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Toxicokinetics of novel psychoactive substances: Characterization of *N*-acetyltransferase (NAT) isoenzymes involved in the phase II metabolism of 2C designer drugs

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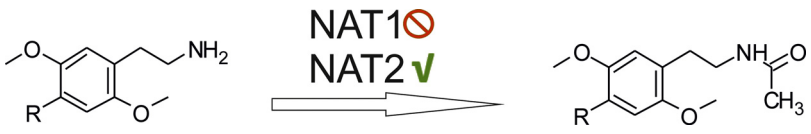
HIGHLIGHTS

- Elucidation of human NATs isoforms involved in 2Cs *N*-acetylation.
- Kinetic profiles of human NAT catalyzed reactions were presented.
- 2C acetylation exclusively catalyzed by NAT2.
- Affinity to human NAT2 increasing with volume of 4-substituent.
- Interaction with NAT2 inhibitors or polymorphisms should be considered.

GRAPHICAL ABSTRACT



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ABSTRACT

The 2,5-dimethoxyphenethylamine-derived designer drugs (so-called "2Cs") recently became of great importance on the illicit drug market as stimulating hallucinogens. They are distributed and consumed as "novel psychoactive substances" (NPS) without any safety testing at the forefront. As previous studies have shown, the 2Cs are mainly metabolized by *O*-demethylation, *N*-acetylation, or deamination. Therefore, the aim of this study was to elucidate the role of the recombinant human *N*-acetyltransferase (NAT) isoforms 1 and 2 in the phase II metabolism of 2Cs. For these studies, cDNA-expressed recombinant human NATs were used and formation of metabolites after incubation was measured using GC–MS. NAT2 could be shown to be the only isoform catalyzing the reaction in vitro, hence it should be the only relevant enzyme for in vivo acetylation. In general, all metabolite formation reactions followed classic Michaelis–Menten kinetics and the affinity to human NAT2 was increasing with the volume of the 4-substituent. In consequence, a slow acetylator phenotype or inhibition of NAT2 could lead to decreased *N*-acetylation and might lead to an increased risk of side effects caused by these novel psychoactive substances.

1. Introduction

The so-called "2C" designer drugs are ring substituted derivatives of β-phenethylamine, which is not a common drug of abuse due to its fast metabolism after consumption (de Boer and Bosman, 2004; Shulgin, 1991). Though, a lot of 2Cs became of great importance in the world wide drugs scene and are distributed as stimulating hallucinogens and so-called "new psychoactive substance" (NPS). First seizures were reported from the United States

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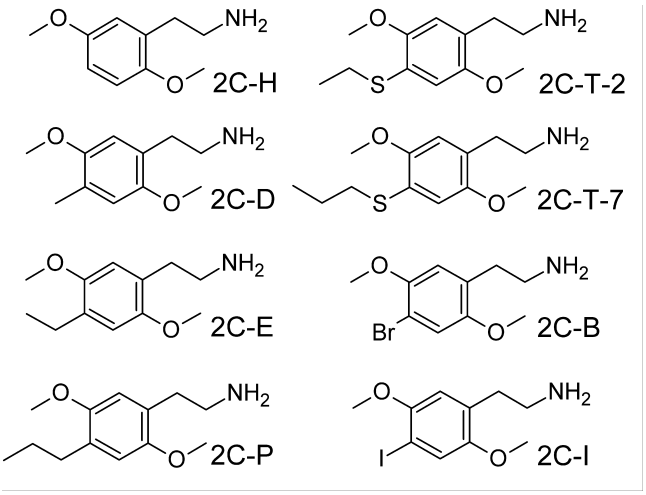

Fig. 1. Chemical structures of the studied 2Cs.

and European countries and since 2009 substances such as 2C-E (4-ethyl-2,5-dimethoxy-β-phenethylamine) and 2C-I (4-iodo-2,5-dimethoxy-β-phenethylamine) have been commonly reported (United Nations Office on Drugs and Crime (UNODC, 2013)). The chemical structures of the most frequently used 2Cs are documented in Fig. 1.

