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Synthesis and Evaluation of Mefway Analogs as Ligands for Serotonin 5HT_{1A} Receptors

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Abstract

¹⁸F-Mefway (*N*-{2-[4-(2'-methoxyphenyl)piperazinyl]ethyl}-*N*-(2-pyridyl)-*N*-(4'-¹⁸F-fluoromethylcyclohexane)carboxamide) was developed and evaluated for use as a PET ligand for imaging 5-HT_{1A} receptors. Ongoing studies of ¹⁸F-Mefway have shown it to be an effective PET radiotracer. We have synthesized isomers of Mefway by changing the position of the methyl-group in attempts to evaluate stability for imaging purposes. 2-Methyl-, 3-methyl-, and 4-methylcyclohexane-1-carboxylic acids and 3-carbomethoxy-, 4-carbomethoxycyclohexane-1-carboxylic acids were coupled with WAY-100634 to provide the methylcyclohexyl derivatives (2-, 3- and 4-methyl). Mefway and 3-Mefway analogs were prepared by reduction of carbomethoxy-derivatives followed by fluorination. In vitro binding affinities for the methylated derivatives in rat brain homogenates was found to be 10.4 nM (2-methyl), 77 nM (3-methyl) and 21.5 nM (4-methyl). Binding affinity of 3-Mefway and 4-Mefway was found to be 17.4 nM and 6.26 nM, respectively. Our results suggest that 3-methyl/3-fluoromethyl substituent has approx. 3-fold lower affinities compared to the 4-methyl/4-fluoromethyl substituent.

Keywords

Mefway; PET imaging; Serotonin

Introduction

Imaging serotonin 5-HT_{1A} receptors has been underway because of their involvement in various CNS functions (Kennedy and Zubiet 2004; Kumar and Mann 2007; Billard et al., 2014). ¹⁸F-Mefway (*N*-{2-[4-(2'-methoxyphenyl)piperazinyl]ethyl}-*N*-(2-pyridyl)-*N*-(4'-¹⁸F-fluoromethylcyclohexane)carboxamide) is a promising PET imaging for serotonin 5-HT_{1A} receptors (Saigal et al., 2013; Wooten et al., 2011a; 2012). The fluoromethyl group in Mefway is incorporated at the 4-position of the cyclohexyl ring. Previous studies on a related analog, ¹⁸F-FCWAY ([*N*-{2-[4-(2-methoxyphenyl)piperazino]ethyl}-*N*-(2-pyridinyl)-4-[¹⁸F]fluorocyclohexanecarboxamide) found changes in the position of the ¹⁸F-radiolabel resulted in a change in the affinity of the compound (Ma et al. 2006). Shifting

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the ^{18}F -radiolabel from the 4 position (^{18}F -FCWAY) to the 3 position (^{18}F -3-FCWAY) had the effect of lowered affinity, faster pharmacokinetics and lower defluorination, giving ^{18}F -3-FCWAY better characteristics for measuring dynamic change in $5\text{-HT}_{1\text{A}}$ receptors (Lang et al. 2006).

In order to evaluate the sensitivity of binding affinity to the position of the methyl substituent on the cyclohexyl ring, we first examined methylated WAY-100635 derivatives (Figure-1). The intent of making the methylated analogs was to understand the structure-activity relationship of methylation of the parent molecule, WAY-100635 and not to develop a carbon-11 radiotracer. We hypothesized that changing the position of the methyl group from the 4 position to the 2 and 3 positions (2-Me, 3-Me respectively) is expected to alter the binding affinity. Thus, three analogs, 2-methyl, 3-methyl and 4-methyl were synthesized and their in vitro binding affinities evaluated. Subsequently, 3-Mefway (N-{2-[4-(2'-methoxyphenyl)piperazinyl]ethyl}-N-(2-pyridyl)-N-(3'-fluoromethylcyclohexane)carboxamide) was synthesized and compared with 4-Mefway.

