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TAMOXIFEN PROMOTES DIFFERENTIATION OF OLIGODENDROCYTE PROGENITORS *IN VITRO*

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Abstract—The most promising therapeutic approach to finding the cure for devastating demyelinating conditions is the identification of clinically safe pharmacological agents that can promote differentiation of endogenous oligodendrocyte precursor cells (OPCs). Here we show that the breast cancer medication tamoxifen (TMX), with well-documented clinical safety and confirmed beneficial effects in various models of demyelinating conditions, stimulates differentiation of rat glial progenitors to mature oligodendrocytes *in vitro*. Clinically applicable doses of TMX significantly increased both the number of CNPase-positive oligodendrocytes and protein levels of myelin basic protein, measured with Western blots. Furthermore, we also found that OPC differentiation was stimulated, not only by the pro-drug TMX-citrate (TMXC), but also by two main TMX metabolites, 4-hydroxy-TMX and endoxifen. Differentiating effects of TMXC and its metabolites were completely abolished in the presence of estrogen receptor (ER) antagonist, ICI182780. In contrast to TMXC and 4-hydroxy-TMX, endoxifen also induced astrogliogenesis, but independent of the ER activation. In sum, we showed that the TMX prodrug and its two main metabolites (4-hydroxy-TMX and endoxifen) promote ER-dependent oligodendrogenesis *in vitro*, not reported before. Given that differentiating effects of TMX were achieved with clinically safe doses, TMX is likely one of the most promising FDA-approved drugs for the possible treatment of demyelinating diseases. Published by Elsevier Ltd. on behalf of IBRO.

Key words: tamoxifen, endoxifen, 4-hydroxy-tamoxifen, oligodendrocyte progenitors, estrogen receptors, astrocytes.

INTRODUCTION

A large number of people suffer from demyelinating conditions, but an effective therapy is still lacking. The central nervous system (CNS) responds to demyelinating insults by inducing proliferation and maturation of oligodendrocytes from dividing oligodendrocyte progenitor cells (OPCs; Fancy et al., 2011). Those progenitors can migrate to demyelinated axons, differentiate to mature oligodendrocytes and remyelinate axons, thus enabling functional recovery. The endogenous OPC population, which comprises up to 8% of the total glial cells in the CNS represents a promising source for the regeneration of oligodendrocytes after demyelinating insults (Franklin and Ffrench-Constant, 2008). However, differentiation of OPCs in demyelinating lesions usually fails, largely due to the failure of the precursor cells to proliferate and differentiate (Wolswijk, 2002; Rosenzweig and Carmichael, 2015). Therefore, the development of therapeutic interventions that can promote the proliferation and differentiation of OPCs is one of the main goals in identifying treatments for demyelinating conditions (Huang et al., 2011).

Estrogen facilitates differentiation of OPCs *in vitro* (Okada et al., 2011), consistent with well-established remyelinating effects of estrogens in multiple sclerosis (Dwosh et al., 2003; Crawford et al., 2010). Despite their neuroprotective effects in various animal models of demyelinating conditions, estrogens have limited therapeutic potential due to their serious systemic adverse effects, such as peripheral feminizing in men, or dangerous pro-proliferative effects in reproductive organs of women (Shao et al., 2012).

The cancer drug tamoxifen (TMX) is a selective estrogen receptor (ER) modulator that can mimic the neuroprotective effects of estrogens in the CNS without the adverse systemic effects. TMX inhibits ERs in the breast tissue, and thus remains the most widely used drug by patients with breast cancer and (ER)-positive tumors (Hoskins et al., 2009). Tamoxifen taken orally metabolizes into two main active compounds, 4-hydroxytamoxifen and endoxifen (*N*-desmethyl-4-hydroxytamoxifen). These metabolites exhibit a 100-fold higher binding affinity to the ER and are more effective in suppressing cancer cell proliferation than TMX (Johnson et al., 2004; Lim et al., 2005). In humans, the conversion from TMX to endoxifen is predominant, and the circulating concentrations of the pro-drug TMX and endoxifen are considerably higher than those of 4-hydroxytamoxifen (Ahmad et al., 2010). However, both

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Abbreviations: 4-OHTMX, 4-hydroxytamoxifen; BBB, blood brain barrier; CNS, central nervous system; DM, differentiation medium; ER, estrogen receptor; EtOH, ethyl alcohol; OPC, oligodendrocyte progenitor cells; PM, proliferation medium; TMX, tamoxifen; TMXC, tamoxifen citrate.

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TMX and its metabolites readily cross the blood–brain barrier (BBB; Zarate et al., 2007; Iusuf et al., 2011), and thus can all significantly affect CNS processes. Although the neuroprotective effects of TMX have been established, the effect of TMX or its metabolites on the differentiation of OPCs has not been studied.

