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Endoxifen is a new potent inhibitor of PKC: A potential therapeutic agent for bipolar disorder

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ABSTRACT

Protein kinase C (PKC) plays a major role in regulation of both pre and postsynaptic neurotransmission. Excessive activation of PKC results in symptoms related to bipolar disorder. Tamoxifen, a widely used breast cancer drug is known to inhibit PKC and demonstrate antimanic properties in human. We describe herein the synthesis of endoxifen, a tamoxifen active metabolite and compared its PKC inhibitory activity with that of tamoxifen. Endoxifen exhibited fourfold higher potency compared to tamoxifen.

Bipolar disorder (BPD) is a chronic debilitating illness characterized by drastic swings in mood, energy and functional ability, that affects adult population.[1] Until recently, not much was known about the molecular and cellular mechanisms of BPD. Recent data suggests that mania is associated with overactive protein kinase C (PKC) intracellular signaling which plays a major role in regulation of both pre and postsynaptic neurotransmission.[2] Interestingly, the current first line therapy of mania, lithium and valproate indirectly inhibit PKC.[3] In addition, recent preclinical and clinical studies with the relatively selective PKC inhibitor *Z*-tamoxifen add support to the relevance of the PKC target in BPD. Animal studies demonstrate that tamoxifen significantly reduced manic behavior in rodents.[4] Preliminary investigations of PKC inhibitor tamoxifen in the treatment of acute mania showed anti-manic effects compared to placebo.[5] Recently, two groups confirmed antimanic properties of tamoxifen in double-blind, placebo controlled clinical studies.[6] Thus, a growing body of work in both preclinical and clinical indicates that PKC signalling may play an important factor in the pathophysiology and treatment of BPD. The development of CNS-penetrant PKC inhibitors such as tamoxifen may have considerable benefit for this devastating illness.[7] However, there is a large inter-individual and ethnic variability in tamoxifen bioavailability and function due to CYP2D6 genetic polymorphism which extensively metabolizes tamoxifen into active metabolites, 4-hydroxy tamoxifen and 4-hydroxy-*N*-desmethyl tamoxifen (endoxifen).[8] Alternative PKC inhibitors that are independent of CYP2D6 action such as endoxifen may provide better opportunity for the treatment of BPD and other diseases. Here we have synthesized endoxifen (Fig. 1) and showed its PKC inhibitory activity in vitro for the first time. We have also compared its PKC activity with known PKC inhibitor tamoxifen.

Previously reported endoxifen synthesis involved demethylation of 4-hydroxytamoxifen.[9] However, there are no reports for its synthesis starting from commercially available starting materials. Here, we report the synthesis of endoxifen starting from commercially available 4-bromophenol, α-phenyl butyric acid and 2-chloroethoxybenzene. The synthetic scheme is depicted in Scheme 1. 1-[4-(2-Chlororethoxy)phenyl]-2-phenyl-butan-1-one **3** was synthesized by refluxing solution of 2-phenylbutyric acid **2** and thionyl chloride in 1,2-dichloroethane (EDC) for an hour before it was cooled to room temperature and added drop wise to a stirring solution of 2-chloroethoxybenzene **1** and anhydrous aluminum chloride in EDC at 0 °C. After the completion of reaction the workup was done by pouring the reaction solution in HCl/H_2O solution (1:1) and extracted with EDC. The crude product was purified by flash chromatography (hexane/ethyl acetate) yielding **3** in 88% yield. Alternatively, **3** was also synthesized in 96% yield by

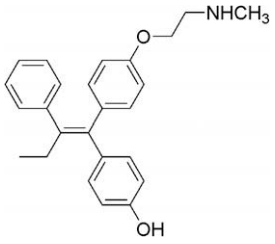

Figure 1. Structure of endoxifen.

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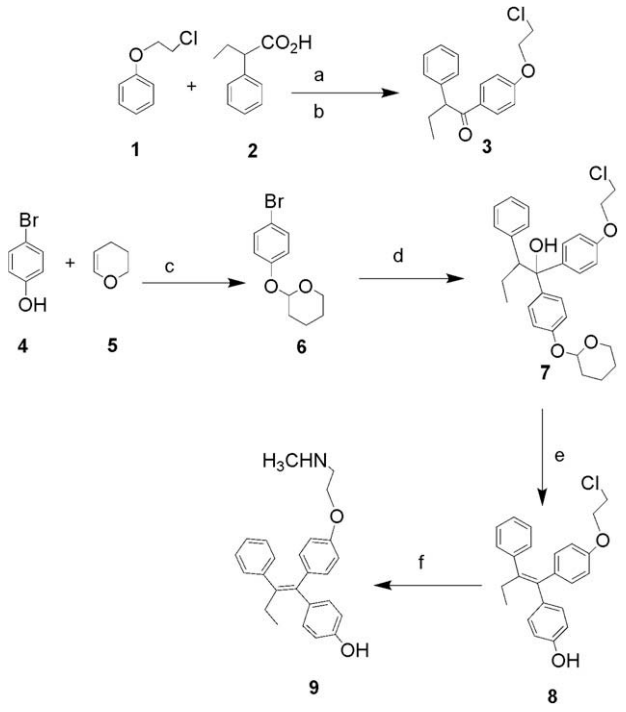

