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Effects of the new psychoactive substances diclofensine, diphenidine, and methoxphenidine on monoaminergic systems

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ABSTRACT

Diclofensine, diphenidine, and methoxphenidine are new psychoactive substances (NPSs) that recently appeared on the illicit drug market. Pharmacological profiling of such newly emerged drugs is crucial for a better understanding of their psychotropic effects and toxicity. We therefore investigated the potential of these NPSs to inhibit the norepinephrine, dopamine, and serotonin transporters in human embryonic kidney cells stably transfected with the respective transporters. In addition, we determined monoamine transporter and receptor affinities for the substances. Diclofensine potently bound to the monoamine transporters in the submicromolar range and had similar inhibition potential for all three transporters in the range of 2.5–4.8 μM. Moreover, diclofensine bound to adrenergic, dopamine, serotonin, and trace amine-associated receptors. Diphenidine was an equipotent inhibitor of the norepinephrine and dopamine transporters in the low micromolar range and a very weak inhibitor of the serotonin transporter. Besides binding to transporters, diphenidine bound to adrenergic α_{1A} and α_{2A} receptors and serotonin 5-hydroxytryptamine 1A (5-HT_{1A}) and 5-HT_{2A} receptors in the range of 4–11 μM. Methoxphenidine bound to all transporters, but considerable inhibition (IC_{50} < 10 μM) was observed only for the norepinephrine transporter. Moreover, methoxphenidine bound to adrenergic α_{2A} and serotonin 5-HT_{2A} and 5-HT_{2C} receptors in the range of 2.5–8.2 μM. None of the test drugs mediated substrate-type efflux of monoamines. These data demonstrate that the monoamine transporter inhibition and receptor interactions most likely mediate the psychoactive effects of diclofensine and possibly play a contributory role for diphenidine and methoxphenidine.

1. Introduction

The emergence of numerous potentially harmful new psychoactive substances (NPSs) in recent years poses a challenge to drug regulatory authorities and health personnel. Case reports are often the only source of information on the toxicity of newly emerged drugs and in vitro screenings are therefore a helpful tool to better understand the pharmacology of such substances. In the current study, we present in vitro pharmacological profiles of three NPSs (Fig. 1) that have recently reached the illicit drug market. Diclofensine was originally developed as an antidepressant and was shown to have potent monoamine transporter inhibition potencies in rat brain synaptosomes (Andersen, 1989; Funke et al., 1986; Gasić et al., 1986; Hyttel and Larsen, 1985; Keller et al., 1982) and to increase extracellular dopamine levels in rats (Nakachi et al., 1995). Diphenidine and its methoxylated derivative 2-methoxydiphenidine (methoxphenidine) are NPSs of the diarylethylamine class, which have previously been associated with adverse events including deaths (Elliott et al., 2015; Gerace et al., 2017; Helander et al., 2015; Hofer et al., 2014; Kusano et al., 2017; Lam et al., 2016; Valli et al., 2017). Diphenidine and methoxphenidine act as *N*-methyl-D-aspartate receptor antagonists (Berger et al., 2009; Wallach et al., 2015), and their effects have been described as being comparable to other dissociative anesthetic drugs, such as ketamine (Helander et al., 2015; Morris and Wallach, 2014).

2. Material and methods

2.1. Drugs

Diclofensine, diphenidine, and methoxphenidine were kindly provided by the Forensic Institute Zürich (Zürich, Switzerland) with > 98% purity. 3,4-Methylenedioxymethamphetamine (MDMA) was purchased from Lipomed (Arlesheim, Switzerland) with high-performance liquid chromatography purity of > 98.5%. Diclofensine was obtained

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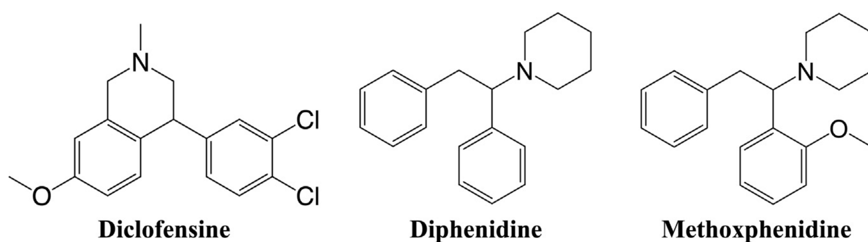

Fig. 1. Structures of NPSs included in the study.

as racemic base, the other drugs were obtained as racemic hydrochloride salts. Radiolabelled [^{3}H]norepinephrine and [^{3}H]dopamine were purchased from Perkin-Elmer (Schwerzenbach, Switzerland). Radiolabeled [^{3}H]serotonin was obtained from Anawa (Zürich, Switzerland).

2.2. Monoamine uptake transporter inhibition

Norepinephrine, dopamine, and serotonin uptake inhibition for the test drugs in the range of 1 nM to 900 µM was assessed in human embryonic kidney (HEK) 293 cells transfected with the human transporter for norepinephrine (hNET), dopamine (hDAT), or serotonin (hSERT) as previously described in detail (Hysek et al., 2012b; Tatsumi et al., 1997), with slight modifications (Luethi et al., 2017b). Briefly, cells were suspended in uptake buffer and incubated with the test drugs for 10 min before [^{3}H]norepinephrine, [^{3}H]dopamine, or [^{3}H]serotonin were added at a final concentration of 5 nM for additional 10 min to initiate uptake transport. Thereafter, the cells were separated from the uptake buffer by centrifugation through silicone oil. The centrifugation tubes were then frozen in liquid nitrogen and the cell pellet was cut into scintillation vials containing lysis buffer. Scintillation fluid was added to the vials and uptake was quantified by liquid scintillation counting. Transporter inhibitors (10 µM nisoxetine for NET, 10 µM mazindol for DAT, and 10 µM fluoxetine for SERT) were added to assess for non-specific monoamine uptake.

