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.

Keywords

Tolvaptan; liver injury; DNA damage; apoptosis; proteasome; autophagy.

Introduction

Tolvaptan (*N*- (4- {[*(5R)*-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 V₂-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.

Materials and Methods

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).

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.

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 293T 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% CO₂.

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 tolcapton 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). 293T 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].

Analysis of CYP3A4-mediated metabolism of tolcapton

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 tolavaptan 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 tolavaptan, 1.3 mM NADPH, 3.3 mM MgCl₂, 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 tolavaptan 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 micro) 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.

The half inhibitory concentration of tolavaptan (IC₅₀) on cell growth

The effects of tolavaptan 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/cm² 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 tolavaptan (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 tolavaptan were replaced at 48 h. At the end of the treatment, MTT assays were performed as previously described [13]. The IC₅₀ values were obtained from the cell growth curves, using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA).

Western blot analysis

At the end of the treatment, the HepG2 cells were lysed in RIPA lysis buffer (Thermo Fisher Scientific Inc.) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The protein concentrations were determined using a BCA protein assay. Twenty micrograms of whole cell lysate protein was separated by SDS polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. The membranes were blocked with 4% bovine serum albumin and incubated with specific primary antibodies against CYP3A4 (1:1,000), cyclin D1 (1:1,000), cyclin D3 (1:1,000), cyclin B1 (1:1,000), CDK1 (1:1,000), CDK2 (1:1,000), CDK4 (1:1,000), CDK6 (1:1,000), γ -H2AX (1:1,000), p-Chk1 (1:1,000), p-Chk2 (1:500), cytochrome C (1:1,000), Bcl-2 (1:1,000), Bad (1:1,000), Bim (1:1,000), p-ERK1/2 (1:1,000), ERK1/2 (1:1,000), p-JNK (1:1,000), JNK (1:1,000), p-p38 (1:1,000), p38 (1:1,000), Bax (1:1,000), Bid (1:1,000), LC3B (1:1,000), GFP (1:1,000), Atg7 (1:1,000), or β -actin (1:2,000), followed by a secondary antibody conjugated to horseradish peroxidase. The blots were then detected by chemiluminescence using Immobilon Western Horseradish Peroxidase Substrate (Millipore Corporation, Billerica, MA), a UVP BioSpectrum AC Imaging System, and VisionWorks LSD Image Acquisition & Analysis Software (UVP LLC, Upland, CA).

Lactate dehydrogenase (LDH) release assay

Cell death was measured by a LDH release assay as previously described [13].

Cell cycle analysis

The effect of tolvaftan on the cell cycle distribution was determined by flow cytometric analysis. At the end of the treatment, the HepG2 cells were pulse-labeled with BrdU by incubating with complete media containing 10 μ M BrdU for 1 h. The cells were then trypsinized. One half of the cells was fixed in 70% ethanol and stained with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody and propidium iodide as previously described [14]. To determine the percentage of cells in M phase, the other half of the cells was fixed in 1% formaldehyde, permeabilized with methanol, and stained with Alexa Fluor 488-conjugated monoclonal antibody against p-H3, a marker of mitotic cells [15]. The stained cells were analyzed on a Becton Dickinson FACS Calibur flow

cytometer with CellQuest software (BD Biosciences).

Alkaline comet assays

Alkaline comet assays, with or without post-incubation with bacterial formamidopyrimidine DNA glycosylase (Fpg) protein, were performed using a Fpg FLARE™ assay kit or a CometAssay kit (Trevigen, Gaithersburg, MD), as previously described [16, 17].

8-Oxoguanine and reactive oxygen species (ROS) determinations

The level of 8-oxoguanine was determined using an OxyDNA assay kit (Millipore) according to the manufacturer's instructions. Briefly, at the end of the treatment, the HepG2 cells were trypsinized, fixed in 1% formaldehyde, permeabilized in 70% ethanol, and incubated with a FITC-conjugated protein that can bind directly to 8-oxoguanine present in oxidized DNA. The stained cells were analyzed on a Becton Dickinson FACS Calibur flow cytometer with CellQuest software. The fluorescent intensity was determined and normalized to control cells.

The level of ROS was determined using 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA; Life Technologies, Grand Island, NY). Briefly, at the end of the treatment, the HepG2 cells were incubated with fresh medium containing 100 µM carboxy-H2DCFDA at 37°C for 1 h. The cells were then trypsinized, washed with PBS, and analyzed on a Becton Dickinson FACS Calibur flow cytometer with CellQuest software. The fluorescent intensity was determined and normalized to control cells.

Caspase 3/7 activity assay

Apoptotic cell death was determined by caspase 3/7 activity, which was measured using a Caspase-Glo 3/7 assay kit (Promega) according to manufacturer's instructions. Briefly, at the end of the treatment, 100 µl of Caspase-Glo 3/7 reagent was added to each well of a 96-well plate containing 100 µl of culture medium per well. After incubation at room temperature for 30 min, luminescence was measured with a BioTek Synergy 2 Reader (BioTek Instruments, Inc., Winooski, VT). An MTT assay was performed in a parallel 96-

well plate. The luminescence was normalized to the MTT assay data, and further normalized to the control.

