

## **Proteasome Inhibitors Bortezomib And Carfilzomib Stimulate the Transport Activity of Human Organic Anion Transporter 1**

Yunzhou Fan and Guofeng You\*

Department of Pharmaceutics, Rutgers, the State University of New Jersey, Piscataway, NJ,  
USA

**Running title:** Regulation of OAT1 by Proteasome Inhibitors

**Corresponding author:** Guofeng You, Department of Pharmaceutics, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA, Tel: 848-445-6349; E-mail: gyou@pharmacy.rutgers.edu

Number of pages: 26

Number of tables: 0

Number of figures: 8

Number of References: 56

Number of words in Abstract: 211

Number of words in Introduction: 504

Number of words in Discussion: 1139

### **Abbreviations**

OAT, organic anion transporter; PAH, p-aminohippuric acid; Ub, ubiquitin; PKC, protein kinase C; PBS, phosphate-buffered saline;  $C_{max}$ , maximum plasma concentration; LDH, lactate dehydrogenase; CI, confidence interval.

## Abstract

Organic anion transporter 1 (OAT1), expressed at the basolateral membrane of renal proximal tubule epithelial cells, mediates the renal excretion of many clinically important drugs. Previous study in our lab demonstrated that ubiquitin conjugation to OAT1 leads to OAT1 internalization from the cell surface and subsequent degradation. The current study showed that the ubiquitinated OAT1 accumulated in the presence of the proteasomal inhibitors MG132 and ALLN rather than the lysosomal inhibitors leupeptin and pepstatin A, suggesting that ubiquitinated OAT1 degrades through proteasomes. Anticancer drugs bortezomib and carfilzomib target the ubiquitin-proteasome pathway. We therefore investigate the roles of bortezomib and carfilzomib in reversing the ubiquitination-induced downregulation of OAT1 expression and transport activity. We showed that bortezomib and carfilzomib extremely increased the ubiquitinated OAT1, which correlated well with an enhanced OAT1-mediated transport of p-aminohippuric acid and an enhanced OAT1 surface expression. The augmented OAT1 expression and transport activity following the treatment with bortezomib and carfilzomib resulted from a reduced rate of OAT1 degradation. Consistent with this, we found decreased 20S proteasomal activity in cells that were exposed to bortezomib and carfilzomib. In conclusion, this study identified the pathway in which ubiquitinated OAT1 degrades and unveiled a novel role of anticancer drugs bortezomib and carfilzomib in their regulation of OAT1 expression and transport activity.

## **Significance Statement**

Bortezomib and carfilzomib are two FDA-approved anticancer drugs, and proteasome is the drug target. In this study, we unveiled a new role of bortezomib and carfilzomib in enhancing OAT1 expression and transport activity by preventing the degradation of ubiquitinated OAT1 in proteasomes. This finding provides a new strategy in regulating OAT1 function, which can be used to accelerate the clearance of drugs, metabolites or toxins, and reverse the decreased expression under disease conditions.

## 1. Introduction

Many clinically important drugs including antiviral therapeutics (e.g. adefovir, acyclovir), antitumor drugs (e.g. methotrexate), antibiotics (e.g. cephaloridine, penicillin G), antihypertensives (e.g. captopril, quinapril), and antiinflammatories ( e.g. salicylate, indomethacin) are eliminated from the kidney through organic anion transporter 1 (OAT1) (Burckhardt, 2012; Liang et al., 2015; You, 2004). OAT1, expressed at the basolateral membrane of the renal proximal tubules, actively transport drugs from blood into tubule cells. followed by the efflux of these drugs to urine by other transporters in the apical membrane (Nigam, 2015; Xu et al., 2016a; You, 2002). The function of OAT1 is the rate-limiting factor in the renal clearance of drugs from the body, affecting drug concentration in the plasma and various tissues, thereby influencing drug therapeutic efficacy and the toxicity.