Materials and Methods

General

All chemicals and solvents used were obtained from Aldrich Chemical Co. WAY-100634 (1-(2-methoxyphenyl)-4-[2-(pyridylamino)ethyl]piperazine) was synthesized using reported procedures (Saigal et al., 2006). Analytical thin-layer chromatography (TLC) was used to monitor reactions (Baker-flex, Phillipsburg, NJ, USA). Electrospray mass spectra were obtained from a Model 7250 mass spectrometer (Micromass LCT). Proton NMR spectra were recorded on a Bruker OMEGA 500-MHz spectrometer. Tritium was assayed by using a Packard Tri-Carb Liquid scintillation counter with 65% efficiency. All animal studies were approved by the Institutional Animal Care and Use Committee of University of California-Irvine.

Synthesis

Synthesis of substituted cyclohexylcarbonyl chlorides (6)—The acids, 0.28 g (2 mmol) of 2-methyl-1-cyclohexanecarboxylic acid (**5**), 0.14 g (1 mmol) of 3-methyl-1-cyclohexanecarboxylic acid or 0.14 g (1 mmol) of 4-methyl-1-cyclohexanecarboxylic acid were treated with oxalyl chloride in a 1:4 ratio of carboxylic acid to oxalyl chloride in separate vials (Figure-2). The reactions were allowed to stir overnight. The mixtures were then heated at 70-80°C for an hour during which time excess oxalyl chloride was removed. The substituted cyclohexylcarbonyl chlorides (**6**) were allowed to cool to room temperature then were immediately used for the next step of the synthesis.

Synthesis of N-{2-[4-(2'-methoxyphenyl)piperazinyl]ethyl}-N-(2-pyridyl)-N-(2/3/4-methyl-cyclohexane)carboxamide (8)—WAY-100634 (**7**) was reacted with the substituted cyclohexylcarbonyl chlorides (**6**) from the previous step. For the synthesis of 2-methyl, 30 mg (0.096 mmol) of WAY-100634; for 3-methyl, 11 mg (0.035 mmol) of WAY-100634; and for 4-methyl, 11 mg (0.035 mmol) of WAY 100634 was dissolved in 1mL of dichloromethane (DCM, CH_2Cl_2) and added to the respective cyclohexylcarbonyl

chlorides (**6**). The mixtures were allowed to stir for 24 hrs. The mixtures were then purified using preparative TLC (9:1 CH₂Cl₂:CH₃OH). The products were isolated from the plates to yield 11 mg (0.023 mmol) of 2-methyl (**4**; >95%), 3 mg (0.006 mmol) of 3-methyl (**3**; >95%) and 6 mg (0.012 mmol) of 4-methyl (**2**; >96%) and were characterized by NMR and mass spectroscopy as described below. The purified derivatives were used for biological assays.

2-Methyl (4): ¹H-NMR (500 MHz, CDCl₃) δ ppm: 8.51-8.52 (d, *J*=3.95, 1H), 7.73-7.77 (t, *J*=3.90, 1H), 7.20-7.35 (m, 2H), 6.95-7.05 (m, 1H), 6.90-6.91 (d, *J*=4.30, 2H), 6.83-6.87 (m, 1H), 3.97-3.99 (m, 1H), 3.85 (s, 3H, OCH₃), 3.73-3.76 (t, *J*=3.50, 2H), 3.45-3.50 (m, 2H), 2.95-3.19 (br, 2H), 2.55-2.70 (br, 2H), 2.64 (s, 1H), 2.17 (s, 1H), 2.01 (s, 1H), 1.65-1.85 (m, 4H), 1.55 (s, 3H, CH₃), 1.15-1.27 (m, 4H), 0.93-0.94 (d, *J*=7, 2H). MS, *m/z*, 437 (100%, [M+H]⁺), 459 (70%, [M+Na]⁺).

