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The synthetic cannabinoid XLR-11 induces *in vitro* nephrotoxicity by impairment of endocannabinoid-mediated regulation of mitochondrial function homeostasis and triggering of apoptosis

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ABSTRACT

Synthetic cannabinoids (SCBs)-related intoxications and deaths have been increasingly reported, turning its widespread recreational use into a major public health concern. Specifically, a direct link between SCBs and acute kidney injury (AKI) has been established. XLR-11 is an SCB commonly found in the toxicological analysis of patients with SCB-associated AKI. However, the pathophysiology of AKI among SCB consumers remains unknown. This work thus represents the first *in vitro* assessment of SCB nephrotoxicity, as a first approach to identify its cellular targets.

We demonstrate that XLR-11, at biologically relevant concentrations (in the nanomolar range), primarily targets mitochondrial function in human proximal tubule (HK-2) cells, inducing a transient hyperpolarization of the mitochondrial membrane and increasing ATP production, accompanied by Bax translocation from cytosol into mitochondria. These phenomena further triggered energy-dependent apoptotic cell death pathways, indicated by increased caspase-3 activity and chromatin condensation. Experiments using SR141716A and SR144258, specific antagonists for CB1 and CB2 receptors, respectively, as well as HEK293T cells (which do not express CBRs) highlighted these processes' dependence on CBR activation. Nevertheless, ATP formation seemed to follow a CBR-independent pathway. Our findings using specific inhibitors of endogenous cannabinoids biosynthesis (*i.e.* MAFP and THL) further evidenced the involvement of the endocannabinoid system in the regulation of these processes, as XLR-11 binding to CBRs seemed to compromise endocannabinoid-mediated preservation of mitochondrial function. Nevertheless, the exact mechanisms involved require further clarification.

1. Introduction

Synthetic cannabinoids (SCBs) are a heterogeneous group of substances chemically designed to bind and activate at least one cannabinoid receptor (CBR), thus mimicking the effects of Δ9-tetrahydrocannabinol (THC), the main psychoactive constituent of cannabis (Castaneto et al., 2014). SCBs were initially developed as research tools for the study of CBR pharmacology or as potential therapeutic agents. As full agonists of cannabinoid receptors, SCBs show higher potency and binding affinity than THC (partial agonist), resulting in stronger psychoactive results, as well as aggravated adverse effects (Castaneto et al., 2014; Tait et al., 2016; Znaleziona et al., 2015). Clandestine variations of SCBs have rapidly surfaced over the past few years, being marketed and used for recreational purposes. In fact, according to the latest European Drug Report, 169 new SCBs have been detected since 2008, 70% of which during the past 5 years (EMCDDA, 2017).

The scarce information available regarding SCBs toxicological profiles, along with several reports of intoxications and deaths, have turned SCB use into a major public health concern (EMCDDA, 2017; Buser et al., 2014). Reported symptoms following SCB consumption usually include anxiety, hallucinations, hypertension, low body temperature, irritability, seizures and tachycardia (Shanks et al., 2015). In particular, a series of clinical data reported acute kidney injury (AKI) among SCB users with no previous signs of renal disease (Bhanushali et al., 2013; Luciano and Perazella, 2014; Thornton et al., 2013). Such patients reported intense nausea, vomiting and abdominal pain, while clinical records showed elevated levels of serum creatinine and blood urea nitrogen. Acute tubular necrosis was also observed in some patients' biopsies (Bhanushali et al., 2013). This evidence led the Centre for Disease Prevention and Control (CDC, USA) to establish a direct link between SCB consumption and AKI (CDC, 2012). Nevertheless, the pathophysiology of SCB-induced AKI remains unknown.

XLR-11, also known as (1-(5-fluoropentyl)-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone or as 5F-UR-144, is a synthetic

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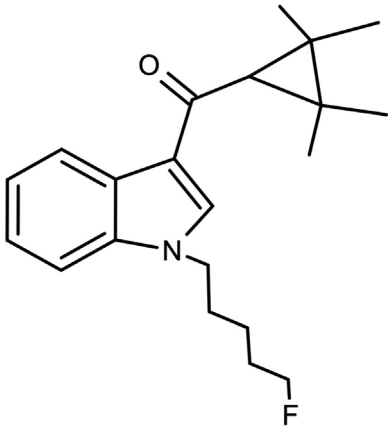

Fig 1. Chemical structure of XLR-11.

cannabinoid that has already been detected in several clinical specimens of patients with SCB-associated AKI (WHO, 2016). The chemical structure of XLR-11, represented in Fig. 1, shares close similarities with UR-144, one of many SCBs developed by Abbott Laboratories that showed high affinity towards the CB2 receptor (Frost et al., 2010). The fluoride group at the end of the pentyl side chain in XLR-11 increases its affinity to CB1 receptor (compared with the non-fluorinated analogue), while providing stability and extending the molecule's half-life (Banister et al., 2015). Wiley and co-workers (2013) have further confirmed the cannabimimetic effects of XLR-11, both *in vitro* and *in vivo*, showing that this molecule is a full agonist of both CB1 and CB2 receptors with Ki equal to 24.0 ± 4.6 nM and 2.1 ± 0.6 nM, respectively. XLR-11 is often found in herbal smoking blends worldwide, including in Europe (WHO, 2016), Japan (Uchiyama et al., 2013), and USA (NFLIS, 2013). Although XLR-11 has already been banned in a few countries, currently it is not legally controlled under the United Nations Drug Control Conventions, still making it readily available in several regions of the globe (WHO, 2016).