The transport activity of OAT1 critically relied on their expression level at basolateral membranes of kidney proximal tubule cells, which may be altered under certain pathological and pharmacological conditions (Burckhardt, 2012; Wang and Sweet, 2013). For example, the OAT1 expression in basolateral membranes was increased in the early phase of acute extrahepatic cholestasis, or chronic administration of furosemide (Brandoni et al., 2006b; Kim et al., 2003); while the expression was decreased 3 days of obstructive cholestasis, bilateral ureteral obstruction, chronic or acute renal failure, administration of  $1\alpha,25$ -dihydroxyvitamin  $D_3$  (the biologically active form of vitamin D) (Brandoni et al., 2006a; Kwon et al., 2007; Kwon et al., 2008; Miao et al., 2013; Monica Torres et al., 2005; Villar et al., 2005).

Our lab previously demonstrated that OAT1 constitutively internalizes from and recycles back to cell surface, and ubiquitin (an 8-kDa polypeptide) conjugation was a precedent step in initiating OAT1 internalization to early endosomes (Zhang et al., 2008; Zhang et al., 2013).

Once in the endosomes, OAT1 is either deubiquitinated and recycles back to cell surface or undergoes proteolytic degradation. We further demonstrated that activation of protein kinase C (PKC) inhibits OAT1 transport activity and reduces the amount of OAT1 at the cell surface by enhancing OAT1 ubiquitination, resulting an accelerated OAT1 internalization from cell surface to intracellular early endosomes and subsequent proteolytic degradation (Xu et al., 2017; Zhang et al., 2008; Zhang et al., 2013). Ubiquitination is an important post-translational mechanism of OAT1 regulation (Xu et al., 2016a; Xu and You, 2017).

The proteasomes and the lysosomes are two major systems, through which cells degrade proteins (Clague and Urbe, 2010). The two proteolytic systems can be differentiated by the sensitivity to corresponding inhibitors. Degradation of polypeptides through the proteasomes can be prevented by proteasomal inhibitors such MG132, while lysosomal proteolysis can be hindered by lysosomal inhibitors such as leupeptin (Goldberg and Rock, 2002; Kisselev and Goldberg, 2001; Lee et al., 2011; Yang et al., 2013). Since ubiquitination leads to OAT internalization and degradation, the alteration of proteasome or lysosome activity can potentially affect the transporter function. In the present study, we investigated the proteolytic system in which the ubiquitinated OAT1 is degraded, and explored the strategies of reversing ubiquitination-dependent OAT1 degradation and its influence on OAT1 expression, and transport activity.

## **2. Materials and Methods**

### **2.1. Materials**

HEK293 cells were purchased from ATCC (Manassas, VA). [<sup>3</sup>H]-labeled p-aminohippuric acid (PAH) was purchased from PerkinElmer (Waltham, MA). Sulfo-NHS-SS-biotin, streptavidin agarose resin and protein G agarose were purchased from Thermo Scientific (Rockford, IL). Mouse anti-Myc antibody (9E10) was purchased from Roche (Indianapolis, IN). Mouse anti-E-Cadherin antibody was purchased from Abcam (Cambridge, MA). Mouse anti-ubiquitin antibody, mouse anti-GAPDH antibody and normal mouse IgG were purchased from Santa Cruz Biotechnology (Dallas, TX). LDH cytotoxicity assay kit and 20S proteasome assay kit were purchased from Cayman Chemical (Ann Arbor, MI). Bortezomib and carfilzomib were purchased from Cell Signaling (Danvers, MA). MG132, ALLN, leupeptin, pepstatin A, probenecid and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

### **2.2. Cell culture**

Parental HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning, Tewksbury, MA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) at 37 °C in 5% CO<sub>2</sub>. HEK293 cells stably expressing human OAT1 (hOAT1) were generated in our lab (Xu et al., 2016b). hOAT1 cells were maintained in DMEM medium supplemented with 0.2 mg/ml G418 (Gibco, Grand Island, NY) and 10% fetal bovine serum.

