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Antidepressants are cytotoxic to rat primary blood brain barrier endothelial cells at high therapeutic concentrations

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ABSTRACT

Antidepressants are commonly employed for the treatment of major depressive disorders and other psychiatric conditions. We investigated the relatively acute cytotoxic effects of three commonly prescribed antidepressants: fluoxetine, sertraline, and clomipramine on rat primary blood brain barrier endothelial cells over a concentration range of 0.1–100 μM. At therapeutic concentrations (0.1 μM) no significant cytotoxicity was observed after 4, 24, or 48 h. At high therapeutic to overdose concentrations (1–100 μM), antidepressants reduced cell viability in proportion to their concentration and exposure duration. At 1 μM, antidepressants significantly reduced mitochondrial membrane potential. At drug concentrations producing ~50% inhibition of cell viability, all drugs significantly reduced cellular oxygen consumption rates, activities of mitochondrial complexes I and III, and triggered a significant increase of lactate production. Fluoxetine (6.5 μM) and clomipramine (5.5 μM) also significantly lowered transcellular transport of albumin. The mechanism of cellular cytotoxicity was evaluated and at high concentrations all drugs significantly increased the production of reactive oxygen species, and significantly increased the activity of the pro-apoptotic caspases-3, 8, and 9. Comet assays revealed that all drugs were genotoxic. Pre-incubation of cells with glutathione significantly ameliorated antidepressant-induced cytotoxicity, indicating the potential benefit of treatment of overdosed patients with antioxidants.

1. Introduction

Major depressive disorder (MDD) is a multifactorial disease affecting an estimated 350 million people worldwide and across all ages (WHO, 2016). Depression is the leading cause of disability and a major contribution to the global burden of disease (Smith, 2014; WHO, 2016). Antidepressants are first-line therapeutic drugs used to treat MDDs, and other conditions including dysthymia, anxiety disorders, obsessive-compulsive disorder (OCD), and also to combat neuropathic pain and migraines. Antidepressants can be loosely categorised into tricyclic antidepressants (TCAs); cyclic antidepressants; mixed action agents; selective serotonin reuptake inhibitors (SSRIs); serotonin-norepinephrine reuptake inhibitors; monoamine oxidase inhibitors; and alternative (non-traditional) antidepressants (Ciraulo et al., 2004).

Many factors influence antidepressant drug selection including the potential of pharmaco-toxicity. Patient trialling of an antidepressant drug may require an adequate dose of drug for several weeks to prove efficacious, and pharmacotherapy then continued for ~6–12 months or indeed indefinitely for recurrently depressed patients. This raises concerns of both acute and chronic drug-induced cytotoxicity.

Over the last two decades there has been upward trend in prescribing of antidepressants in the USA and UK (Mojtabai and Olfson, 2014; Mars et al., 2017); which for the UK was driven by an increase in prescribing SSRIs such as Fluoxetine (FLX) and Sertraline (SER). FLX is

Abbreviations: AB, Alamar Blue; ANOVA, Analysis of variance; BBB, Blood brain barrier; CLM, Clomipramine; FCCP, Carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone; DCFDA, 3,7-Dichlorodihydrofluorescein diacetate; FLX, Fluoxetine; GSH, Glutathione; IC_{50}, Inhibitor concentration producing 50% inhibition; MC-I, Mitochondrial complex I; MC-III, Mitochondrial complex-III; MDD, Major depressive disorders; MMP, Mitochondrial membrane potential; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MW, Molecular weight; OCD, Obsessive-compulsive disorder; OCRs, Oxygen consumption rates; rCMECs, rat cerebral microvascular endothelial cells; Rh-123, Rhodamine-123; ROS, Reactive oxygen species; SER, Sertraline; SSRIs, Selective serotonin reuptake inhibitors; TCAs, Tricyclic antidepressants

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now the first choice antidepressant used to treat children and adolescents as a monotherapy of unipolar depression, and is approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMEA) (Masi et al., 2010; Taurines et al., 2011). SSRIs are thought to relieve depression symptoms by a number of mechanisms that include increasing serotonin (5-hydroxytryptamine) levels in the brain. SSRIs inhibit serotonin reuptake transporters at presynaptic terminals and thereby sustain levels of serotonergic neurotransmitter levels at postsynaptic clefts (Benfield et al., 1986; Murdoch and McTavish, 1992; MacQueen et al., 2001; Ciraulo et al., 2004; Perez-Caballero et al., 2014).