2.3. Drug-induced monoamine efflux

To assess whether the test drugs act as pure transporter inhibitors or as transporter substrates, drug-induced monoamine efflux was assessed at a drug concentration of 100 µM in hNET-, hDAT-, or hSERT-transfected HEK 293 cells as previously described (Simmler et al., 2013) with slight modifications. Briefly, cells were cultured in a poly-D-lysine coated microplate and preloaded with 10 nM [^{3}H]norepinephrine, [^{3}H]dopamine, or [^{3}H]serotonin for 20 min at 37 °C. The cells were then washed twice, and subsequently treated with the test drugs for 15 min (DAT and SERT) or 45 min (NET) at 37 °C on a rotary shaker. Thereafter, 300 µl of the assay buffer was transferred into scintillation vials, scintillation fluid was added, and the amount of monoamine efflux was then quantified by liquid scintillation counting. Transporter inhibitors (10 µM nisoxetine for NET, 10 µM mazindol for DAT, and 10 µM citalopram for SERT) were included to determine “pseudo-efflux” caused by nonspecific monoamine efflux and subsequent reuptake inhibition (Scholze et al., 2000). The assay set-up was based on previous kinetic evaluation of the efflux-over-time curves for monoamine transporter substrates (Hysek et al., 2012b; Simmler et al., 2014a). The transporter substrate MDMA was used as positive control.

2.4. Monoamine receptor and transporter binding affinities

Radioligand binding affinities for transporters and receptors were assessed as previously described in detail (Luethi et al., 2017c). Briefly, membrane preparations that overexpressed the respective transporters (Tatsumi et al., 1997) or receptors (human genes, with the exception of rat and mouse genes for trace amine-associated receptors (Revel et al., 2011)) were incubated with the radiolabeled selective ligands at concentrations equal to K_d, and ligand displacement by the compounds was measured. Specific binding of the radioligand to the target receptor was defined as the difference between the total binding and nonspecific binding that was determined in the presence of the selected competitors at a concentration of 10 µM. The following radioligands and competitors, respectively, were used: *N*-methyl-[^{3}H]nisoxetine and indatraline (NET), [^{3}H]WIN35,428 and indatraline (DAT), [^{3}H]citalopram and indatraline (SERT), [^{3}H]8-hydroxy-2-(di-*n*-propylamine)tetralin and pindolol (serotonin 5-HT_{1A} receptor), [^{3}H]ketanserin and spiperone (serotonin 5-HT_{2A} receptor), [^{3}H]mesulgerine and mianserin (serotonin 5-HT_{2C} receptor), [^{3}H]prazosin and chlorpromazine (α_1 adrenergic receptor), [^{3}H]rauwolscine and phentolamine (α_2 adrenergic receptor), [^{3}H]spiperone and spiperone (dopamine D_2 receptors), and [^{3}H]RO5166017 and RO5166017 (trace amine-associated receptors 1).

2.5. Activity at the serotonin 5-HT_{2A} receptor

Mouse embryonic fibroblasts (NIH-3T3 cells) expressing the human serotonin 5-HT_{2A} receptor were incubated in HEPES-Hank's Balanced Salt Solution (HBSS) buffer (Gibco, Zug, Switzerland; 70,000 cells/100 µl) for 1 h at 37 °C in 96-well poly-D-lysine-coated plates. Thereafter, 100 µl of dye solution (fluorescence imaging plate reader [FLIPR] calcium 5 assay kit; Molecular Devices, Sunnyvale, CA, USA) was added to each well, and the plates were again incubated for 1 h at 37 °C. The plates were then placed in a FLIPR, and 25 µl of the test drugs that were diluted in HEPES-HBSS buffer containing 250 mM probenicid was added online. The increase in fluorescence was measured, and EC_{50} values were derived from the concentration-response curves using nonlinear regression.

2.6. Activity at the serotonin 5-HT_{2B} receptor

HEK 293 cells expressing the human serotonin 5-HT_{2B} receptor were incubated in growth medium at a density of 50,000 cells per well at 37 °C in poly-D-lysine-coated 96-well plates overnight. The growth medium was then removed by snap inversion, and 100 µl of the calcium indicator Fluo-4 solution (Molecular Probes, Eugene, OR, USA) was added to each well. The plates were incubated for 45 min at 31 °C, the Fluo-4 solution was then removed by snap inversion, and 100 µl of Fluo-4 solution was added a second time for 45 min at 31 °C. The cells were washed with HBSS and 20 mM HEPES (assay buffer) using an EMBLA cell washer, and 100 µl assay buffer was added. The plates were then placed in a FLIPR, and 25 µl of the test substances that were diluted in assay buffer was added online. The increase in fluorescence was measured, and EC_{50} values were derived from the concentration-response curves using nonlinear regression.

2.7. Cytotoxicity

Adenylate kinase release was measured with the ToxiLight BioAssay Kit from Lonza (Basel, Switzerland) as marker for cytotoxicity to confirm cell integrity under assay conditions. Briefly, 25,000 hSERT-, hDAT-, or hNET-transfected HEK 293 cells per well were seeded in a 96-well plate. The following day, the cells were treated with 100 µl of the

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