### **2.3. Transport measurement**

The transport activity was measured using the method published in our lab (Zhang et al., 2008). The uptake solution consisted of phosphate-buffered saline (PBS)/Ca<sup>2+</sup>/Mg<sup>2+</sup> (PBS/CM) and [<sup>3</sup>H]-PAH (20 μM) was added to each well. After 3 min, uptake process was stopped by aspirating the uptake solution and rapidly washing the cells with ice-cold PBS solution. The cells

were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquotted for liquid scintillation counting (Beckman LSC LS650).

#### **2.4 Lactate dehydrogenase (LDH) cytotoxicity assay**

Cytotoxicity of bortezomib and carfilzomib was assayed using LDH cytotoxicity assay kit (Cayman Chemical) according to the manufacturer's protocol. After treatment with bortezomib or carfilzomib for 12 h, 100  $\mu$ l supernatant of hOAT1 cells was transferred to a 96-well assay plate, 100  $\mu$ l LDH reaction solution was added and incubated for 30 min at 37°C. Absorbance at 490 nm was read using a microplate reader (Molecular Devices Spectramax M3). 10% Triton X-100-treated and untreated cells were used as positive control and negative control, respectively.

#### **2.5. Measurement of 20S proteasome activity**

Proteasome activity was assayed using 20S proteasome assay kit (Cayman Chemical) according to the manufacturer's protocol. After treatment with MG132, bortezomib, or carfilzomib, hOAT1 cells were washed with 200  $\mu$ l assay buffer and lysed with 100  $\mu$ l lysis buffer. Then, 90  $\mu$ l supernatant was transferred to a black 96-well plate, 10  $\mu$ l assay buffer and 10  $\mu$ l substrate solution (SUC-LLVY-AMC) was added and incubated for 1 h at 37°C. Fluorescence intensity (excitation 360 nm, emission 480 nm) was measured using a microplate reader (Molecular Devices Spectramax M3).

#### **2.6. Cell surface biotinylation**

Cell surface expression level of hOAT1 was examined using the method published in our lab (Zhang et al., 2013). hOAT1 cells was incubated with 1 ml of freshly made sulfo-NHS-SS-biotin (0.5 mg/ml in PBS/CM) in two successive 20 min incubations on ice with very gentle shaking. After biotinylation, each dish was briefly rinsed with 3 ml of PBS/CM containing 100 mM glycine then incubated with the same solution for 20 min on ice, to ensure complete



quenching of the unreacted sulfo-NHS-SS-biotin. The cells were then lysed on ice for 50 min in 400  $\mu$ l of lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100) with 1% proteinase inhibitor cocktail. The cell lysates were cleared by centrifugation at 16,000 g at 4 °C. 40  $\mu$ l of streptavidin agarose resin was then added to the supernatant to isolate cell membrane proteins. hOAT1 was detected in the pool of surface proteins by SDS-PAGE and immunoblotting using anti-Myc antibody 9E10.

## 2.7. Immunoprecipitation

The ubiquitination of hOAT1 was detected using the Immunoprecipitation method published in our lab (Zhang et al., 2013). hOAT1 cells were lysed with lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, and 1 mM NaF) with 1% of proteinase inhibitor cocktail and 20 mM N-ethylmaleimide. Cell lysates were precleared with protein G agarose to reduce nonspecific binding at 4 °C for 2 h. Anti-Myc antibody was incubated with 30  $\mu$ l of protein G agarose at 4 °C for 2 h. The precleared protein sample was then mixed with antibody-bound protein G agarose and underwent end-over-end rotating at 4 °C overnight. Proteins bound to the protein G agarose were eluted with urea buffer containing  $\beta$ -mercaptoethanol and analyzed by immunoblotting with indicated antibodies.

## 2.8. Degradation assay

The degradation of hOAT1 was assayed using the method published in our lab (Wang et al., 2016). hOAT1 cells were first biotinylated, then the labeled cells were incubated in DMEM containing with or without bortezomib and carfilzomib at 37 °C. Treated cells were collected at 0, 4, 8 and 12 h and lysed in lysis buffer with 1% protease inhibitor cocktail. The cell lysates were cleared by centrifugation at 16,000  $\times$  g at 4 °C. 40  $\mu$ l of streptavidin agarose resin were then added to the supernatant to isolate cell membrane proteins. Samples were loaded on 7.5% SDS-PAGE minigels and analyzed by immunoblotting with anti-Myc antibody.