Mitochondrial membrane potential determination

Mitochondrial membrane potential was determined using the mitochondrial membrane potential-sensitive dual-emission probe JC-1 (Sigma-Aldrich). JC-1 forms aggregates that display orange fluorescence in healthy mitochondria. When the mitochondrial membrane potential decreases, JC-1 exists as a monomer and displays green fluorescence. The fluorescence ratio of JC-1 aggregates to JC-1 monomers is an indication of mitochondrial membrane potential.

At the end of the treatment, HepG2 cells, seeded in black 96-well plates, were incubated with 100 µl fresh medium containing 2.5 µg/ml of JC-1. After incubation at 37°C for 20 min, the cells were washed three times with PBS. The fluorescence of the JC-1 aggregates and monomers was measured on a Biotek Synergy 2 Reader using filter pairs of 530 nm/590 nm and 485 nm/538 nm, respectively. The ratio of fluorescence of aggregates to monomers was calculated and normalized to control cells.

Mitochondria isolation

The separation of mitochondria from cytosolic components was performed using Pierce's mitochondria isolation kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The isolated mitochondria were lysed in RIPA lysis buffer. The protein concentrations were determined with a BCA protein assay.

Confocal microscopic analysis

HepG2/GFP-LC3B cells were seeded on coverslips in 24-well plates and then treated with tolvaftan or vehicle for 24 h. At the end of the treatment, the coverslips were washed with PBS and incubated in 4% paraformaldehyde for 15 min, followed by another wash with PBS. The presence of GFP-LC3B puncta was determined using an Olympus FV1000 confocal microscope (Olympus, Center Valley, PA) by observing GFP fluorescence after mounting the

coverslips onto glass slides using Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA).

GFP fluorescence intensity determination

At the end of the treatment, the HepG2/GFP-LC3B cells were trypsinized and washed with PBS. GFP fluorescence intensity was analyzed on a Becton Dickinson FACS Calibur flow cytometer with CellQuest software and normalized to the control.

RNA interference of Atg7 in HepG2 cells

Human Atg7 siRNA and negative control siRNA were purchased from Cell Signaling Technology. Transfection of siRNA into HepG2 cells was conducted by using Lipofectamine RNAiMAX (Life Technologies) following the manufacturer's instructions. The knock-down efficiency of the human Atg7 siRNA was determined using western blot analysis 96 h after the siRNA transfection.

Statistical analyses

Data are presented as the mean $\pm$ standard deviation of three independent experiments. Comparisons amongst concentrations were conducted by one-way analysis of variance, with pairwise comparisons versus control group being performed by Dunnett's method. When necessary, the data were log transformed to maintain an equal variance or normal data distribution. The results were considered significant at $P < 0.05$.

Results

Tolvaptan exhibited similar cytotoxicity in HepG2, HepG2/vector, and HepG2/CYP3A4 cells

The effect of tolvaptan on the growth of HepG2 cells was initially screened using an MTT assay. As shown in Fig. 2A, tolvaptan decreased the growth of the HepG2 cells in a concentration- and time-dependent manner, with the IC₅₀ values being > 100, 52.2, 33.0, and 27.1 µM after 24, 48, 96, and 168 h of exposure, respectively (Fig. 2C). Previous studies have suggested that tolvaptan is metabolized primarily by CYP3A4 [4]. To determine the effect of CYP3A4 on the cytotoxicity of tolvaptan, a stable HepG2 cell line that overexpressed human CYP3A4 (HepG2/CYP3A4 cells) was generated. As shown in Fig. 2B (upper left panel), when assessed by western blot analysis, CYP3A4 was readily detected in HepG2/CYP3A4 cells but not in HepG2 and HepG2/vector cells. Likewise, the enzymatic activity of CYP3A4 against luciferin-IPA was much higher in HepG2/CYP3A4 cells compared to HepG2 and HepG2/vector cells (Fig. 2B, upper right panel). Incubation of tolvaptan with HepG2/CYP3A4 cell lysates at 37°C for 1.5 h led to the formation of at least seven metabolites of tolvaptan that eluted from the HPLC column at retention times ranging from 17.8 to 32.4 min, as shown in Fig. 2B (lower panel), and a decrease in the tolvaptan substrate by 11.5 ± 0.6 %. None of the metabolites were detected in reactions using HepG2 or HepG2/vector cell lysates (Fig. 2B, lower panel). A comparison of the effect of tolvaptan on cell growth showed comparable IC₅₀ values among three cell lines (Fig. 2C), indicating that CYP3A4 had little effect on tolvaptan-induced cytotoxicity in HepG2 cells.

Tolvaptan inhibited cell growth and induced cell death in HepG2 cells

Since there was no significant difference in the cytotoxicity of tolvaptan between HepG2 and HepG2/CYP3A4 cells, subsequent mechanistic studies were conducted with HepG2 cells. Based on the IC₅₀ values obtained above, HepG2 cells were incubated with five concentrations (20, 40, 60, 80, and 100 µM) of tolvaptan for 24 or 48 h. Each of the concentrations of tolvaptan caused a significant decrease in the cell growth, in a both a time- and concentration-dependent manner (Fig. 3A). Starting from 40 µM, tolvaptan

increased LDH release, indicative of enhanced cell death (Fig. 3B). These data further confirmed that tolavaptan inhibited cell growth and caused cell death in HepG2 cells.