This work represents the first assessment of XLR-11's nephrotoxicity, as a first approach to identify its cellular targets and how they may account for this SCB-induced kidney damage. Different toxicological parameters were evaluated in a human proximal tubule cell line (HK-2), in which endogenous expression of cannabinoid receptors has already been described (Jenkin et al., 2010). Also, these cells are commonly used to study different processes related to renal toxicity, including mitochondrial function (Peraza et al., 2006). Noteworthy, the proximal tubule represents the primary target for processes like local interstitial drug accumulation, active clearance and reabsorption, assuming particular relevance for nephrotoxicity studies (Jang et al., 2013; Weber et al., 2016). Moreover, activation of CBRs in human proximal tubules has already been reported to play a crucial role in the pathogenesis of kidney disease (Jenkin et al., 2013; Lecru et al., 2015). In this regard, the potential involvement of CBRs in XLR-11-induced nephrotoxicity was ascertained in the presence of specific CB1 and CB2 antagonists and by analyzing XLR-11's effects on the HEK293T cell line, which does not express CBRs (Atwood et al., 2011). Inhibitors of endocannabinoids biosynthesis were also used to analyze their role on SCB-mediated nephrotoxicity.

2. Materials and methods

2.1. Chemicals

XLR-11 ((1-(5-fluoropentyl)-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone) was kindly supplied by Dr. Ana Santos Carvalho (Center for Neurosciences and Cell Biology, University of Coimbra, Portugal). Relative purity of XLR-11 samples was determined by High-Performance Liquid Chromatography coupled to Mass Spectrometry (HPLC–MS) to be 99.6%. However, it should be noted that this analytical method does not account for the presence of other smaller impurities (*e.g.* ions) and that it is possible that isomers of XLR-11 (regarding the position of the fluoride in the fluoropentyl chain) may be also present in the mixture.

SR141716A and SR144258, antagonists of CB1 and CB2 receptors, respectively, were obtained from Tocris Bioscience (Bristol, UK). Stock solutions of XLR-11 and CBR antagonists were prepared in dimethylsulfoxide (DMSO) and sequentially diluted in Hank's balanced salt solution (HBSS) prior to cell exposure in order to attain a final DMSO concentration below 0.5% DMSO, previously described as non-toxic to HK-2 cells (Adler et al., 2016). Heat-inactivated fetal bovine serum (FBS), 0.25% trypsin/EDTA, antibiotic (10 000 $U.ml^{-1}$ penicillin, 10 000 $\mu g.ml^{-1}$ streptomycin), Hank's balanced salt solution (HBSS) and phosphate buffered saline (PBS) were purchased from Gibco Laboratories (Lenexa, KS, USA). 2′,7′-dichlorodihydrofluorescein diacetate (DCFH2-DA) was obtained from Invitrogen (Eugene, OR, USA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA), unless stated otherwise.

2.2. Cell culture

The immortalized proximal tubule cell line from normal adult human kidney (HK-2) was obtained from the American Type Cell Collection (ATCC® CLR-2190™) and routinely cultured in 75 cm^2 flasks containing RPMI-1640 culture medium. Human embryonic kidney cells (HEK293T), kindly provided by Prof. Andreia Gomes (University of Minho, Braga, Portugal), were cultured in Dulbecco's Modified Eagle's Medium (DMEM). Both cell culture media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin, and were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Contrarily to HK-2, HEK293T cells do not express cannabinoid receptors mRNA (Atwood et al., 2011), thus representing a suitable complementary model to study CBR-mediated regulatory effects. Both cell lines were sub-cultured once they reached 70–80% confluence by trypsinization using a 0.25% trypsin/EDTA solution.

Cells were seeded at 3.5×10^4 cells/ml in 96-well plates for cell viability assays or in 100 mm diameter tissue culture dishes for protein expression analysis. For mitochondrial integrity and intracellular ROS/RNS experiments, cells were seeded at 8×10^4 cells/ml in 96-well plates. Densities of 1×10^5 and 3×10^5 cells/well, seeded in 24-well or 12-well plates, were used to assay ATP production and caspase-like activity, respectively. To assess chromatin condensation, cells were seeded at 1.5×10^4 cells/well in 96-well plates.

For cannabinoid receptor inhibition assays, HK-2 cells were incubated with 500 nM SR141716A or SR144258, antagonists for CB1 and CB2 receptors, respectively, for 20 min (37 °C, 5% CO_2) before adding XLR-11, according to a protocol previously described (Ross et al., 2000). Inhibition of endocannabinoid synthesis was attained by incubating HK-2 cells with 100 µM methyl arachydonyl fluorophosphonate (MAFP) and 1 µM tetrahydrolipstatin (THL) for 20 and 5 min (37 °C, 5% CO_2), respectively, prior to the addition of XLR-11 (Bisogno et al., 2006; Petersen and Hansen, 1999). MAFP and THL respectively inhibit N-acylphosphatidylethanolamine-PLD (NAPE-PLD) and diacylglycerol lipase (DGL), the enzymes responsible for the synthesis of anandamide and 2-arachidonoylglycerol.

2.3. Cell viability assays

2.3.1. MTT reduction assay

Cells' metabolic activity was measured with the MTT reduction assay, as previously described (Barbosa et al., 2014). This method is based on the reduction of the tetrazolium salt MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) to its insoluble formazan crystals in the presence of NAD(P)H-dependent cellular