman 2005; Schwartz et al. 2008; Koren-Michowitz et al. 2014). In a study by Reissig, 11 of out 12 participants rated dextromethorphan as a classic hallucinogenic after receiving doses of 400mg/70kg BM (Reissig et al. 2012). Because of its antitussive properties, dextromethorphan is a common ingredient in many over-the-counter cough medicines in a range that spans 5-30mg DXM HBr per formula. Cheap and easily accessible when compared to other drugs, recreational abuse of dextromethorphan (called "robo-tripping", after cough medicine-producing brand Robitussin) is no rarity (Wikipedia 2018a). The FDA issued a warning against DXM abuse in 2005 after five teenagers died from consuming pure, powdered dextromethorphan (FDA 2005). DXM abuse has been in decline ever since, though the prevalence of cough medicine abuse among 12th graders still amounts to 4%, one in twenty-five students (NIDA 2015).

6.1.5 Dextrorphan

Dextrorphan is a metabolite of dextromethorphan derived from CYP2D6-mediated O-demethylation of the hydroxyl group at the phenolic C3 (Vengurlekar et al. 2002). The dextro-rotatory enantiomer of levorphanol, dextrorphan is not, strictly speaking, an opioid as it does not bind to opioid receptors. It does bind to N-methyl-D-aspartate (NMDA) receptors at the phencyclidine site, though, making it a NMDA receptor antagonist (Davis 2009d). Competitive binding assays in rat forebrain showed that the IC_{50} of dextrorphan lay below that of ketamine, levorphanol, and dextromethorphan (Franklin and Murray 1992). Dextrorphan can be converted into 3-hydroxymorphinan through CYP3A4-mediated N-demethylation (Vengurlekar et al. 2002).

6.1.6 Fentanyl

Fentanyl is a 4-anilidopipderidine derivative and a highly lipophilic compound, with an analgesic potency estimated to be nearly 100 times that of morphine. Due to its high lipid solubility, it can pass the brain-blood barrier and even the skin, allowing for fentanyl formulations that are unique among the opioids. These include TTS (transdermal therapeutic systems- "patches"), buccal tablets, sublingual and nasal sprays, and the "lozenge", which is basically a fentanyl-containing lollipop (Mandel and Carunchio 2011; Bornemann-Cimenti et al. 2013). These rapid-onset preparations of fentanyl are popular in the treatment of

breakthrough cancer pain: the application is non-invasive and bypasses the liver, so that ultimately a higher amount of active fentanyl reaches the brain (Bornemann-Cimenti et al. 2013). About 50 to 75% of orally ingested fentanyl undergoes first-pass metabolism to norfentanly, which is then excreted by the kidneys (Bornemann-Cimenti et al. 2013). Though short-acting in single doses, fentanyl can accumulate within the cells and adipose tissue if given repeatedly. When given continuously, it no longer qualifies as a short-acting opioid (Brayfield 2014).

Fentanyl is currently the most commonly applied opioid worldwide (International Narcotics Control Board 2011).

6.1.7 Hydrocodone

Hydrocodone is a semi-synthetic derivative of codeine with approximately the same analgesic potency as morphine. It is, however, considered a weak opioid as it is only commercially available as a compound analgesic, usually with ibuprofen or acetaminophen (Davis 2009a). CYP2D6-mediated O-demethylation of hydrocodone yields the more potent hydromorphone (analogously to codeine and morphine), while CYP3A4-mediated transformation and reduction yield small quantities of norhydrocodone and 6β-/6α-1-hydromorphol, which also activate MOP (Davis 2009a). Ultra-rapid metabolizers of CYP2D6 are reported to be at greater risk of suffering from adverse reactions against hydrocodone, probably a result of overdosing since greater amounts of the potent hydromorphone will be produced within a shorter time.

6.1.8 Hydromorphone

A metabolite of hydrocodone, hydromorphone is a potent congener of morphine used in moderate to severe pain. It has been used for the treatment of cancer pain since 1932 (Quigley and Glare 2009). Analogously to morphine, hydromorphone is glucuronidated to hydromorphone-3-glucuronide and excreted via the kidneys, and like morphine-3-glucuronide, HM3G is suspected of being neurotoxic (Quigley and Glare 2009).