Several intoxication cases have been reported in recent years describing psychosis and serotonin syndrome after 2C intake (Ambrose et al., 2010; Drees et al., 2009; Huang and Bai, 2011; Miyajima et al., 2008). The 2Cs were described as agonists or antagonists of the 5-HT_2 receptor and their psychoactive effects have been reported to be dose dependent, ranging from mere stimulant effect at lower doses, with hallucinogenic and entactogenic effects at higher doses (Acuna-Castillo et al., 2002; Huang and Bai, 2011; Theobald and Maurer, 2007; Villalobos et al., 2004).

In general, all 2Cs have several common chemical properties responsible for their hallucinogen-like activity such as two methoxy groups and a lipophilic substituent in position 4 of the ring system (alkyl, halogen, or alkylthio) (Shulgin, 1991). In previous studies, the metabolism of several 2C's was studied mainly in rats (Kanamori et al., 2002, 2003, 2007; Theobald et al., 2005a,b, 2006; Theobald and Maurer, 2006a,b), but also in humans (de Boer et al., 1999), mice (Carmo et al., 2004), and hepatocytes of different species (Carmo et al., 2005; Kanamori et al., 2005). The major metabolic steps were *O*-demethylation in position 2 or 5, deamination or *N*-acetylation. The role of isoenzymes of the monamine oxidase (MAO)- and cytochrome P450 (P450)-type in the metabolism of the 2C's has also been described (Theobald and Maurer, 2007). The investigated compounds showed a slightly higher affinity to MAO-A and the affinity is becoming more dominant with an increasing 4-substituent size. Concerning the isoenzymes of the P450-type, only P450 2D6 showed activity in metabolism of 2C-D (4-methyl-2,5-dimethoxy-β-phenethylamine), 2C-E, 2C-T-2 (4-ethylthio-2,5-dimethoxy-β-phenethylamine), and 2C-T-7 (4-propylthio-2,5-dimethoxy-β-phenethylamine), but with low formation rate. However, the role of the *N*-acetyltransferase (NAT) isoforms in the metabolism of the 2Cs is still unclear. The NATs are among the major hepatic phase II enzymes involved in drug metabolism and play an important role in the *N*-acetylation of various amines. Human NATs, especially NAT2, are polymorphically expressed and slow and rapid acetylators have been identified (Grant et al., 1991, 1997). Therefore, the aim the study was to determine the relevance of NAT1 and NAT2 in metabolism of the eight most frequent 2Cs. K_m and V_{max} values were determined for calculating the apparent intrinsic clearances (Cl_{int}) in order to compare the influence of the different substituents of the 2Cs in relation to their NAT affinity.

2. Materials and methods

2.1. Chemicals and reagents

Hydrochlorides of 2C-D, 2C-E, and 2C-P (4-propyl-2,5-dimethoxy-β-phenethylamine) were provided by Dejachem (Schwendi, Germany), 2C-T-2 hydrochloride by Bundeskriminalamt (Wiesbaden, Germany), 2C-T-7 hydrochloride by Lipomed (Weil am Rhein, Germany), 2C-B tartrate by Hessisches Landeskriminalamt (Wiesbaden, Germany), 2C-I hydrochloride by Landeskriminalamt Baden-Württemberg (Stuttgart, Germany), 2C-H (2,5-dimethoxy-β-phenethylamine) hydrochloride by Institute of Forensic Research (Krakow, Poland). Sulfamethazine, carnitine-acetyl-transferase, acetyl coenzyme A sodium salt, and acetyl-DL-carnitine hydrochloride were obtained from Sigma–Aldrich (Taufkirchen, Germany). All other chemicals and reagents were from VWR (Darmstadt, Germany) and of analytical grade. Baculovirus infected insect cell-expressed NAT1 (human arylamine *N*-acetyltransferase 1*4, wild-type allele) and NAT2 (human arylamine *N*-acetyltransferase 2*4, wild-type allele) were from BD Biosciences (Heidelberg, Germany). Activity of enzymes was documented using the known NAT1 and NAT2 substrates para-aminosalicylic acid and sulfamethazine, respectively. After delivery, the enzymes were thawed at 37 °C, aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C until use.