In contrast to SSRIs, the number of prescriptions of TCAs in the UK, such as Clomipramine (CLM), has remained stable over the same time course (Mars et al., 2017). TCAs mainly exert their effects via inhibition of presynaptic neural reuptake of serotonin and nor-epinephrine (McTavish and Benfield, 1990; Ciraulo et al., 2004; Gillman, 2007). Although prescribing CLM is associated with a greater benefit compared to placebo than that seen for SSRIs for treating paediatric OCD, it is not recommended for first-line use because of its adverse effects and limited tolerability (Varigonda et al., 2016).

To exert their cellular effects, antidepressants need to cross the blood brain barrier (BBB). The BBB is comprised of endothelial cells that are associated together by tight and adherens junctions. This interlinking of endothelial cells with little fenestration limits the ability of molecules to cross them from the blood to enter brain tissue. The basement membrane of the endothelial cells (and resident pericytes) is also in close association with foot processes of brain glial cells such as astrocytes. Hence the BBB provides a protective cell layer that separates the CNS from circulatory blood and is able to regulate the uptake of endogenous molecules and xenobiotics into the brain (Schinkel, 1999; Abbott et al., 2010; O'Brien et al., 2012). Certain small lipophilic molecules (< 400 Da in size) are able to cross the BBB by passive diffusion, whereas other (larger) molecules and certain drugs including antidepressants, may be uni-directionally transported from the brain across the BBB to the blood driven by the consumption of ATP (Schinkel and Jonker, 2003; Horstmann and Binder, 2009; O'Brien et al., 2012). These luminal membrane energy-dependent efflux pumps include proteins of the multidrug resistance-associated (MDR) gene family such as P-glycoprotein (P-gp), and act to provide resistance to drug toxicity (Regina et al., 1998; Schinkel, 1999; Schinkel and Jonker, 2003; Horstmann and Binder, 2009; Abbott et al., 2010; O'Brien et al., 2012).

A number of established protocols exist for production of BBB cells that use isolated monocultures of endothelial cells of primary or tumorigenic origin, or mixed co-cultures that include glial cells such as astrocytes (Abbott et al., 1992; Perrière et al., 2005; Roux and Couraud, 2005; Weksler et al., 2005). The ability to culture cell lines continually provides convenience and initial reproducibility, but ultimately progressive genetic and phenotypic cell changes may arise and lead to dedifferentiation and selection of rapidly proliferating clones. Furthermore, lab-specific culturing conditions promote culture outgrowth. Human primary cells provide physiologically relevant cells, but ethical clearance and cost may be prohibitive to use. By contrast, although there are limitations associated with species specific responses, laboratory rats provide a useful source of primary cells to model drug effects on BBB endothelial cells.

Thus the ability to isolate and culture primary rat endothelial cells of the BBB provides an in vitro model able to evaluate local cytotoxicity that could impact upon BBB integrity and functionality. The objective of this study was to assess the cytotoxicity of two of the widely prescribed antidepressants from the SSRIs class, FLX and SER, and CLM from the TCAs class, to rat cerebral microvascular endothelial cells (rCMEC), and examine the molecular mechanism of any drug-induced cytotoxicity.