3-Methyl (3): ¹H-NMR (500 MHz, CDCl₃) δ ppm: 8.52 (d, *J*=3.90, 1H), 7.70-7.77 (t, *J*=3.90, 1H), 7.20-7.32 (m, 2H), 6.98 (br, 1H), 6.89-6.91 (d, *J*=4.55, 2H), 6.85-6.85 (m, 1H), 3.84 (s, 3H, OCH₃), 3.73-3.76 (t, 2H), 3.45-3.48 (m, 2H), 2.98-3.02 (br, 2H), 2.61 (br, 2H), 2.17 (s, 1H), 2.01 (s, 1H), 1.65-1.85 (m, 4H), 1.84-1.88 (m, 2H), 1.55 (s, 3H, CH₃), 1.18-1.30 (m, 4H), 0.84-0.90 (d, 2H). MS, *m/z*, 437 (100%, [M+H]⁺), 459 (70%, [M+Na]⁺).

4-Methyl (2): ¹H-NMR (500 MHz, CDCl₃) δ ppm: 8.53 (d, *J*=4.25, 1H), 7.75 (t, *J*=4.5, 1H), 7.20-7.32 (m, 2H), 6.98 (m, 1H), 6.89-6.91 (d, *J*=4.1, 2H), 6.83-6.85 (m, 1H), 3.98 (br, 1H), 3.84 (s, 3H, OCH₃), 3.73-3.76 (t, *J*=6.65, 2H), 3.47-3.49 (m, 2H), 3.02 (br, 1H), 2.98 (br, 2H), 2.62 (br, 2H), 2.17 (s, 1H), 2.01 (s, 1H), 1.65-1.85 (m, 4H), 1.56 (s, 3H, CH₃), 1.15-1.27 (m, 4H), 0.93-0.94 (d, 2H). MS, *m/z*, 437 (100%, [M+H]⁺), 459 (70%, [M+Na]⁺).

Synthesis of N-{2-[4-(2-methoxyphenyl)piperazinyl]ethyl}-N-(2-pyridyl)-N-(3/4-fluoro-methylcyclohexane)carboxamide (9,10, Figure-3)—Using the previous two steps described for (**6**), 372 mg (2.62 mmol) of 3-carboxymethyl-1-cyclohexanecarboxylic acid (**11**) was converted to its corresponding substituted cyclohexylcarbonyl chloride (**12**). The ester (**12**; 57 mg, 0.4 mmol) was then diluted with 3 mL of diethyl ether and placed into a reaction flask and was reacted with 80 mg (0.26 mmol) of WAY-100634 for 24 hrs. The isolated yield of 44 mg (0.09 mmol; >95% pure) of ester (**13**) was obtained.

The anhydrous ether solution of the ester (**13**; 39 mg; 0.08 mmol 3 mL of diethyl ether) was stirred in an ice bath for 15 minutes to bring the solution temperature down to 0°C. Once the solution reached 0°C, 0.08 mL (0.08 mmol) of 1M LiAlH₄ in diethyl ether was slowly added to the flask and allowed to stir for 30 minutes. Subsequently the reaction was quenched with 1N hydrochloric acid to pH ~7. The reaction mixture was then taken up in ethyl ether. The ether layer was then isolated and dried to yield 24 mg of crude product. It was then purified using preparative TLC (9:1 CH₂Cl₂:CH₃OH). The product was isolated from the preparative TLC plate and filtered to yield 8 mg (0.018 mmol) of alcohol (**14**; >90% pure) and 20 mg of recovered reactant ester (**13**).