6.1.9 Levorphanol

Levorphanol (levo-3-hydroxy-N-methylmorphinan) is the levo-rotatory enantiomer of dextrorphan (Davis 2009d). Its analgesic potency is four to eight times that of morphine, and it has an especially high affinity for KOP (Prommer 2007). Like dextromethorphan and its metabolites, levorphanol acts antagonistically upon NMDA receptors. It has also been noted

for causing spasm of the sphincter of Oddi and for stimulating the hypothalamic-pituitary axis (Davis 2009d). Analogously to morphine and related compounds, levorphanol is mainly metabolized to levorphanol-3-glucuronide (L3G). L3G is thought to be inactive and undergoes enterohepatic circulation in mice (Davis 2009d). Ultimately, all metabolites are excreted via the kidneys, and only small amounts of levorphanol are excreted unchanged (Prommer 2007).

6.1.10 Meptazinol

Meptazinol is a synthetic opioid developed in the early nineteen-eighties with an analgesic potency ten times lower than that of morphine. A mixed opioid agonist-antagonist, it may precipitate withdrawal symptoms in opioid-dependent patients and seems to have cholinergic effects, too (Holmes and Ward 1985; Brayfield 2014). Though Meptazinol can cross the placenta –which may occur when the drug is administered as an analgesic to women in labor— it is quickly eliminated from the neonate (Franklin et al. 1981). Only 10% of meptazinol is excreted unchanged via the kidneys, the rest is metabolized by the liver, mainly via glucuronidation (Franklin et al. 1975).

6.1.11 Methylnaltrexone

Methylnaltrexone is a selective MOP antagonist administered as an adjuvant to palliative care patients suffering from opioid-induced constipation (Rodrigues et al. 2013; Brayfield 2014). Due to a quaternary nitrogen atom, it is polar and does not cross the brain-blood barrier (Leppert 2015). Methylnaltrexone undergoes sulfation to methylnaltrexone-3-sulfate and reduction to different methylnaltrexols, though apparently no demethylation to naltrexone (Leppert 2015). Approximately 40% are excreted unchanged by the kidneys; another 20% with the feces (Leppert 2015).

6.1.12 Morphine

Morphine is an opiate and the prototypic opioid. Analgesic potency is scaled with reference to morphine (analgesic potency of 1) and, along with codeine, it is one of two opioids declared essential drugs by the WHO (Glare 2009a; WHO 2015). Despite the wide offer of alternative opioids, morphine remains the most popular opioid in pediatrics and is the opioid of choice in the treatment of cancer pain (Brayfield 2014). Its main metabolites, morphine-3-glucuronide and morphine-6-glucuronide, are formed via UDP-glucuronosyltransferase (UGT). Morphine and all of its metabolites are excreted by the kidneys, which is why

morphine should be administered with caution in patients with decreased renal clearance(Glare 2009a). Like codeine and pethidine, morphine is known to trigger the release of histamine from mast cells, resulting in erythrema and itching (Blunk et al. 2004). It can also cause spasms of the sphincter of Oddi (Helm et al. 1988).

6.1.13 Naltrexone

In contrast to methylnaltrexone, naltrexone is an opioid receptor antagonist that is active peripherally as well as centrally (Barnett et al. 2014). Naltrexone undergoes first-pass metabolism in the liver to 6-β-naltrexol, which, too, has antagonist properties (Barnett et al. 2014). It is employed in the long-term treatment of cholestatic pruritus, but may counteract the analgesic effects of co-administered opioids and cause typical withdrawal symptoms (Barnett et al. 2014). Administered as an adjunct in the management of alcohol dependence, naltrexone reduces the amounts of alcohol ingested (Volpicelli et al. 1992; Brayfield 2014). It is further under investigation as a therapeutic drug in eating disorders, and may be beneficial in autistic children with self-injurious behavior (Brayfield 2014).

6.1.14 N-Desmethyltramadol

N-Desmethyltramadol (M2) is a CYP3A4 and CYP2B6-derived metabolite of tramadol (Leppert 2011). Its effects are unknown.

6.1.15 Norfentanyl

Norfentanyl is produced by CYP3A4-mediated N-demethylation of fentanyl (Hall and Hardy 2009). It is considered an inactive metabolite.

6.1.16 Noroxycodone

Noroxycodone is a weak opioid receptor agonist derived from oxycodone through CYP3A4-mediated N-demethylation (Smith 2011). Its impact on analgesia, however, is negligible. CYP2D6-mediated O-demethylation of noroxycodone can yield noroxymorphone, but most of it will be excreted by the kidneys unchanged (Glare and Davis 2009; Smith 2011).