Tolvaptan disturbed cell cycle progression

Growth inhibition could be due to delayed cell cycle progression. The effects of tolavaptan on cell cycle progression were examined using BrdU incorporation and a flow cytometric technique [14]. As shown in Fig. 4A, there was a concentration-dependent increase in the percentage of G0/G1 phase cells, with a concomitant decrease in the percentage of S phase cells, following 24 or 48 h of exposure to 20 – 60 μ M tolavaptan. Tolavaptan at a concentration of 60 – 100 μ M also resulted in a significant increase in the percentage of G2/M phase cells (Fig. 4A). To discriminate cells in G2 phase from cells in M phase, the percentage of p-H3 positive cells was determined using a flow cytometric assay. As shown in Fig. 4B, treatment with tolavaptan for 24 or 48 h caused a concentration-dependent decrease in the percentage of p-H3 positive cells, implying that tolavaptan treatment caused cell cycle arrest at the G2 phase.

Cell cycle progression is regulated by a number of molecules, including cyclin family proteins and CDKs [18]. Cyclin D1, cyclin D3, CDK2, CDK4, and CDK6 are involved in the regulation of G1 phase progression and G1/S phase transition [19], and cyclin B1 and CDK1 participate in the regulation of M phase entry [18]. A concentration-dependent decrease in the protein levels of cyclin D1, cyclin D3, cyclin B1, CDK1, CDK2, CDK4, and CDK6 was observed in HepG2 cells following exposure to tolavaptan for 24 or 48 h (Fig. 4C). These data demonstrate that tolavaptan disturbed cell cycle progression in HepG2 cells and that this was associated with the down-regulation of a number of cyclins and CDKs.

Tolvaptan caused DNA damage and activated cell cycle checkpoint

The altered cell cycle progression following tolavaptan treatment raised the question of whether or not this was in response to DNA damage. To evaluate if tolavaptan induced DNA damage, alkaline comet assays were performed. In the comet assay conducted without Fpg, a

1 significant increase in the tail moment was observed in the HepG2 cells exposed to 60 – 100
2 μ M tolvaaptan for 24 or 48 h (Fig. 5A).
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6 Fpg is an *E. coli* glycosylase that has been widely used to assess oxidative DNA damage in
7 comet assays [20]. In a comet assay conducted with Fpg, HepG2 cells treated with 60 – 100
8 μ M tolvaaptan for 24 or 48 h displayed an increase in tail moment compared to control cells,
9 suggesting that tolvaaptan also caused oxidative DNA damage (Fig. 5A). Furthermore, the
10 level of 8-oxoguanine, a representative type of oxidative DNA damage, and the formation of
11 ROS were significantly increased in the HepG2 cells following exposure to 60 – 100 μ M
12 tolvaaptan (Fig. 5B and 5C). The presence of DNA damage in tolvaaptan-treated HepG2 cells
13 was further confirmed by an increased level of γ -H2AX, a sensitive marker of DNA double
14 strand breaks [21] (Fig. 5D).
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27 Checkpoint kinase 1 and 2 (Chk1 and chk2) are two essential molecules involved in cell
28 cycle checkpoint control. These kinases are phosphorylated and activated by ATM (ataxia
29 telangiectasia mutated protein)/ATR (ATM and Rad3-related) in response to DNA damage
30 [22]. As shown in Fig. 5D, an increase in the levels of p-Chk1 and p-Chk2 was observed in
31 the HepG2 cells treated with tolvaaptan for 24 or 48 h, with the effect being greater with p-
32 Chk2. These data suggest that tolvaaptan caused DNA damage and activated cell cycle
33 checkpoints in the HepG2 cells.
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44 **Tolvaaptan induced apoptosis**

45 The activation of caspase3/7 is a key step in the execution of cell apoptosis [23];
46 accordingly, caspase 3/7 activity was measured as a marker for the induction of apoptosis.
47 At concentrations $\geq 40 \mu$ M, tolvaaptan caused a significant increase in caspase 3/7 activity
48 after 24 or 48 h of exposure, suggesting tolvaaptan induced apoptosis in the HepG2 cells
49 (Fig. 6A). The levels of poly (ADP-ribose) polymerase (PARP), a well-known substrate of
50 active caspase 3/7, were analyzed using a Western-blot assay. Consistent with caspase 3/7
51 activation, tolvaaptan treatment induced an increase in PARP cleavage (Fig. 6B). Although
52 the intensity of the cleaved PARP fragment was low, the concomitant decrease in the level
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of full length PARP indicated the occurrence of PARP cleavage.

Apoptosis can be triggered primarily through two pathways, namely a mitochondria-mediated intrinsic pathway or a death receptor-mediated extrinsic pathway [24-26].

Tolvaptan decreased the mitochondrial membrane potential (Fig. 6C) and enhanced the release of cytochrome C from mitochondria to the cytosol after a 48 h exposure (Fig. 6D), suggesting that the mitochondria-mediated intrinsic pathway was involved in tolvaptan-induced apoptosis.

It is well-established that the balance between the pro-apoptotic and anti-apoptotic Bcl-2 family members regulates mitochondria-mediated intrinsic apoptosis [27-29]. The protein levels of several Bcl-2 family members were examined using western blot assay. Tolvaptan caused a dose-dependent decrease in the level of the anti-apoptotic protein Bcl-2, and increase in the levels of the pro-apoptotic proteins Bad and Bim in HepG2 cells following 24 or 48 h of exposure (Fig. 6B). Taken together, these data suggest that tolvaptan disrupted the balance between pro-apoptotic and anti-apoptotic proteins in the Bcl-2 family, enhancing cytochrome C release and triggering apoptosis.