The alcohols (4.5 mg; 0.01 mmol) (**14**) were reacted with diethylaminosulfur trifluoride (DAST) (4 μL; 0.03 mmol) in 1 mL of DCM at room temperature for 24 hrs. The reaction mixture was washed with saturated sodium bicarbonate (NaHCO₃) and dried over

magnesium sulfate. The crude product in the organic layer was purified using preparative TLC (9:1 CH₂Cl₂:CH₃OH) to yield 2 mg (0.004 mmol; >90% pure) of 4-Mefway (**9**) and 2 mg (0.004 mmol; >90% pure) of 3-Mefway (**10**) (Figure-4). 4-Mefway was characterized as described previously (Saigal et al, 2006). For 3-Mefway (**10**): ¹H-NMR (500 MHz, CDCl₃) δ ppm: 8.52 (d, *J*=4, 1H), 7.77 (t, *J*=7.5, 1H), 7.74-7.79 (m, 2H), 7.05 (br, 1H), 6.80-6.93 (m, 2H), 4.10-4.21 (dd, *J*=5.5, *J*_{H,F}= 48, 2H, CH₂F), 3.86 (s, OCH₃), 3.74 (t, 2H), 3.40-3.49 (m, 2H), 3.25 (br, 1H), 3.02 (br, 2H), 2.70 (br, 2H), 2.45 (s, 1H), 1.84-1.88 (m, 1H), 1.50-1.70 (m, 4H), 1.19-1.27 (m, 4H), 0.84-0.88 (d, 2H). MS, *m/z*, 455 ([M+H]⁺), 477 ([M+Na]⁺).

Homogenate Binding Assay

Binding affinities of methyl derivatives of WAY-100635 were measured using rat brain homogenate assays. Rat brain homogenates were labeled with ³H-WAY 100635 were carried out to measure the binding affinity of 2-methyl, 3-methyl, 4-methyl-WAY-100635 and were compared with WAY-100635 to 5-HT_{1A} receptor. The cerebrum (0.85 g of tissue per assay) of each male rats was isolated and homogenized in 30 mL of assay buffer for 30 secs (50 mM Tris-HCl, pH 7.6) using Tekmar tissumizer (15 secs, half maxima speed). The homogenate was centrifuged at 27,000g for 20 minutes at 4 °C and the supernatant was discarded. The resulting pellet was resuspended in 30 mL of buffer, homogenized, and then centrifuged at 27,000g for 20 minutes at 4 °C. Once again the supernatant was removed and the pellet was diluted with incubation buffer. A fixed concentration of ³H-WAY 100635 (1 nM) was incubated with the rat homogenate in the presence of various concentrations of 2-methyl, 3-methyl, 4-methyl-WAY-100635, 4-Mefway, 3-Mefway and WAY-100635 (10⁻¹² to 10⁻⁴ M) in the assay buffer (50 mM Tris-HCl, pH 7.6). Nonspecific binding was determined by including 10 μM of WAY-100635. Total assay volume was 0.5 mL. To start, 0.2 mL of the rat brain homogenate was added to each test tube containing ³H-WAY-100635, different concentrations of drugs and 10 μM of WAY-100635 for nonspecific binding. The test tube samples were incubated for 1 hour in a 37 °C water bath. After incubation a rapid vacuum filtration was implemented through Whatman GF/C filter paper (pre-soaked in 0.1% polyethylamine in 10 mL of millipore water) using Brandel tissue harvester. The filter was washed three times with 5 mL of cold buffer and was transferred to vials with 5 ml Bio-Safe II scintillation cocktail and counted for 10 mins in a scintillation counter. The same procedures were followed when performing the binding assay for WAY-100635 as the test compound. Data was analyzed using following procedure: (a) the non-specific binding of ³H-WAY-100635 was subtracted for all samples; (b) the specific binding was normalized to 100% (no competitive ligand) and (c) the binding isotherms were fit to the Hill equation (KELL BioSoft software (v 6), Cambridge, U.K.).

Results and Discussion

¹⁸F-Mefway is a promising PET radioligand for 5-HT_{1A} receptors. In order to further understand how the binding properties are affected based on the position of the methyl and fluoromethyl group, four new compounds were investigated. For this study, the methyl group was placed on the 2, 3 and 4 positions (**2**, **3**, **4**) and the fluoromethyl group was placed on the 3 and 4 positions (**9**, **10**).

