

Can tolvaptan usage cause cytotoxicity? An in vitro study

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ABSTRACT

Objectives: Tolvaptan is a nonpeptide V2 (vasopressin) receptor antagonist which is commonly used for treatment of hypernatremia. Besides it is mostly used for rescue strategies of mutant V2 receptors which are responsible for congenital type of Nephrogenic Diabetes insipidus (NDI) as a pharmacological chaperone (PC) treatment. Tolvaptan is metabolized by CYP3A4 and usage of tolvaptan may cause cytotoxicity which can be prevented by antioxidants. The aim of this study is investigating cytotoxic effect of tolvaptan on COS-1 cells and preventing it via antioxidants such as Vitamin C and N-acetyl cysteine (NAC).

Methods: To measure cytotoxicity of tolvaptan, COS-1 cells were separated in three groups; tolvaptan, tolvaptan+Vitamin C and tolvaptan+NAC. 24 h after cells were seeded in 96-well plates, they were treated with different concentrations of tolvaptan, tolvaptan+Vitamin C and tolvaptan+NAC. After 24 h incubation, the (3-(4,5-Dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide) [MTT] analysis were performed and GraphPad Prism 5.01 for Windows was used for statistical analysis.

Results: According to results of MTT assay, treatment with tolvaptan did not decrease cell viability except that treatment of 10^{-5} M tolvaptan showed significantly decrease on cell viability compared to control group. At the concentration of 10^{-9} M, there was significantly different cell viability between treated with tolvaptan and tolvaptan+Vitamin C.

Conclusions: Tolvaptan may show its cytotoxic effects when it is used for the treatment of hyponatremia than its usage of as a PC. Since low concentrations of tolvaptan for a short time treatment is enough for its PC role, it may not show any cytotoxic effect on cells which is coherent with our results.

Keywords: Tolvaptan, cytotoxicity, cell culture, hyponatremia, nephrogenic diabetes insipidus, pharmacological chaperone

Arginine vasopressin (AVP) is a small peptide hormone that plays a critical role in body water and sodium homeostasis and also regulating serum osmolality [1]. AVP shows its effects on V1a, V1b and V2 receptors (vasopressin receptors) which are mainly seen in the heart and blood vessels, anterior pituitary

and collecting ducts in the kidney, respectively. In physiological conditions, when urine osmolality increase and plasma serum level decrease, AVP is secreted from posterior pituitary which causes increased water reabsorption resulting in an increase of total body water. AVP levels are excessively increased in several clinical



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cal situations such as syndrome of inappropriate antidiuretic hormone (SIADH), polycystic kidney disease (PKD), liver cirrhosis and heart failure [2, 3]. Hyponatremia is seen as the most common electrolyte disorder in hospitalized patients [4, 5] and many studies showed that hyponatremia can increase mortality in patients with liver cirrhosis, congestive heart failure and neurological diseases [6-9]. Traditional treatment of hyponatremia is fluid restriction with or without the usage of lithium, demeclocycline and urea can be remained incomplete in clinical practice because different pathophysiological mechanisms have multiple causes [3, 10]. Usage of vasopressin antagonists known as vaptans can induce electrolyte free excretion of water; therefore, they provide a more effective option to treat hyponatremia [3, 4, 10, 11]. Vaptans are non-peptide vasopressin receptor antagonists which can be usable both orally and intravenously. Tolvaptan, which is a selective V2 receptor antagonist, is an orally active molecule that is commonly used for the treatment of hyponatremia. Tolvaptan was evaluated in many studies such as "Study of Ascending Levels of Tolvaptan in Hyponatremia" (SALT-1 and 2), "The Efficacy of Vasopressin Antagonism in Heart Failure Outcome Study with Tolvaptan" (EVEREST) and autosomal dominant PKD [5, 11-14]. On the other hand, V2 vasopressin antagonists mostly have been studied in functional analysis studies on Nephrogenic Diabetes insipidus which is characterized imbalance of body water homeostasis that is a related pathway mentioned above. V2 receptor mutations can affect maturation of receptor protein and they can be trapped by Endoplasmic reticulum (ER) quality control mechanism in the cell. Therefore, they cannot perform their function properly in the kidney. Last years, many vasopressin agonists and antagonists (tolvaptan belong to this group) have been used as a pharmacological chaperone (PC) to rescue these mutant receptors from ER. PCs are small cell-permeable molecules that bind specifically to a misfolded protein and help its stabilization throughout decreasing the folding energy barrier [15, 16]. As a PC, tolvaptan has a potential to rescue ER-trapped misfolded protein and helps it to become functional again [17-19]. Studies of functional analysis and PC rescue about GPCRs mostly use COS-1 or COS-7 cell lines which were derived from the CV-1 fibroblast cell line (kidney cell of an adult African green monkey) in addition to other cell line