Activation of p38 contributed to tolvaptan-induced apoptosis

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that regulate a variety of cellular activities [30, 31], and their involvement in the regulation of apoptosis has been well-documented [32]. The most extensively studied MAPKs are extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNK), and p38 kinase [30]. The activation of ERK1/2, JNK, and p38 is mediated by the dual phosphorylation of threonine and tyrosine residues [31]. Western blot analysis was used to monitor the phosphorylation of JNK, ERK1/2, and p38 as an indication of kinase activation. Tolvaptan, at concentrations ≥ 60 μ M, induced the activation of ERK1/2 and p38 when assessed after 4 or 24 h of exposure (Fig. 7A). The activation of JNK was not observed under the same conditions (data not shown).

To investigate whether or not the activation of ERK1/2 and p38 contributed to tolavaptan-induced apoptosis, specific inhibitors were used. Pre-treatment with the p38 inhibitor SB239063 for 1 h significantly decreased tolavaptan-induced apoptosis and partially attenuated its cytotoxicity after a 24 h exposure (Fig. 7B and 7C). In contrast, pre-treatment with the MEK1/2 inhibitor PD184352 did not decrease, but even slightly potentiated tolavaptan-induced apoptosis and its cytotoxicity, suggesting that the activation of ERK1/2 was not the cause of tolavaptan-induced apoptosis. Pre-treatment with SB239063 also partially reversed the tolavaptan-mediated decrease in the level of Bcl-2, but had minimal effects on the levels of Bad and Bim (Fig. 7D). These data suggest that the activation of p38 contributed to tolavaptan-induced apoptosis and that this occurs through the modulation of Bcl-2.

Proteasome inhibition blocked tolavaptan-mediated degradation of cyclin D3 and CDK4, and altered tolavaptan-induced cell cycle deregulation

The ubiquitin-proteasome pathway plays a pivotal role in the regulation of cell cycle progression [33, 34] and apoptosis [35, 36]. To determine whether or not tolavaptan-induced proteasomal degradation was responsible for the decrease in the protein levels of cyclins and CDKs, the specific proteasome inhibitor MG-132 was used. As shown in Fig. 8A, co-treatment of HepG2 cells with MG-132 and tolavaptan for 24 h markedly blocked the tolavaptan-mediated decrease in the protein levels of cyclin D3 and CDK4, exacerbated the tolavaptan-mediated decrease in the level of cyclin D1, and had no effect on CDK1, CDK2, or CDK6. These data suggest that tolavaptan caused proteasomal degradation of cyclin D3 and CDK4.

The effect of MG-132 on tolavaptan-mediated alterations in cell cycle progression was also examined. MG-132 alone did not have any effects on the cell cycle progression after a 24 h exposure (Fig. 8B). When HepG2 cells were treated with tolavaptan in the presence of MG-132 for 24 h, the percentage of G0/G1 phase cells was decreased compared with cells treated with tolavaptan only, suggesting that the tolavaptan-mediated G0/G1 phase cell arrest was partially reversed (Fig. 8B). This effect possibly resulted from the restoration of the

1 levels of cyclin D3 and CDK4, two key molecules controlling G1/S phase transition.
2 Furthermore, there was a significant increase in the percentage of G2/M phase cells, with a
3 concomitant decrease in the percentage of S phase cells in the HepG2 cells co-treated with
4 MG-132 and tolvaaptan for 24 h, compared with the HepG2 cells treated with tolvaaptan
5 alone (Fig. 8B). These data suggest that proteasome inhibition altered tolvaaptan-mediated
6 cell cycle deregulation.
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13 **Proteasome inhibition enhanced tolvaaptan-induced apoptosis and cytotoxicity**

14 The effect of MG-132 on tolvaaptan-induced apoptosis was also investigated. As shown in
15 Fig. 9A, MG-132 dramatically enhanced the tolvaaptan-mediated increase in caspase 3/7
16 activity after a 24 h exposure. In the presence of MG-132, tolvaaptan at concentrations as low
17 at 10 μ M caused an increase in caspase 3/7 activity, whereas in the absence of MG-132, an
18 increase in caspase 3/7 activity only occurred at ≥ 60 μ M tolvaaptan (Fig. 9A). PARP
19 cleavage was also enhanced in HepG2 cells co-treated with tolvaaptan and MG-132 (Fig. 9B).
20 The increase in tolvaaptan-induced apoptosis was accompanied by an increased cytotoxicity in
21 HepG2 cells co-treated with MG-132 for 24 h (Fig. 9C). MG-132 alone did not increase
22 caspase 3/7 activity or exhibit cytotoxicity at the concentration tested (100 nM).
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37 MG-132 exhibited a marginal effect on the tolvaaptan-mediated decrease in the level of Bcl-2
38 and increase in the level of Bad (Fig. 9B), and there was no difference in the level of Bim in
39 cells co-treated with MG-132 and tolvaaptan for 24 h, compared with cells exposed to
40 tolvaaptan alone (Fig. 9B).
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48 Bid and Bax are two additional pro-apoptotic proteins belonging to Bcl-2 family. Cleavage
49 of Bid generates the active form of Bid, which translocates from the cytosol to mitochondria,
50 activates Bax, and contributes to apoptosis [37, 38]. Cleaved Bax has been shown to possess
51 more potent activity in enhancing cell death than its parent form [39]. As shown in Fig. 9C,
52 co-treatment of the HepG2 cells with MG-132 and tolvaaptan for 24 h induced the cleavage of
53 Bid and Bax, which did not occur in cells treated with either tolvaaptan or MG-132 alone.
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60 Taken together, these data suggest that proteasome inhibition enhanced tolvaaptan-induced
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apoptosis and cytotoxicity, and that the modulation of Bcl-2 family proteins was involved in this process.