Synthesis

Synthesis of 2-methyl, 3-methyl and 4-methyl-WAY-100635 took place in 2 steps. At the start of the synthesis, we used the alternate mechanism of an acid chloride coupling (Choi et al., 2010) instead of the BOP catalyst as outlined by Saigal et al. (2006). The reaction of the 2-/3-/4-methyl-1-cyclohexanecarboxylic acid (**5**, **6**, **7**) in the presence of oxalyl chloride produced the corresponding substituted cyclohexylcarbonyl chlorides which were coupled with WAY-100364 to give a modest yield of products in the range of 17-34%. The yield provided by the coupling of the acid chlorides was modest and not necessarily better than the previously reported BOP coupling method (Saigal et al., 2006). However, the reactions from the acid chloride pathway led to fewer impurities.

Synthesis of 3-Mefway and 4-Mefway took place in 5-steps. Using acid chlorides of carboxymethyl esters (**12**) as shown in Fig-5, the respective carboxamides (**13**) were produced in modest yields (approx. 30%). Reduction of the esters (**13**) proceeded with the use of LiAlH_4 resulting in a very modest yields of the alcohols (10%-20%, **14**). The alcohols (**14**) were treated with DAST, resulting in the formation of 3- and 4-Mefway (**9a**, **10a**) in yields of approx. 40%.

Binding Affinity

2-Methyl, 3-methyl, 4-methyl-WAY-100635 and WAY-100635 exhibited an IC_{50} of 10.4 nM, 77 nM, 21.5 nM, and 3.97 nM, respectively (Figure-5). As shown in Table-1, relative affinities of 2-methyl, 3-methyl and 4-methyl were all lower than the affinity of WAY with affinities of 2-methyl and 4-methyl being better and 3-methyl having the lowest affinity (19-fold weaker). The decrease in affinity appears to be regioselective with the order being 2-Me < 4-Me < 3-Me. Thus, it appears that in vitro binding assays show that a methyl group on the cyclohexyl ring of WAY-100635 decreases binding affinity. It must be noted however, that the methyl derivatives were a mixture of *cis/trans* isomers. Our previous work has shown differences in the binding profile of *cis*- versus the *trans*-isomer for ^{18}F -Mefway (Wooten et al., 2011b). Thus it is possible that the affinities of the pure isomers may be different and provide more accurate information.

In the case of the fluorinated derivatives, 3-Mefway had an IC_{50} of 17.4 nM (about 4.4 times weaker than WAY-100635) while 4-Mefway had an IC_{50} of 6.26 nM (about 1.6 times weaker than WAY-100635) (Figure-6). The ratio between the two derivatives is approximately similar to the results found for the 3-methyl and 4-methyl derivatives. The presence of the fluorine on the methyl group increases the binding affinity of the compounds (by approximately 3-fold). The *trans*-isomer of 4-Mefway was found to be the most potent amongst the compounds studied in this paper, with an IC_{50} of 3.11 nM. This high affinity is confirmed by PET studies showing the distinct binding pattern of 4- ^{18}F -Mefway in serotonin 5-HT_{1A} receptor-rich areas in the brain of rats and monkeys (Saigal et al., 2013; Wooten et al., 2011a). Hippocampus (dorsal and ventral) and entorhinal cortex were the high binding regions followed by the cortex. Striatum and cerebellum showed little binding consistent with the lack of receptors in these brain regions. Preliminary comparative in vivo studies of 3- ^{18}F -Mefway and 4- ^{18}F -Mefway have been carried out in monkeys. The lower binding seen in the brain regions in PET studies using 3- ^{18}F -Mefway confirms the lower in

vitro binding affinity of 3-Mefway compared to 4-Mefway for the serotonin 5HT_{1A} receptors (Wooten et al., 2014).