6.1.17 Nortilidine

Nortilidine is the active metabolite of tilidine, produced by CYP3A4-mediated N-demethylation in the liver (Grun et al. 2012). With an opioid receptor affinity ten times that of the parent drug, nortilidine is about equianalgesic to morphine (Hajda et al. 2002). Apart

from being an opioid receptor agonist, nortilidine is also an NMDA receptor antagonist and dopamine reuptake inhibitor (Brayfield 2014).

6.1.18 Oxycodone

Oxycodone is a thebaine congener with approximately the same analgesic potency as morphine (Glare and Davis 2009). Due to its extensive and varied metabolization, oxycodone is sometimes regarded as a prodrug, exerting its analgesic effects through oxymorphone and noroxymorphone (Smith 2011). CYP2D6-mediated O-demethylation of oxycodone yields oxymorphone; however, CPY3A4-mediated N-demethylation to noroxycodone dominates *in vivo* (Smith 2011). Along with fentanyl, hydromorphone, and morphine, oxycodone is one of the most commonly applied opioids worldwide (International Narcotics Control Board 2011). Its popularity may be partly grounded in the fact that is has been in use since 1917, and decades of clinical experience have made a wide array of formulations, among them sustained-release tablets, available

6.1.19 Oxymorphone

Oxymorphone is a semi-synthetic opioid congener and a metabolite of oxycodone. It is more than six times as potent as morphine, and immediate-release and extended-release tablets made available in 2006 paved the way for more wide-spread use of oxymorphone in the management of cancer pain (Glare 2009b). Oxymorphone has an oral availability of 10%, and analogously to morphine, it is metabolized to and excreted as oxymorphone-3-glucuronide, though small amounts of alpha- and beta-6-oxymorphol are produced (Glare 2009b; Smith 2011).

6.1.20 Pethidine (Meperidine)

Pethidine (synonym: meperidine) is a synthetic opioid and a peperidine, like fentanyl, and equally lipophilic (Davis 2009c). It has been in clinical use since 1939 (Corbett et al. 2006). Its major metabolite, meperidinic acid, is produced by hydrolysis and then glucuronidated before excretion, while another metabolite, normeperidine, is synthesized through CYP3A4-mediated N-demethylation (Smith 2011). Liver and kidney disease lead to an accumulation of these metabolites and a build-up of, a neurotoxic agent known to cause tremors, myoclonus and seizures (Szeto et al. 1977; Kaiko et al. 1983). Adverse side effects were also more common in patient-controlled analgesia (Brayfield 2014). In 2011, the FDA expanded the list of contraindications, highlighting the increased awareness of pethidine side effects

(FDA 2011). Additionally, pethidine is a weak serotonin reuptake inhibitor, and in combinations with monoamino oxidase (MAO) inhibitors it has been reported to cause fatal serotonin toxicity (Gillman 2005). Although the use of pethidine is currently being discouraged -more so as alternative opioids with less pronounced side effects are available- it is still among the seven most commonly administered opioids worldwide(Latta et al. 2002; Davis 2009c; International Narcotics Control Board 2011). It is widely used in obstreterix (Brayfield 2014).

6.1.21 Sufentanil

Sufentanil is a highly lipophilic congener of fentanyl and about 500 times as potent as morphine, making it the most potent opioids administered to humans (Hall and Hardy 2009). Because of its rapid inset and recovery, sufentanil is used as an adjunct in anesthesia, postoperative pain, and labor pain, and, like fentanyl, it may accumulate when given repeatedly, especially in obese patients. About 2% are eliminated unchanged via the kidneys, the rest undergoes N-dealkylation and O-demethylation in the liver and small (Hall and Hardy 2009).

6.1.22 Tapentadol

Tapentadol is a relatively new opioid that was approved for use by the FDA in 2008 (Singh et al. 2013). It is nearly completely metabolized to tapentadol-O-glucuronide and other minor metabolites, such as N-desmethyltapentadol (Singh et al. 2013). Apart from acting upon opioid receptors, tapentadol inhibits noradrenaline reuptake (Tzschentke et al. 2011). The higher concentrations of noradrenaline contribute to the overall analgesic effect, so that tapentadol, despite a 44-lower binding affinity to MOP than morphine, has an analgesic potency of 0.4 (Tzschentke et al. 2011).

6.1.23 Tilidine

Tilidine is a synthetic opioid and prodrug of the active compound nortilidine. Classified as weak opioid, it is employed as analgesic in moderate acute and chronic pain. CYP3A4 and CYP 2C19-mediated demethylation yield nortilidine and bisnortilidine (Grun et al. 2012). It is available as single formulation or in combination with naloxone, an opioid receptor antagonist, to prevent abuse.

7 References

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