Tolvaptan induced autophagy

Autophagy is a catabolic process responsible for the degradation of unnecessary or dysfunctional cellular components [40]. Execution of autophagy involves the formation of a double-membrane autophagosome, subsequent fusion of the autophagosome with lysosome to become an autolysosome, and the degradation of intra-autophagosomal components by lysosomal hydrolases [41]. Autophagy occurs in response to a variety of stimuli, and can promote either cell survival or cell death under different conditions [42]. The role of autophagy in drug-induced liver toxicity is beginning to be recognized [43-45]. Microtubule-associated protein light chain 3 (LC3) is a widely used marker to monitor autophagy [46]. Upon the induction of autophagy, cytosolic LC3-I is converted to autophagosome membrane-bound LC3-II. LC3-II in the autolysosomal lumen is then degraded. Thus, LC3-II is an autophagosomal marker and the lysosomal turnover of LC3-II reflects the autophagic process [46].

Western blot analysis revealed a concentration-dependent increase in the level of LC3B-II by tolvaptan treatment for 24 or 48 h (Fig. 10A). Using HepG2/GFP-LC3B cells that stably express GFP-LC3B fusion protein, tolvaptan-induced autophagosome formation was examined morphologically. As shown in Fig. 10B, GFP-LC3B has a diffuse localization in the control cells, whereas in cells treated with tolvaptan for 24 h, GFP-LC3B had a punctate structure indicative of an autophagosome. In addition, the lysosomal turnover of GFP-LC3B was determined by analyzing for free GFP, which is resistant to lysosomal degradation after being cleaved from GFP-LC3B in the autolysosomes [41]. Tolvaptan treatment for 24 or 48 h increased the amount of free GFP in a concentration-dependent manner (Fig. 10A). The increase in the lysosomal turnover was further confirmed by a decrease in the GFP fluorescence intensity in cells treated with tolvaptan for 24 or 48 h (Fig. 10C) because GFP fluorescence is quenched in the acidic environment of lysosomes [47]. Taken together, these data demonstrate that tolvaptan induced autophagy in HepG2 cells.

Tolvaptan-induced autophagy prevented apoptosis in HepG2 cells

The role of autophagy in tolvaptan-induced apoptosis was determined by knocking-down Atg7, an important autophagy-related gene that controls autophagosome formation [48]. At 96 h after transfection, HepG2 cells transfected with Atg7 siRNA exhibited a dramatic decrease in the protein level of Atg7 compared with non-transfected HepG2 cells or cells transfected with negative control siRNA (Fig. 11A). The inhibition of autophagy by knocking-down Atg7 was reflected by a decrease in the level of LC3B-II either in the presence or absence of tolvaptan treatment for 24 h (Fig. 11A). In addition, knocking-down Atg7 resulted in an increase in caspase 3/7 activity (Fig. 11B) and a decrease in cell viability (Fig. 11C) in cells treated with tolvaptan for 24 h compared to control cells treated with the same concentration of tolvaptan. These data demonstrate that inhibition of autophagy sensitized the HepG2 cells to tolvaptan-induced apoptosis and cytotoxicity, indicating that autophagy promotes cell survival after tolvaptan treatment.

Discussion

In 2009, tolvaptan was approved by the U.S. FDA for the treatment of hyponatremia. Most published studies on tolvaptan have focused on its pharmacological effects and metabolic profile. Subsequently, tolvaptan was found to be associated with an increased risk of liver injury [3]. To the best of our knowledge, our study is the first to investigate the mechanisms underlying the potential hepatotoxicity of tolvaptan. We found that tolvaptan inhibited cell growth, delayed cell cycle progression, caused DNA damage, and triggered apoptosis in HepG2 cells. We also demonstrated a critical role of p38 activation, modulation of Bcl-2 family proteins, the ubiquitin-proteasome pathway, and autophagy in tolvaptan-induced cytotoxicity. Nevertheless, it should be noted that most of the data presented in the current study were obtained in cells following 24 or 48 h exposure, and it is not known whether these changes will persist during a longer exposure.

HepG2 cells have been demonstrated to be of great value in toxicological studies [49-53]. In addition, their capacity for genetic modifications, such as overexpressing and silencing

genes of interest, is advantageous for in-depth mechanistic studies at the molecular level. Although human primary hepatocytes might be more relevant, the limited availability and high variability among different donors restrict their use [11]. Thus, HepG2 cells represent a useful model for mechanistic studies of hepatotoxicity.

CYP3A4 plays an important role in the phase I metabolism of tolavaptan [4, 5].

Unexpectedly, we found that the overexpression of human CYP3A4 did not significantly affect tolavaptan-induced cytotoxicity compared with HepG2 cells and HepG2/vector cells.