Several recent efforts have been made to study effects of replacing the cyclohexyl group in WAY-100635 with other cyclic rings. One study examined the effects of bridgehead fluoromethyl group (cubyl, adamantly and others) with some success (Al Hussainy et al., 2011). RWAY- which contains a cycloheptyl has been studied with ¹¹C-RWAY, although human studies have indicated interference by a lipophilic metabolite (Zhang et al., 2007). More recently, a rather large derivative has been shown to have some promising properties in vivo. Among the various fluorine-18 derivatives reported thus far, it appears that ¹⁸F-Mefway has comparable properties to ¹¹C-WAY-100635, in rodents and monkeys (Saigal et al., 2013). Studies on demethylated ¹¹C-WAY-100635 (¹¹C-DWAY-100635) have shown higher brain uptake in humans (Pike et al., 1998); however, despite this higher uptake of ¹¹C-DWAY-100635, not many studies have reported using it. It may be anticipated that demethylated ¹⁸F-Mefway may mimic the increased brain uptake in humans.

In conclusion, amongst the three methylated derivatives investigated in this study, the 3-methyl has the weakest affinity for PET studies. Thus, the 2- and 4-methyl derivatives are more optimal. Our ongoing PET imaging studies with 4-¹⁸F-Mefway in different species confirms this suitability (Bajwa et al., 2014).

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Figure 1.
Chemical Structures of WAY-100635 (**1**), 4-methyl-WAY-100635 (**2**), 3-methyl-WAY-100635 (**3**) and 2-methyl-WAY-100635 (**4**).

Figure 2.

Synthesis scheme of methyl-WAY-100635. Starting with the conversion of the carboxylic acids (5) to the corresponding cyclohexanoyl chlorides (6), the amine WAY-100634 (7) was converted to the corresponding carboxamides (8).

Figure 3.
Chemical Structures of 4-Mefway (**9**), 3-Mefway (**10**), 4- ^{18}F -Mefway (**9a**) and 3- ^{18}F -Mefway (**10a**).

Figure 4.

Synthesis scheme of Mefway. Starting with the conversion of the carboxylic acids (**11**) to the corresponding cyclohexanoyl chlorides (**12**), the amine WAY-100634 (**7**) was converted to the corresponding carboxamides (**13**). Reduction of the carboxylic acid esters with lithium aluminum hydride (LiAlH_4) gave the corresponding alcohols (**14**) which were subsequently fluorinated with DAST to provide 4-Mefway (**9**) and 3-Mefway (**10**).

Figure 5. Binding affinity curves of methyl-WAY-100635 derivatives. Competition experiments on rat brain cerebrum labeled with ^3H -WAY 100635 showing higher affinity of WAY-100635 > 2-methyl-WAY-100635 > 4-methyl-WAY-100635 > 3-methyl-WAY-100635.

Figure 6. Binding affinity curves of Mefway. Competition experiments on rat brain cerebrum labeled with ³H-WAY 100635 showing higher affinity of 4-Mefway compared to 3-Mefway.

Table 1
Binding Affinities for Serotonin 5HT_{1A} Receptor

Serial Number	Compound	IC ₅₀ (nM) ^a	Relative Affinity ^b
1	WAY-100635	3.97	1
2	2-Methyl (<i>cis/trans</i>)	10.4	2.62
3	3-Methyl (<i>cis/trans</i>)	77	19.4
4	4-Methyl (<i>cis/trans</i>)	21.5	5.42
5	3-Mefway (<i>cis/trans</i>)	17.4	4.38
6	4-Mefway (<i>cis/trans</i>)	6.26	1.58
7	4-Mefway (<i>trans</i>)	3.11	0.78

^a Affinity measures from competition assay in rat brain homogenates labeled with ³H-WAY-100635 (1nM).

^b Relative affinity compared to WAY-100635.