The *N*-acetylated derivatives of all 2Cs were synthesized in-house according to a previously published procedure with the following modifications (Maurer et al., 2011; Meyer et al., 2013a): 2Cs at concentrations from 0.09 to 100 μM in 100 μL methanol were evaporated to dryness and 100 μL of a mixture of pyridine/acetic anhydride (2:3 v/v) were added and put into a microwave oven at 300 W for 2 min. Samples were then evaporated and reconstituted with 100 μL supernatant of a blank incubation mixture (see below), diluted with 100 μL acetonitrile for GC–MS or 200 μL acetonitrile for LC–MS/MS. Finally, 100 μL were transferred to an autosampler vial and injected onto the LC–MS (10 μL) and GC–MS (1 μL) apparatus for identity and purity analysis. Individual calibration curves were recorded at concentrations ranging from 0.09 μM to 100 μM.

2.2. Initial activity studies with recombinant human NAT isoenzymes

Incubations were performed at 37 °C with the substrates 2C-B, 2C-I, 2C-H, 2C-D, 2C-E, 2C-P, 2C-T-2, and 2C-T-7 (final concentrations 32–50 μM) and recombinant human NAT1 or NAT2 (final concentration 0.05 mg/mL) for 30 min. Besides substrate and enzymes, incubation mixtures (final volume, 100 μL) consisted of triethanolamine (TEA) buffer pH 7.5 (TEA 100 mM, ethylenediaminetetraacetic acid 500 mM, and dithiothreitol 50 mM) and CoA-system (final concentrations acetyl-CoA 0.1 mM, acetyl-carnitin 2.3 mM, and carnitin-acetyltransferase 0.008 U/μL). The incubation mixture was preincubated for 10 min at 37 °C and the reactions were started by addition of the substrate and stopped with 50 μL of acetonitrile. The solution was centrifuged for 5 min at 14,000 × *g*, 100 μL of the supernatant phase was transferred to an autosampler vial, and 1 μL injected onto the GC–MS apparatus for analysis.

To identify the metabolites after initial activity studies, all analytes were separated by GC and identified by their electron ionization (EI) mass spectra published elsewhere (Maurer et al., 2011). For enzyme kinetic studies, all analytes were separated by GC, identified by their EI mass spectra, and quantified by reconstructed mass chromatography.

2.3. Enzyme kinetic studies with recombinant human NAT isoenzymes

Enzyme kinetic studies were performed in accordance to a previously published strategy (Meyer et al., 2013b) with the following modifications. Incubation time and enzyme concentration for all incubations were chosen to be within a linear range of metabolite formation (non saturating conditions). Kinetic constants were derived from incubations ($n = 2$ each) with the following final substrate concentration ranges (μM), final protein concentrations (human NAT2, mg/mL), and incubation times (min), respectively: 2C-B, 3.2–634, 0.02, 4; 2C-I, 25–1000, 0.02, 15; 2C-H, 21–833, 0.02, 15; 2C-D, 5–150, 0.02, 4; 2C-E, 5–800, 0.02, 10; 2C-P, 22–860, 0.02, 10; 2C-T-2 5–400, 0.02, 20; and 2C-T-7, 4.4–700, 0.02, 10. Besides enzymes and substrate, incubation mixtures (final volume: 100 μL) consisted of TEA buffer pH 7.5 and CoA-system as described above. The incubation mixture was preincubated for 10 min at 37 °C and reactions were started by addition of the substrate and stopped with 20 μL chloroform and 200 μL ice cold acetonitrile containing 25 μM of diphenhydramine as internal standard. The mixtures were then shaken for 2 min and then centrifuged for 5 min at 14,000 × *g*. Afterwards, the organic phase (100 μL-aliquot) was diluted with acetonitrile (100 μL), transferred to an autosampler vial, and injected (1 μL) onto the GC–MS apparatus for analysis.

For data analysis, enzyme kinetic constants were estimated by non-linear curve-fitting using GraphPad Prism 5.00 software (San Diego, CA). The Michaelis–Menten