Although the difference was not significant, tolavaptan was slightly less toxic in HepG2/CYP3A4 cells compared to the other two cell lines, with the IC_{50} values being approximately 10% higher (Fig. 2C). This difference is consistent with the finding that there was only limited (12%) metabolism of tolavaptan in HepG2/CYP3A4 cells (Fig. 2B). Thus, these data suggest that tolavaptan rather than the metabolites resulting from CYP3A4 metabolism is responsible for the observed toxicities.

Delayed cell cycle progression is a frequent cause of cell growth inhibition [18]. Tolavaptan at 20 – 60 μ M induced cell cycle arrest at the G0/G1 phase (Fig. 4A). Several key regulators of G1/S phase transition, including cyclin D1, cyclin D3, CDK4, and CDK6 were down-regulated upon tolavaptan treatment (Fig. 4C). Among these, the greatest decrease was observed with cyclin D3 and CDK4, and these changes occurred at as low as 20 μ M tolavaptan, implying that the decrease in the levels of cyclin D3 and CDK4 may be responsible for tolavaptan-induced G0/G1 phase arrest. This notion was further supported by the finding that proteasome inhibition blocked the tolavaptan-mediated degradation of cyclin D3 and CDK4 and attenuated the tolavaptan-induced increase in G0/G1 phase cells (Fig. 8B).

Tolavaptan at concentrations ≥ 60 μ M induced cell cycle arrest at G2/M phase (Fig. 4A and 4B), which correlated with a decrease in the levels of cyclin B1 and CDK1 (Fig. 4C), an increase in DNA damage (Fig. 5), and the activation of Chk2 (Fig. 5C). The control of G2/M phase transition involves two critical molecules, cyclin B1 and CDK1, the down-regulation of which has been demonstrated to result in the delay of M phase entry [54-57]. Furthermore, it

1 is well-established that DNA damage can trigger cell cycle arrest, which allows cells time to
2 repair the damage [22]. Therefore, it is possible that both the down-regulation of cyclin B1
3 and CDK1 and the induction of DNA damage contributed to G2/M phase cell arrest resulting
4 from higher concentrations of tolvaaptan (60 – 100 μ M).
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10 The delicate balance between pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family is
11 critical in controlling mitochondria-mediated intrinsic apoptosis [27, 28]. Tolvaaptan was
12 found to down-regulate anti-apoptotic protein Bcl-2 and up-regulate pro-apoptotic proteins
13 Bad and Bim. In addition, enhanced apoptosis in HepG2 cells co-treated with tolvaaptan and
14 the specific proteasome inhibitor MG-132 was associated with the cleavage of the pro-
15 apoptotic proteins Bid and Bax. These findings suggest a critical role of the modulation of
16 Bcl-2 family proteins in tolvaaptan-induced apoptosis.
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27 MAPKs regulate a variety of cellular activities, including apoptosis [30, 31]. We
28 demonstrated that tolvaaptan activated p38 and ERK1/2, but not JNK, and, using specific
29 inhibitors of MAPKs, that only the activation of p38 contributed to tolvaaptan-induced
30 apoptosis. Down-regulation of anti-apoptotic protein Bcl-2 was involved in p38-mediated
31 apoptosis. Of note, several findings suggest that p38 activation is not solely responsible for
32 tolvaaptan-induced apoptosis. First, the activation of p38 occurred at ≥ 60 μ M tolvaaptan,
33 while apoptosis occurred at ≥ 40 μ M tolvaaptan; second, inhibition of p38 activity with
34 SB239063 did not completely block tolvaaptan-mediated apoptosis; and third, although
35 SB239063 reversed the tolvaaptan-mediated decrease in Bcl-2, it had no significant effect on
36 the level of Bad and Bim. These data imply that tolvaaptan-induced apoptosis involves other
37 currently unidentified pathways.
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52 One interesting finding of this study is that proteasome inhibitor MG-132 sensitized HepG2
53 cells to tolvaaptan-induced apoptosis and cytotoxicity. This deserves attention because some
54 proteasome inhibitors are used clinically as prescription drugs. For instance, bortezomib is
55 the first therapeutic proteasome inhibitor approved for treating relapsed multiple myeloma
56 and mantle cell lymphoma [58]. Carfilzomib, a second-generation proteasome inhibitor, is
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also used to treat patients with multiple myeloma who have received at least two prior therapies [59]. MG-132 is a potent and specific proteasome inhibitor widely used in *in vitro* studies. Although MG-132 is not a clinical drug, the increased cytotoxicity of tolavaptan in the presence of MG-132 raise concerns about potential adverse effects resulting from drug-drug interactions between tolavaptan and proteasome inhibitors, especially considering the fact that the concentration of MG-132 used in this study was in the nanomolar range (100 nM) and not cytotoxic. When co-treated with MG-132, apoptosis and decreased cell viability was observed in cells exposed to 10 μ M tolavaptan, which is approaching to the plasma levels of tolavaptan found in humans. It is possible that patients who take both tolavaptan and proteasome inhibitors might have a higher risk of developing liver injury.