EXPERIMENTAL PROCEDURES

Oligodendrocyte progenitor cells

Purified rat glial restricted precursor cells were purchased from *MTI-Global Stem* (GSC-8040) and plated (3×10^4 cells/cm^2) into poly-L-Ornithine-coated T75 dishes. Cells were kept in proliferation medium (**PM**) for 2 days: *Neural-X*® serum-free NSC medium with growth factors; PDGF, EGF, and FGF, 2% GS22 Supplement (*Global Stem*), 1% 100x Glutamax-I Supplement. Cells were split into laminin and poly-L-ornithine coated plates and treated with differentiation medium (**DM**): *Neural-Q*® Basal medium, containing 1% Fetal Bovine Serum and appropriate treatment for 5 days with or without the following treatments.

Treatments.

(1) TMX citrate (TMXC pro-drug; *Clayman Chemicals*),
(2) 4-hydroxytamoxifen (4-OHTMX; *Sigma*), or
(3) endoxifen (*Sigma*).

These three TMX forms were tested because 4-OHTMX and endoxifen are the main TMX metabolites, and because 4-OHTMX, endoxifen, and TMX citrate are not only present in high levels in the circulation, but they all cross the blood–brain barrier (Iusuf et al., 2011). In addition to their clinical relevance, 4-OHTMX and TMXC are also extensively used in conditional mutagenesis experiments (Jokela and Vainio, 2007).

We tested 3 concentrations of each TMX form: 4 μM, 0.8 μM, 0.4 μM. Those concentrations were chosen as clinically relevant, because they closely correspond to the brain concentration (~1 μM) of TMX (and its metabolites) in breast cancer patients taking TMX orally (Lien et al., 1991; Iusuf et al., 2011).

(4) As a positive control, we used 1 μM triiodothyronine (T3, *Sigma*) a known inducer of OPC differentiation (Billon et al., 2001). T3 concentration was based on Deshmukh et al. (2013).
(5) ER antagonist, ICI182780 (*Santa Cruz Biotechnology*), was used as a potent inhibitor of ERα and ERβ (Xiao et al., 2012; Karki et al., 2013) at 1 μM, either alone or in combination with TMXC or TMX metabolites. The concentration of ICI18278 was based on Xiao et al. (2012).

Controls.

(a) cells exposed for 2d to the PM, or
(b) cells exposed for 5d to the DM

Since TMXC, 4-OHTMX, endoxifen and ICI182780 were dissolved in sterile 100% ethanol (EtOH), control groups contained the corresponding concentration of EtOH that was added to the DM:

(c) 1, 0.2 or 0.1 μL/mL EtOH in controls for three different concentrations of TMXC, 4-OHTMX or endoxifen (4, 0.8 and 0.4 μM)
(d) 0.5 μL/mL EtOH in controls for ICI182780 alone
(e) 0.6 μL/mL EtOH in controls for ICI182780 + TMX (TMXC, 4-OHTMX, or endoxifen; 0.4 μM).

Western blot analysis

Whole-cell lysates were harvested after 5 days of treatment. Isolated proteins (20–80 μg) were boiled at 90 °C with SDS–Page buffer and 0.5% beta-mercaptoethanol. Western blot method is described in Guptarak et al. (2014). Antibodies used: mouse anti-CNPase (*Abcam*; 1:300); mouse anti-MBP (*Abcam*; 1:100); rabbit anti-estrogen receptor α (*Abcam*; 1:300); rabbit anti-estrogen receptor β (*Abcam*; 1:300); rabbit anti-GFAP (*Invitrogen*; 1:500); mouse anti-β-actin (*Sigma*; 1:5000) and mouse anti-NeuN (*Millipore*; 1:100). NeuN, a neuronal marker, was not detected in Western blots or by immunolabeling, suggesting the absence of neurogenesis in cells exposed to control or other treatments.

Final detection was made by incubating membranes with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Goat IgG; *BioRad*; 1:3000) with Luminol/H_2O_2. Western blot images were captured and analyzed with ChemiDoc + XRS chemiluminescent imaging system (*BioRad*) using *Quantity-One* software. Protein levels of β-actin were used as a loading control in all Western blots.

Immunofluorescent labeling

Cells were fixed with 4% paraformaldehyde (*Electron Microscopy Sciences*) and blocked with 2% Bovine Serum Albumin, 5% Normal Donkey Serum, and 1× Dulbecco's Phosphate-Buffered Saline solution containing Ca^{2+} and Mg^{2+}. Antibodies used: mouse anti-A2B5 (*Invitrogen*; 1:500); rabbit anti-NG2 (*Millipore*; 1:200); mouse anti-CNPase (*Abcam*; 1:500); rabbit anti-estrogen receptor α (*Abcam*; 1:500); rabbit anti-estrogen receptor β (*Abcam*; 1:500). Secondary antibodies (goat; *Invitrogen*; 1:1000) were diluted in blocking solution. To label nuclei, we used 4′,6-diamidino-2-phenylindole (DAPI; 1:1000). Images were captured the *FSX-BFX* image capturing software.

Oligodendrocyte quantification

Cells were stained with antibody that recognizes 2′,3′-cyclic-nucleotide,3′-phosphodiesterase (CNPase), a myelin-associated enzyme expressed in pre-oligodendrocytes or mature oligodendrocytes (Girolamo et al., 2010). Morphological determination of mature oligodendrocytes was based on the method reported by Sperber and

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