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Mechanisms of tolvaptan-induced toxicity in HepG2 cells☆

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ABSTRACT

Tolvaptan, a vasopressin receptor 2 antagonist used to treat hyponatremia, has recently been reported to be associated with an increased risk of liver injury. In this study, we explored the underlying mechanisms of hepatotoxicity of tolvaptan using human HepG2 cells. Tolvaptan inhibited cell growth and caused cell death in a concentration- and time-dependent manner. Tolvaptan treatment led to delayed cell cycle progression, accompanied by decreased levels of several cyclins and cyclin-dependent kinases. Tolvaptan was found to cause DNA damage, as assessed by alkaline comet assays; this was confirmed by increased levels of 8-oxoguanine and phosphorylation of histone H2AX. Exposure of HepG2 cells to tolvaptan enhanced cytochrome C release and triggered apoptosis by modulating Bcl-2 family members. The activation of p38 contributed to tolvaptan-mediated apoptosis via down-regulation of Bcl-2. Proteasome inhibition altered tolvaptan-induced cell cycle deregulation and enhanced tolvaptan-induced apoptosis and cytotoxicity. Moreover, tolvaptan treatment induced autophagy. Inhibition of autophagy by knocking-down an autophagy-related gene increased tolvaptan-induced apoptosis and cytotoxicity. Taken together, our findings suggest that the cytotoxicity of tolvaptan results from delayed cell cycle progression, the induction of DNA damage, and the execution of apoptosis. In addition, a number of signaling pathways were perturbed by tolvaptan and played an important role in its cytotoxicity.

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1. Introduction

Tolvaptan (*N*-(4-{[(5*R*)-7-chloro-5-hydroxy-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl]carbonyl}-3-methylphenyl)-2-methylbenzamide, Fig. 1) is a selective vasopressin V_2-receptor antagonist that has been approved in U.S. for the treatment of clinically significant hypervolemic and euvolemic hyponatremia in patients with heart failure, cirrhosis, and the syndrome of inappropriate antidiuretic hormone secretion (SIADH) [1,2]. In Europe, tolvaptan is also approved for the treatment of patients with hyponatremia, secondary to SIADH.

In recent large clinical trial, which tested the use of tolvaptan in patients with autosomal dominant polycystic kidney disease, tolvaptan was reported to be associated with a high risk of liver injury [3]. Three cases of serious liver injury and an increased incidence of elevated serum alanine aminotransferase were observed. As a consequence, the U.S. FDA issued a warning that tolvaptan "should not be used for longer than 30 days and should not be used in patients with underlying liver diseases" (http://www.fda.gov/Drugs/DrugSafety/ucm350062.htm). However, the mechanisms underlying its potential hepatotoxicity are currently unknown.

In vitro studies have demonstrated that cytochrome P450 3A4 (CYP3A4) is the major CYP isozyme that metabolizes tolvaptan [4]. For example, co-administration of grapefruit juice [4], which interferes with CYP3A4 activity, or the CYP3A4 inhibitor ketoconazole [5], increased the mean maximal plasma concentration (C_{max}) and the area-under-the-curve (AUC) of tolvaptan in human subjects, while co-administration of CYP3A4 inducer rifampicin reduced the C_{max} and AUC of tolvaptan [5].

In this study, using human HepG2 cells, we investigated the mechanisms underlying tolvaptan-mediated cytotoxicity by examining the effects of tolvaptan on the cell growth, cell cycle progression, DNA damage, and apoptosis. In addition, we determined the role of mitogen-activated protein kinase (MAPK) activation, proteasomal degradation, and autophagy in these processes.

☆ The views presented in this article do not necessarily reflect those of the U.S. Food and Drug Administration.

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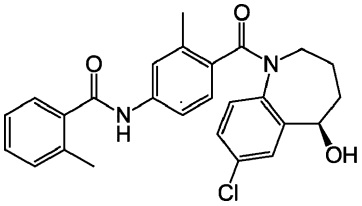

Fig. 1. The chemical structure of tolvaptan.

2. Materials and methods

2.1. Chemicals and reagents

Tolvaptan, dimethyl sulfoxide (DMSO), Williams' Medium E, sodium pyruvate, non-essential amino acid solution, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (Bis-Tris), and thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). PD184352, a highly potent and specific inhibitor of MEK1/2 [6], a kinase that phosphorylates the threonine and tyrosine residues of ERK1/2, thus, activating ERK1/2 [7], was also acquired from Sigma-Aldrich, as were SB239063, a specific inhibitor of p38 [8], and MG-132, a potent and specific proteasome inhibitor [9]. These inhibitors were used to investigate the role of the activation of ERK1/2 and p38 and proteasomal degradation in tolvaptan-induced apoptosis. Dulbecco's Modified Eagle Medium (DMEM), penicillin–streptomycin solution, and 2.5% trypsin were purchased from Thermo Fisher Scientific Inc. (Pittsburgh, PA). Fetal bovine serum (FBS) was acquired from Atlanta Biologicals (Lawrenceville, GA).