The clinical dosage of tolavaptan for the treatment of hyponatremia ranges from 15-60 mg once daily. Pharmacokinetic studies have shown that the average therapeutic $C_{\max}$ of tolavaptan is around 0.7 μ M in Japanese [60, 61] and 1.0 μ M in Caucasians [61] following a single oral dose of 60 mg tolavaptan. Co-administration of grapefruit juice with 60 mg tolavaptan increased the $C_{\max}$ value by 1.9 fold, from 0.7 μ M to 1.3 μ M [4]. Co-administration of ketoconazole with 30 mg tolavaptan increased the $C_{\max}$ value by 3.4 fold, from 0.4 μ M to 1.3 μ M [5]. Although the cytotoxic concentrations of tolavaptan reported here are higher than the plasma concentration in humans, we believe they were reasonable in the present mechanistic study for two reasons. First, the concentration of tolavaptan in human liver is currently unknown, and there are many uncertainties in extrapolating plasma concentrations to hepatocyte concentrations. Second, it is well-known that individual susceptibility to drugs can be affected by a number of factors, including genetic variability, age, drug-drug interactions, and pre-existing diseases [62-64]. To identify drugs with potential idiosyncratic hepatotoxicity, it has been suggested that a concentration equivalent to 100-fold of the therapeutic $C_{\max}$ should be tested [65]. The cytotoxic concentrations of tolavaptan presented in this study (10 – 100 μ M) are within the range of 100 times of the therapeutic $C_{\max}$ (70 – 130 μ M).

In conclusion, tolavaptan delayed cell cycle progression, caused DNA damage, and induced

apoptosis in human HepG2 cells. Activation of p38 and modulation of Bcl-2 family members contributed to tolcapant-induced apoptosis and cytotoxicity, while the ubiquitin-proteasome pathway and autophagy seemed to be pro-survival mechanisms.

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Figure legends

Fig. 1 The chemical structure of tolavaptan.

Fig. 2 Tolavaptan exhibited similar cytotoxicity in HepG2, HepG2/vector, and HepG2/CYP3A4 cells. (A) Representative cell growth curves of HepG2, HepG2/vector, and HepG2/CYP3A4 cells treated with tolavaptan (1.56 – 100 μ M) for 24, 48, 96, or 168 h. The results shown are the mean and standard deviation of three independent experiments. (B) The protein level of CYP3A4 (upper left panel), the enzymatic activity of CYP3A4 against luciferin-IPA (upper right panel), and HPLC analysis of tolavaptan metabolites (indicated using Arabic numbers) by CYP3A4 (lower panel) in HepG2, HepG2/vector, and HepG2/CYP3A4 cells. β -Actin was used as a loading control. The enzymatic activity of CYP3A4 against CYP3A4 substrate luciferin-IPA was determined using a P450-Glo CYP3A4 assay kit (Promega, Madison, WI) according to manufacturer's instructions. (C) The IC₅₀ values obtained from the cell growth curves shown in (A) using GraphPad Prism 6.0. Values in the parenthesis were 95% confidence intervals of the IC₅₀.

Fig. 3 Tolavaptan inhibited cell growth and induced cell death in HepG2 cells. HepG2 cells were treated with tolavaptan (0 – 100 μ M) for 24 or 48 h. (A) Cell viability and (B) cell death was assessed by MTT and LDH release assays, respectively. The results shown are the mean and standard deviation of three independent experiments. *: significantly ($p < 0.05$) different from 0 μ M tolavaptan.

Fig. 4 Tolavaptan disturbed cell cycle progression. HepG2 cells were treated with tolavaptan (0 – 100 μ M) for 24 or 48 h. (A) Cell cycle progression was determined as described in Materials and Methods. The results shown are the mean and standard deviation of three independent experiments. *: significantly ($p < 0.05$) different from 0 μ M tolavaptan. (B) Percentage of p-H3 positive cells. The results shown are the mean and standard deviation of three independent experiments. *: significantly ($p < 0.05$) different from 0 μ M tolavaptan. (C) Representative western blot images of cyclin D1, cyclin D3,