Scheme 1. Reagents and conditions: (a) method; (i) **2**, $SOCl_2$, EDC; **3**, $AlCl_3$, EDC; (b) method; (ii) TFAA, rt; (c) concd H_2SO_4, rt; (d) (i) **6**, Mg, THF; reflux→rt; (ii) add **3** in THF, reflux; (e) concd HCl, MeOH, reflux; (f) methylamine, 2-propanol, reflux.

mixing **1**, **2**, and trifluoroacetic anhydride (TFAA) and stirring for 10 h. The reaction solution was neutralized with saturated sodium bicarbonate solution before it was extracted with ethyl acetate and concentrated. The crude product after crystallization in hexane at 0 °C provided **3** in 96% yield. The tetrahydopyran (THP) protecting group was introduced into 4-bromophenol **4** by stirring with 3 M equiv of 3,4-dihydro-2*H*-pyran **5** and catalytic amount of concentrated sulfuric acid at 0 °C under nitrogen for 30 min. The reaction solution was diluted with hexane–water. After workup, the product was isolated in 91% yield by crystallization in small amount of cold hexane. Compounds **3** and **6** were reacted under Grignard's reaction conditions (Mg/THF) to provide an intermediate **7** which was heated under acidic condition (HCl and methanol) to undergo simultaneously dehydration and deprotection reaction. After conventional workup, the crude product was purified on silica gel column and eluted with dichloromethane–hexane mixture (1:1) to afford 1-[4-(2-chloroethoxy)phenyl-(4-hydroxyphenyl)-2-phenyl but-1-ene] **8** as *E*- and *Z*-geometric isomers in 86.5% yield. Compound **8** was then transformed into compound **9** by treatment with 40% methylamine (aqueous) in isopropyl alcohol and refluxed for 48 h. The solvent was removed and the residue was extracted with ethyl acetate and washed with 5% aq sodium bicarbonate solution followed by water and brine solution.

After drying over anhydrous sodium sulfate, the organic extract was concentrated in vacuo and subsequently triturated with ethyl acetate/hexane (1:1) to provide a mixture (*E:Z*, 5.8:4.2, HPLC) of geometric *E*- and *Z*-isomers of endoxifen in 52% yield. *Z*-Endoxifen was isolated in pure form by fractional crystallization in benzene. The product and the intermediates were characterized[10] by ^{1}H NMR, ^{13}C NMR, high resolution Mass spectrometry. The *Z*-configuration was assigned based on the characteristic chemical shift[9b,11] of OCH_2 in ^{1}H NMR. In all tamoxifen derivatives studies, the *Z*-isomers (*trans*), the OCH_2 protons of the side chain have a chemical shift (δ) less than 4.00 ppm owing to the through-space shielding influence of the vicinal 2-phenyl substituents. Based on this, the isomer having the triplet at δ 3.86 was assigned as *Z*-isomer and the isomer with δ 4.03 was identified as *E*-isomer.

Effect of synthesized endoxifen on PKC activity was evaluated in vitro and compared with that of tamoxifen using a PKC kinase activity ELISA assay kit (Assay Designs, Ann Arbor, MI) which contained a PKC Substrate Microtiter Plate, Active PKC, ATP, Phosphospecific Substrate Antibody, Anti-Rabbit IgG-HRP conjugate, TMB substrate, Stop solution, Wash and Dilution Buffer. The assay was carried out according to manufacturer's instructions. Briefly, 50 μl of a reaction mix containing Kinase Assay Dilution Buffer, 0.025, 0.05, 0.1, or 0.2 mM endoxifen and 10 ng PKC was added to each well of a presoaked PKC Substrate Microtiter Plate. To compare PKC inhibition activity with tamoxifen, 50 μl of the Dilution Buffer reaction mix containing 0.025, 0.05, 0.1, or 0.2 mM tamoxifen (Toronto Research Chemicals, North York, ON, Canada) and 10 ng PKC was added to another set of wells. Fifty microliter of Kinase Assay Dilution Buffer alone or with 10 ng PKC was used as negative and positive controls, respectively. The reaction was initiated by adding 10 μl of diluted ATP to each well. Following incubation at 30 °C for up to 90 min, the reaction was stopped by emptying contents of the plate. Forty microliters of Phosphospecific Substrate Antibody was added to each well followed by 60 min incubation at room temperature. All wells were washed four times with Wash Buffer and 40 μl of diluted Anti-Rabbit IgG: HRP Conjugate was added. After 30 min incubation at room temperature, wells were again washed and 60 μl of TMB Substrate was added. Following further incubation at room temperature for 30 min, 20 μl of Stop Solution was added and absorbance was read at 450 nm in a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA). The results were analyzed using SoftMax® Pro 5 software.