2. Materials and methods

2.1. Chemicals and reagents

Fluoxetine (*N*-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy] propan-1-amine) ($C_{17}H_{18}F_3NO$; MW = 309.13 g/mol), Sertraline (1*S*,4*S*)-4-(3,4-dichlorophenyl)-*N*-methyl-1,2,3,4-tetrahydronaphthalen-1-amine ($C_{17}H_{17}Cl_2N$; MW = 306.23 g/mol), and Clomipramine (3-(2-chloro-5,6-dihydrobenzo[*b*][1]benzazepin-11-yl)-*N*,*N*-dimethylpropan-1-amine) ($C_{19}H_{23}ClN_2$; MW = 314.85), and all other reagents were purchased from Sigma (St. Louis, MO, USA) unless detailed otherwise. For cell treatments, drugs were dissolved in DMSO and used at the final concentrations specified in Figures. Cell culture reagents were bought from Gibco BRL (Grand Island, NY, USA). Mitotracker green was purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA).

2.2. Animals

Male Albino rats (~250 g body weight) were used for this study. All experiments were approved by the Mansoura Faculty of Medicine Ethical Committee. Rats were sacrificed under sodium pentobarbital anaesthesia.

2.3. Isolation and characterisation of rat cerebral microvascular endothelial cells (rCMECs).

Isolation of rCMECs was based upon published methods (Abbott et al., 1992; Regina et al., 1998; Perrière et al., 2005; Weksler et al., 2005; Calabria et al., 2006; Nakagawa et al., 2009; Liu et al., 2014). After isolation, cells were purified using a 33% continuous Percoll gradient. Cells were washed and then plated on 35 mm collagen IV/fibronectin-coated plates (0.1 mg/ml). Isolated cells were maintained in Endothelial Cell Growth Medium (Sigma 211–500) supplemented with 4 μg/ml puromycin and 100 mg/ml heparin and maintained in 5% CO_2 at 37 °C. After 3 days, puromycin was removed from the media (Liu et al., 2014). Primary BBB endothelial cells were of the typical elongated spindle-like morphology (Abbott et al., 1992), and were essentially homogeneous when visualized by light microscopy (Supplementary Fig. S1), consistent with the ~99% purity estimates detailed by others (Perrière et al., 2005; Calabria et al., 2006). Under these culturing conditions, BBB endothelial cells display improved barrier properties in part mediated by increased expression of tight junction proteins (Calabria et al., 2006). Further purity confirmation was established via expression of the BBB endothelial cell transport proteins P-glycoprotein (P-gp) and breast cancer resistant protein (BCRP), and the puromycin-induced expression of the tight junction proteins Zonula occludens-1 (ZO-1) and Occludin by Western blotting. (Regina et al., 1998; Schinkel, 1999; Abbott et al., 1992; Schinkel and Jonker, 2003; Perrière et al., 2005; Weksler et al., 2005; Calabria et al., 2006; Horstmann and Binder, 2009; O'Brien et al., 2012).

Cell homogenates were resolved using a 4% acrylamide/bisacrylamide gel, proteins transferred to a nitrocellulose membrane, and then membranes washed and incubated in blocking buffer according to previous publications (Erdozain et al., 2014; Carter et al., 2015). Blocked membranes were incubated with primary antibodies: anti-P-gp monoclonal antibody (C219, Signet Laboratories, Dedham, MA, USA) at 1:200 dilution, anti-BCRP (Bxp-53, Enzo Life Sciences, Farmingdale, NY, USA) at 1:5000, anti-ZO-1 rabbit polyclonal (Invitrogen) at 1:1000, and anti-Occludin rabbit monoclonal (Abcam) at 1:10,000, overnight at 4 °C. Washed membranes were then incubated for 2 h at room temperature with the secondary antibodies (anti-mouse IgG-HRP (Dako Cytomatics, Glostrup, Denmark) at 1:1000 dilution or anti-rat IgG at 1:10,000 dilution (Protein Tech group, Chicago, USA), respectively) in blocking buffer. Membranes were washed and immunoreactive proteins visualized using enhanced chemiluminescence (ECL kit, Amersham,