types, and COS-1 and COS-7 are different from each other in terms of early region of SV40 DNA [15, 17, 20-26]. COS cell line was used in this study because we have been functionally analyzed and showed rescue potential of mutant V2 receptors via PCs in our previous and ongoing studies and therefore it was aimed to show cytotoxic effects of tolvaptan on COS-1 cells. In addition to PC studies, many of the other studies showed that tolvaptan is successful to increase serum sodium levels in patients with hyponatremia related with heart failure, cirrhosis and SIADH [27, 28]. On the contrary, in the present time, tolvaptan are not recommended for routine use in patients with PKD. Also, FDA restricts the maximum usage period of tolvaptan as 30 days because of the increased level of liver enzymes may cause hepatic toxicity with prolonged use [11, 29, 30]. PC studies show that small concentrations of tolvaptan can be enough to rescue mutant protein [18]. Even so it could cause toxicity in the cell. For this reason, it is important to decrease cytotoxicity of tolvaptan when it is used as a PC in case it has a toxic effect. Consequently, the aim of this study is to show cytotoxic effects of tolvaptan on cell viability and to prevent these effects by antioxidants such as vitamin C and N-acetyl cysteine (NAC) in case it shows cytotoxicity.

METHODS

Chemicals

Tolvaptan (Sigma-Aldrich) were prepared as 1 mM stock solution in DMSO (Cell culture grade, AppliChem GmbH). Vitamin C (Basel Kimyevi Mad. ve İlaç San. Tic. A.Ş.) was prepared from 500 mg/ml injection solution as 1 mM in DMEM and NAC (Bayer Türk Kimya San. Ltd. Şti.) was prepared from 600 mg effervescent tablet which was first dissolved in 50 ml cell culture grade water then, was made as 1 mM stock solution in DMEM. Stock solutions of Vitamin C and NAC were freshly prepared before usage. Dulbecco's modified Eagle's medium (DMEM) High Glucose with stable Glutamine and sodium pyruvate (Biowest SAS France facility) supplemented with 10% fetal bovine serum (originated in South America), 100 U/ml penicillin and 10 µg/ml streptomycin was used as a medium in the cell culture.

Cell Culture Studies

COS-1 cell line was purchased from AddexBio Technologies (T0014001, Lot number: 0013255) in 2017 for our previous studies and they have been kept as stocks in liquid nitrogen. For this study, they cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 10 µg/ml streptomycin, in 5% CO₂ in air, at 37°C. Cells were seeded on 96-well plates as 50.000 cells/well as a three group. After 24 h, media were removed and one group was treated with only tolvaptan in different concentrations (between 10⁻⁵ and 10⁻⁹ M). Other two groups were treated as tolvaptan together with Vitamin C, and tolvaptan together with NAC (as the same concentrations of both). After 24 h incubation, the (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [MTT] assay was performed.

MTT Assay

MTT is a reagent that is commonly used to define cell viability in cell culture studies. Formation of MTT formazan crystals is used to determine viable cells in this colorimetric method [28]. To measure the viability of the cells, after the incubation with tolvaptan and antioxidants of the cells, medium was removed and freshly prepared 100 µl 0.5 mg/ml MTT was added to the well and plates were incubated for 4 hours in 37°C in dark. Then, the MTT reagent was removed and replaced with 100 µl isopropanol to solubilize the converted purple dye in wells. Absorbance at 570 nm was measured with EnSight Multimode Plate Reader (Perkin Elmer).

Statistical Analysis

For statistical analysis, tolvaptan treatment was compared with control group, tolvaptan+Vitamin C group and tolvaptan+ NAC group, separately. Two-way ANOVA was used as a statistical analysis to determine the prevention of cytotoxicity of tolvaptan with using Vitamin C and NAC. The level of significance was taken as $p < 0.05$ in all instances. Statistical analysis was performed by using GraphPad Prism 5.01 for Windows (GraphPad Software).

RESULTS

For all comparisons, untreated COS-1 cells were set to 100% as a control group. According to the percentages of MTT assay results, it was seen that increased tolvaptan treatment caused increased cytotoxicity since the percentages of cell viability decreased (Table 1). In addition, Vitamin C and NAC treatment did not increase the cell viability when it is compared with the group of only treated with tolvaptan. In other words, the percentage values were not increased with the treatment of antioxidants (Table 1). However, when these results were statistically analyzed, it was seen that cells treated only with 10⁻⁵ M tolvaptan showed significantly decreased cell viability compared to the control group ($p = 0.0023$) (Table 2). Also, at the concentration of 10⁻⁹ M, there was significantly different cell viability between treated with tolvaptan and tolvaptan together with Vitamin C ($p = 0.0129$) (Table 2).