1 cyclin B1, CDK1, CDK2, CDK4, and CDK6 in the HepG2 cells treated with tolvaptan. β -
2 Actin was used as a loading control.
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6 **Fig. 5 Tolvaptan caused DNA damage and activated cell cycle checkpoint in HepG2**
7 **cells.** HepG2 cells were treated with tolvaptan (0 – 100 μ M) for 24 or 48 h. (A) Tail
8 moment in alkaline comet assays with or without post-incubation with Fpg. The results
9 shown are the mean and standard deviation of three independent experiments. *:
10 significantly ($p < 0.05$) different from 0 μ M tolvaptan. (B) Relative levels of 8-oxoguanine
11 and (C) ROS in the HepG2 cells treated with tolvaptan. The results shown are the mean and
12 standard deviation of three independent experiments. *: significantly ($p < 0.05$) different
13 from 0 μ M tolvaptan. (D) Representative western blot images of γ -H2AX, p-Chk1, and p-
14 Chk2 in the HepG2 cells treated with tolvaptan. β -Actin was used as a loading control.
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27 **Fig. 6 Tolvaptan induced apoptosis.** (A) Apoptosis was determined using caspase 3/7
28 activity assay. The results shown are the mean and standard deviation of three independent
29 experiments. *: significantly ($p < 0.05$) different from 0 μ M tolvaptan. (B) Representative
30 western blot images of PARP, Bcl-2, Bad, and Bim in HepG2 cells treated with tolvaptan. β -
31 Actin was used as a loading control. (C) Mitochondrial membrane potential was determined
32 using JC-1 assay. The results shown are the mean and standard deviation of three
33 independent experiments. *: significantly ($p < 0.05$) different from 0 μ M tolvaptan. (D)
34 Representative western blot images of cytochrome C in mitochondria and cytosol of the
35 HepG2 cells treated with tolvaptan for 48 h. β -Actin was used as a loading control.
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48 **Fig. 7 Activation of p38 contributed to tolvaptan-induced apoptosis.** (A) Representative
49 western blot images of p-p38, p38, p-ERK1/2, and ERK1/2 in HepG2 cells treated with
50 tolvaptan (0 – 100 μ M) for 4 or 24 h. β -Actin was used as a loading control. (B - D) HepG2
51 cells were pre-treated with the p38 inhibitor SB239063 (20 μ M), the MEK1/2 inhibitor
52 PD184352 (2 μ M), or vehicle (0.1% DMSO) for 1 h and then exposed to tolvaptan (0 – 80
53 μ M) for 24 h. Apoptosis (B) and cell viability (C) were determined using capsase 3/7 activity
54 and MTT assays, respectively. The results shown are the mean and standard deviation of
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three independent experiments. *: significantly ($p < 0.05$) different from the respective 0 μ M tolvaaptan group. (D) Representative western blot images of Bcl-2, Bad, and Bim in HepG2 cells treated with 80 μ M tolvaaptan in the presence or absence of the p38 inhibitor SB239063 (20 μ M). β -Actin was used as a loading control.

Fig. 8 Proteasome inhibition blocked tolvaaptan-mediated degradation of cyclin D3 and CDK4 and altered tolvaaptan-induced cell cycle deregulation. HepG2 cells were treated with tolvaaptan (0, 40, or 80 μ M) in the presence or absence of the proteasome inhibitor MG-132 (100 nM) for 24 h. (A) Representative western blot images of cyclin D1, cyclin D3, CDK1, CDK2, CDK4, and CDK6. β -Actin was used as a loading control. (B) Cell cycle distribution. The results shown are the mean and standard deviation of three independent experiments. *: significantly ($p < 0.05$) different from cells without MG-132 treatment.

Fig. 9 Proteasome inhibition enhanced tolvaaptan-induced apoptosis and cytotoxicity. HepG2 cells were treated with tolvaaptan (0 – 80 μ M) in the presence or absence of the proteasome inhibitor MG-132 (100 nM) for 24 h. (A) Apoptosis, as determined by measuring capsase 3/7 activity. The results shown are the mean and standard deviation of three independent experiments. *: significantly ($p < 0.05$) different from 0 μ M tolvaaptan. (B) Representative western blot images of PARP, Bid, cleaved Bid, Bax, cleaved Bax, Bcl-2, Bad, and Bim. β -Actin was used as a loading control. (C) Cell viability, as determined by MTT assays. The results shown are the mean and standard deviation of three independent experiments. *: significantly ($p < 0.05$) different from 0 μ M tolvaaptan.

Fig. 10 Tolvaaptan induced autophagy in HepG2 cells. (A) HepG2 or HepG2/GFP-LC3B cells were treated with tolvaaptan for 24 or 48 h. Representative western blot images LC3B and free GFP. β -Actin was used as a loading control. (B) HepG2/GFP-LC3B cells were exposed to tolvaaptan (0 – 80 μ M) for 24 h. GFP-LC3B puncta (red arrows) were observed by confocal laser microscope. (C) HepG2/GFP-LC3B cells were treated with tolvaaptan (0 – 100 μ M) for 24 or 48 h. GFP fluorescence intensity was determined using flow cytometry. The

1 results shown are the mean and standard deviation of three independent experiments. *:
2 significantly ($p < 0.05$) different from 0 μ M tolvaptan.
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6 **Fig. 11 Tolvaptan-induced autophagy prevented apoptosis in HepG2 cells.** HepG2
7 cells were transiently transfected with control siRNA or Atg7 siRNA. At 72 h after siRNA
8 transfection, cells were exposed to the indicated concentration of tolvaptan for 24 h. (A)
9 Representative western blot images of Atg7 and LC3B. β -Actin was used as a loading
10 control. (B) Apoptosis and (C) cell viability were determined using capsase 3/7 activity
11 and MTT assays, respectively. The results shown are the mean and standard deviation of
12 three independent experiments. *: significantly ($p < 0.05$) different from cells transfected
13 with control siRNA.
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*Graphical Abstract (for review)

Fig. 1

Fig. 2A

Fig. 2B

B

Fig. 2C

C

	IC ₅₀ (μM)		
	HepG2 cells	HepG2/vector cells	HepG2/CYP3A4 cells
24 h	>100	>100	>100
48 h	52.2 (46.4 – 58.6)	56.4 (50.8 – 62.7)	59.5 (55.0 – 64.3)
96 h	33.0 (30.4 – 35.9)	34.7 (32.0 – 37.6)	37.7 (35.5 – 40.0)
168 h	27.1 (25.4 – 28.9)	26.8 (25.9 – 27.8)	29.1 (27.3 – 31.0)

Fig. 3

A

Fig. 5

Fig. 6

Fig. 7

A

B

C

D

Fig. 8

Fig. 9

A

C

B

Fig. 10

A

B

C

Fig. 11