2.2. Antibodies

Mouse monoclonal antibodies against cyclin D1, cyclin D3, cyclin-dependent kinase (CDK) 4 (CDK4), and CDK6, rabbit monoclonal antibodies against CDK2, phosphorylated checkpoint kinase 1 at Ser345 (p-Chk1), Bcl-2, Bad, Bim, Bax, phosphorylated-ERK1/2 at Thr202/Tyr204 (p-ERK1/2), ERK1/2, phosphorylated-JNK at Thr183/Tyr185 (p-JNK), JNK, phosphorylated-p38 at Thr180/Tyr182 (p-p38), and p38, and rabbit polyclonal antibodies against phosphorylated checkpoint kinase 2 at Thr68 (p-Chk2), cytochrome C, and Bid were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibody against phosphorylated histone H2AX at Ser139 (γ-H2AX) was obtained from Millipore (Billerica, MA). Mouse monoclonal antibodies against CYP3A4 and β-actin, rabbit polyclonal antibodies against cyclin B1, CDK1, green fluorescent protein (GFP), and goat polyclonal antibody against Atg7 were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against microtubule-associated protein light chain 3 isoform B (LC3B) was purchased from Sigma-Aldrich. Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody (clone B44) was obtained from BD Biosciences (San Jose, CA). Alexa Fluor 488 conjugated rabbit monoclonal antibody against phosphorylated histone H3 at Ser10 (p-H3) was acquired from Cell Signaling Technology.

2.3. Cell culture

Human hepatoma HepG2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Williams' Medium E supplemented with 10% FBS and penicillin–streptomycin solution. The 293 T cell line used for lentivirus packaging was purchased from Biosettia (San Diego, CA) and maintained in DMEM supplemented with 10% FBS, sodium pyruvate, non-essential amino acids, and penicillin–streptomycin solution [10]. Both cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.4. Generation of HepG2 cells overexpressing human CYP3A4 and GFP-LC3B

HepG2 cells lack of expression of CYP3A4 [11]. To examine the effects of CYP3A4-mediated metabolism of tolvaptan on its cytotoxicity, a stable HepG2 cell line that overexpresses human CYP3A4 was generated. The coding sequence of human CYP3A4 was cloned into the cDNA expression lentiviral vector pLV-EF1a-MCS-IRES-Puro (Biosettia). 293 T cells were co-transfected with the lentiviral packaging mix and the generated human CYP3A4 expression vector or empty vector to produce lentivirus particles. HepG2 cells were then infected with the lentivirus particles carrying the human CYP3A4 expression vector or empty vector. Puromycin (2 μg/ml) was used to select HepG2 cells with stable expression of human CYP3A4 (HepG2/CYP3A4 cells) or the empty vector (HepG2/vector cells).

HepG2/GFP-LC3B cells, which stably express a fusion protein of GFP and LC3B, were constructed as described in Chen et al. [12].

2.5. Analysis of CYP3A4-mediated metabolism of tolvaptan

HepG2, HepG2/vector, and HepG2/CYP3A4 cells were lysed in buffer containing 5 mM Bis-Tris and 0.1 mM EDTA, pH 7.0, followed by sonication, and the protein concentrations were determined using a BCA protein assay (Thermo Fisher Scientific, Inc.). The metabolic activity of CYP3A4 against tolvaptan was assayed in a 100 μl reaction volume using 400 μg cell lysate protein incubated for 1.5 h at 37 °C with 31.25 μM tolvaptan, 1.3 mM NADPH, 3.3 mM $MgCl_2$, and 100 mM potassium phosphate (pH 7.4). Reactions were terminated by the addition of an equal volume of ice-cold methanol. Precipitated material was removed by centrifugation (5 min, 14,000 × *g*), and the supernatants were analyzed for tolvaptan metabolites using a Waters HPLC system consisting of a 600 Controller, a 996 Photodiode Array detector, and a 717 Plus autosampler (Waters Corporation, Milford, MA). The samples were injected onto a 4.6 × 250 mm C18 (5 μm) Luna column (Phenomenex, Torrance, CA) and eluted with a linear gradient elution at 1 ml/min between acetonitrile (solvent A) and 100 mM ammonium acetate, pH 8.0 (solvent B) as follows: a linear gradient of 10 to 30% solvent A in 10 min, then another linear gradient to 45% solvent A over 30 min, and then a linear gradient to 100% A in 3 min. The column was washed with 100% solvent A for 15 min and equilibrated for 15 min with 90% solvent B after every run.

2.6. The half inhibitory concentration of tolvaptan (IC_{50}) on cell growth

The effects of tolvaptan on the growth of HepG2, HepG2/vector, and HepG2/CYP3A4 cells were assessed using an MTT assay. Briefly, cells were plated into 24-well plates at cell densities of 6×10^4, 4×10^4, and 2×10^4 cells/cm^2 culture surface for 48, 72, and 120 h incubations, respectively. The cells were allowed to attach to the bottom of the wells for 24 h before being treated with various concentrations of tolvaptan (3.125, 6.25, 12.5, 25, 50, and 100 μM) for 24, 48, or 96 h. Control cells were fed with complete culture medium containing 0.1% (v/v) DMSO, which had no effect on cell growth. For the 96 h treatment, fresh medium and tolvaptan were replaced at 48 h. At the end of the treatment, MTT assays were performed as previously described [13]. The IC_{50}