Table 1. MTT assay results as percentages are seen

Concentration	Tolvaptan		Tolvaptan + Vitamin C		Tolvaptan + NAC	
	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
10 ⁻⁵ M	67.9	2.7	68.3	6.8	63.0	5.2
10 ⁻⁶ M	79.5	9.9	77.0	11.1	78.3	15.8
10 ⁻⁷ M	88.1	6.5	83.5	6.2	84.9	5.7
10 ⁻⁸ M	89.5	9.5	89.5	8.8	87.2	13.3
10 ⁻⁹ M	92.5	3.5	97.3	2.9	94.5	7.7

The data are given as mean ± S.D. of independent experiments (n = 3). For all concentrations, untreated sample of COS-1 cells was set to 100% as a control. MTT = the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, NAC = N-acetyl cysteine, SD = standard deviation

Table 2. Comparison of groups

Groups	p value
10 ⁻⁵ M Tolvaptan vs Control	0.0023
10 ⁻⁹ M Tolvaptan vs 10 ⁻⁹ M Tolvaptan + Vitamin C	0.0129

Significant p values between the groups were analyzed. All groups were compared between each other and also with the control group using with two-way ANOVA and only significant P values were presented in this table. The level of significance was taken as $p < 0.05$ in all instances. Statistical analysis was performed by using GraphPad Prism 5.01 for Windows (GraphPad Software).

DISCUSSION

Vaptans are nonpeptide V2 receptor antagonists and they are resembling AVP. Tolvaptan, one of these vaptans, is an orally usable and non-peptide V2 receptor antagonist [3]. It has approximately 2-fold greater affinity to V2 receptor than its specific ligand, AVP [31]. Tolvaptan has an ability to increase urine free water excretion causing decreased urine osmolality and increased sodium level [31].

At the first double-blind study, the effects of 30 mg, 45 mg and 60 mg of tolvaptan usage once in a daily for 25 days was investigated in patients with chronic heart failure (CHF) and it was found that CHF patients tolerated tolvaptan well and their serum sodium levels were normalized compared to the placebo group [32]. According to the SALT-1 and SALT-2 trials, which were about evaluation of tolvaptan usage effects on hyponatremia associated with congestive heart failure, cirrhosis of the liver and SIADH, serum sodium levels of patients were improved but after one week of discontinuation, reverted to hyponatremic conditions [5]. Prolonged use of tolvaptan (804 days) caused normalization of serum sodium levels [33]. The results of the EVEREST trials showed that tolvaptan usage made normalize of serum sodium levels in patients with congestive heart failure but it did not demonstrate any survival benefit for patients [12, 34-36]. At the TEMPO trials which were about evaluation of tolvaptan treatment in patients with autosomal dominant PKD, progression of PKD was delayed but transaminases were seen as increased more than 3 times in patients with tolvaptan group [2]. After that, US Food Drug Administration (FDA) restricted the usage of tolvaptan more than 30 days be-

cause of the probability of liver toxicity. At all those trials, the doses of tolvaptan were approximately between 15 mg and 60 mg in a day. Above this dosage and also prolonged usage were reported to cause liver dysfunction via toxicity because tolvaptan is mainly metabolized by CYP3A4 [11, 37]. All these trials were conducted with higher concentrations of tolvaptan treatment in patients; therefore, our results of cellular toxicity cannot be compared with these trials because our results are more meaningful for very small dosages of tolvaptan which could be measured in the cell culture system. A study about determining the mechanism of hepatotoxicity of tolvaptan in HepG2 cells showed that tolvaptan inhibited cell cycle progression, induced DNA damage and executed apoptosis [9]. Also, they found that tolvaptan affected many signaling pathways that cause cytotoxicity [9]. 24 h treatment of HepG2 cells with different tolvaptan concentrations from 1.56 to 100 μ M showed that short time as a 24 h-treatment slightly decreased cell viability. The most obvious decrease which was about 40-50% was seen in 100 μ M concentration of tolvaptan [9]. During 24 h treatment, cell viability decrease was seen at 25 μ M concentration which can be compatible with the results of this study because the difference between even in 10 μ M-treated cells and the control group of cell viability was found significant ($p = 0.0023$) (Table 2). More than 10⁻⁵ M concentration of tolvaptan could cause cytotoxicity with more than 24 h which is known through the clinical studies. However, in this study the experiments were performed for only 24 h because PC studies (which were mentioned in detailed below) mostly treat cells with PCs for 18-24 h and short-time treatment is mostly enough to rescue of mutant protein using with PCs [18, 38].