Endoxifen inhibited PKC activity in concentration dependent manner. The percentage PKC inhibition ranged between 12% and 78% with endoxifen concentration between 0.025 and 0.2 mM, respectively. In comparison, tamoxifen was found less potent PKC inhibitor at 0.1 and 0.2 mM resulting 14% and 25% PKC inhibition, respectively; lower concentrations of tamoxifen (0.025 and 0.05 mM) showed negligible PKC inhibition. Figure 2 shows endoxifen and tamoxifen induced PKC inhibition at 0.2 mM. The study demonstrated that endoxifen is about fourfold more potent PKC inhibitor than tamoxifen, and suggests its pivotal role in BPD.

In summary, we have synthesized endoxifen that inhibited PKC significantly in comparison with tamoxifen. Preclinical and clinical evidence demonstrate that PKC is an important target-perhaps the first mechanistically distinct drug target for BPD and tamoxifen is a relatively selective PKC inhibitor. This is the first report demonstrating endoxifen to be potentially a superior candidate for BPD treatment especially for those with defects in activating enzymes

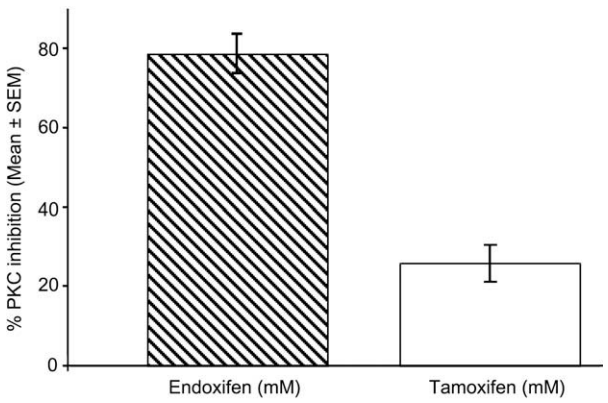

Figure 2. Percentage inhibition of PKC activity by 0.2 mM endoxifen and tamoxifen in vitro.

for tamoxifen metabolism. Repeated daily oral administration of endoxifen up to 4 mg/kg and 8 mg/kg body weight in rats and mice, respectively, for 28 days showed no signs of toxicity (unpublished observation). Efficacy studies with endoxifen will be the subject of further investigation.

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10. *Compound* **6**: mp 56–57 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.68 (m, 3H), 1.83 (m, 2H), 1.98 (m, 1H), 3.57 (m, 1H), 3.85 (m, 1H), 5.36 (t, J = 3.2 Hz, 1H), 6.93 (AB, J = 8.79, J = 2.14, 2H), 7.35 (AB, J = 8.79, J = 2.14 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.6, 25.1, 30.2, 61.9, 96.5, 113.8, 118.3, 132.2, 156.2; HRMS (ESI), expected 278.991, observed 278.9984 ($M+Na^+$). *Compound* **3**: mp 68–70 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (t, J = 7.5 Hz, 3H), 1.85 (m, 1H), 2.18 (m, 1H), 3.78 (t, J = 5.9 Hz, 2H), 4.22 (t, J = 5.9 Hz, 2H,), 4.38 (t, J = 7.26 Hz, 1H), 6.86 (AB, J = 9 Hz, J = 2.14 Hz, 2H), 7.18 (m, 1H), 7.28 (m, 4H), 7.96 (AB, J = 8.78 Hz, J = 1.93 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.3, 27.1, 41.5, 55.1, 67.9, 114.2, 126.8, 128.2, 128.8, 130.6, 130.9, 139.9, 161.7, 198.5. HRMS (ESI), expected 325.0966, observed 325.0976 ($M+Na^+$). *Compound* **9** (***Z*-endoxifen**): mp 139–143 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (t, J = 7.24 Hz, 3H), 2.29 (s, 3H), 2.42 (q, J = 7.2 Hz, 2 H), 2.74 (t, J = 5.59 Hz, 3H), 3.86 (t, J = 5.56 Hz, 2H), 6.58 (d, J = 8.78 Hz, 2H), 6.71 (d, J = 8.56 Hz, 2H), 6.75 (d, J = 8.68 Hz, 2H), 6.98 (d, J = 8.2 Hz, 2H), 7.08–7.13 (m, 3H), 7.15–7.19 (m, 2H), 9.38 (br, 1H); HRMS (ESI), expected 374.2115, observed 374.2116 ($M+H^+$).
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