The disease nephrogenic syndrome of inappropriate antidiuresis (NSIAD) is caused by gain-of-function mutations in the V2 receptor gene (arginine vasopressin receptor 2, AVPR2) [39]. Mutations of Phe229 and Arg137 in AVPR2 were found that cause gain-of-function in the V2 receptor and consequently, it was reported that infants who had these mutations showed clinical symptoms related with hyponatremia [39]. In another study showed that I130N mutation in AVPR2 cause gain-of-function in the V2 receptor which is responsible of constitutive activity of cAMP production [40]. They showed that tolvaptan blocked this basal activity of cAMP production through its inverse ago-

nist property and they proposed that tolvaptan could be a treatment for hyponatremia in patients with NSIAD who have I130N mutation in AVPR2 [40]. On the other hand, many studies showed that loss-of-function mutations in AVPR2 are responsible of NDI. NDI is characterized as an imbalance of body water homeostasis which is normally controlled by antidiuretic hormone AVP via kidney [41]. Functional analysis studies about these loss-of-function mutations in patients with NDI revealed that presence of a mutation may show its effect on function at different levels. Instead, it can cause the mutant V2 receptor is retained in the ER quality control mechanism. Therefore, even if these mutant V2 receptors are functional, they cannot escape from the ER and locate on plasma membrane where they function [18, 38]. To correct this trafficking problem and make mutant V2 receptors functional again, PCs can be used because they are nonpeptide and cell-permeable ligands that bind specifically to the mutant receptor and stabilize receptors conformation [42]. In this way, PCs help mutant V2 receptors to be rescued and located where they are functional again [38, 43, 44]. Tolvaptan is a kind of PC that commonly used in these studies because its rescue potential which was reported by many researches has an importance on to develop new treatment strategies for NDI [17-19]. The treatment dosages of tolvaptan were mostly 1 μ M or 10 μ M at these studies and they all reported that this low level of tolvaptan usage (and also for a short time like 24 h, etc.) was enough to rescue and make functional mutant V2 receptor. They did not report any cytotoxic effect of tolvaptan in cell culture studies [17-19]. In this study, the maximum concentration of tolvaptan was 10 μ M and it was seen just a slight decrease on cell viability (Table 1). Therefore; it can be said that tolvaptan is not cytotoxic for the cells when it is used as a low dose-PC to rescue mutant V2 receptors. Because of this result, treatment of equimolar dose of Vitamin C and NAC with tolvaptan did not show any significant difference about cell viability than treated with only tolvaptan, except the treatment of equimolar dose of 10^{-9} M Vitamin C and tolvaptan ($p = 0.0129$) (Table 2). If it was seen very low cell viability in the group of treated with just tolvaptan, it could be said that tolvaptan treatment might show its cytotoxic effects through the oxidative stress. Since the tolvaptan is metabolized by the cytochrome P450 system, it

might be said that reactive oxygen species (ROS) could be occurred and consequently oxidative stress might show itself [10, 45, 46]. At these kinds of situations, antioxidants such as Vitamin C and NAC may help to reduce oxidative stress in the cell. Vitamin C and NAC may mediate their antioxidant roles via scavenging ROS [45]. Thus, Vitamin C and NAC could be used as antioxidants if it was found a significant cytotoxic effect of tolvaptan. However, there was no cytotoxicity observed. Just at very low concentration (10^{-9} M) of tolvaptan and Vitamin C treatment together, it was seen a significant antioxidant effect of Vitamin C compared to the only tolvaptan treated group ($p = 0.0129$) (Table 2). Even if it was found a significant difference between the group of treated with tolvaptan only and the group of treated with tolvaptan and Vitamin C together, according to the results, it was concluded that the usage of low concentration of tolvaptan did not show any significant effect on cytotoxicity. However, prevention of the cytotoxicity of high dosages of tolvaptan usage, which is known through the clinical studies, using with antioxidants such as Vitamin C and NAC will shed light into the future studies.

CONCLUSION

As a conclusion, tolvaptan is a very successful V2 receptor antagonist for treatment of the hyponatremia. It also can be used as a PC to rescue of misfolded mutant V2 receptor protein from ER to the plasma membrane of the kidney where it shows its function. Tolvaptan may show its cytotoxic effects when it is used for the treatment of hyponatremia than its usage of as a PC. Since low concentrations of tolvaptan for a short time treatment is enough for its PC role, it may not show any cytotoxic effect on the cells which is coherent with our results. In accordance with that, it was seen in this study, the cytotoxic effects of higher concentrations of tolvaptan cannot be prevented by Vitamin C and NAC. However, when tolvaptan was used as low concentration as like a PC, we showed that cytotoxicity of the tolvaptan was significantly decreased with using Vitamin C.

Authors' Contribution

Study Conception: BET; Study Design: BET; Su-

pervision: BET; Funding: BET; Materials: BET; Data Collection and/or Processing: BET; Statistical Analysis and/or Data Interpretation: BET; Literature Review: BET; Manuscript Preparation: BET and Critical Review: BET.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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