## **2.9. Electrophoresis and immunoblotting**

The electrophoresis and immunoblotting were performed using the method published in our lab (Zhang et al., 2008). Protein samples were resolved on 7.5% SDS-PAGE minigels and electroblotted on to polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS-0.05% tween 20, washed, and incubated overnight at 4 °C with appropriate primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The signals were detected by SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Rockford, IL). Nonsaturating, immunoreactive protein bands were quantified by scanning densitometry with the FluorChem 8000 imaging system (Alpha Innotech Corp., San Leandro, CA).

## **2.10. Data analysis**

Each experiment was repeated a minimum of three times. The statistical analysis was from multiple experiments. Among multiple treatments, one-way ANOVA or two-way ANOVA Tukey's test was applied by using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). A P value of <0.05 was considered statistically significant. ns: not statistically significant.

### 3. Results

#### 3.1. Effects of protease inhibitors on the accumulation of ubiquitinated OAT1

To determine the intracellular degradation system for ubiquitinated OAT1, we treated OAT1-expressing cells with proteasome inhibitors MG132 or ALLN, and lysosome inhibitors leupeptin or pepstatin A for 2 h. The treated cells were lysed, and OAT1 was pulled down by anti-Myc antibody (epitope Myc was tagged to OAT1 to facilitate immuno-detection) or with mouse IgG (as negative control), followed by immunoblotting (IB) with anti-ubiquitin antibody (anti-Ub) to detect ubiquitinated OAT1. Our results (Fig. 1, top panel) revealed that although incubation of the cells with the lysosomal inhibitors leupeptin and pepstatin A were without any effect, incubation with proteasomal inhibitors MG132 and ALLN both led to a substantial accumulation of ubiquitinated OAT1, suggesting that ubiquitinated OAT1 degrades through proteasomes rather than lysosomes. The change of ubiquitinated OAT1 was not resulted from the difference of OAT1 immunoprecipitated as the amount of OAT1 pulled down was similar among all samples (Fig. 1, bottom panel).

#### 3.2. Effects of proteasomal inhibitors bortezomib and carfilzomib on the accumulation of ubiquitinated OAT1

Bortezomib and carfilzomib are FDA-approved anti-cancer drugs acting as selective proteasome inhibitors. We therefore examined the effects of bortezomib and carfilzomib on the accumulation of ubiquitinated OAT1. OAT1-expressing cells were treated with bortezomib or carfilzomib for 12 h. The treated cells were lysed, and OAT1 was pulled down by anti-Myc antibody, followed by immunoblotting (IB) with anti-ubiquitin antibody (anti-Ub) to detect ubiquitinated OAT1. Our results (Fig. 2, top panel) revealed that incubation of the cells with bortezomib or carfilzomib led to a substantial accumulation of ubiquitinated OAT1. The

change of ubiquitinated OAT1 was not resulted from the difference of OAT1 immunoprecipitated as the amount of OAT1 pulled down was similar among all samples (Fig. 2, bottom panel; The full blot of which is included in supplemental Fig. 1).

### **3.3. Cis-effect of bortezomib or carfilzomib on OAT1-mediated uptake of [<sup>3</sup>H]-p-aminohippuric acid**

As OAT1 is a multi-substrate transporter, we examined whether bortezomib and carfilzomib are inhibitors for OAT1 by carrying out a *cis*-inhibition experiment (Fig. 3). We measured 3-min uptake of [<sup>3</sup>H]-p-aminohippuric acid (PAH, 20 μM) into OAT1-expressing cells with 20 μM probenecid, 3 μM bortezomib or carfilzomib being present in the same solution as PAH. Probenecid is a well-known competitive inhibitor for OAT (Nigam et al., 2015; Vallon et al., 2012; Wang et al., 2014). Under such condition, probenecid inhibited OAT1-mediated transport of [<sup>3</sup>H]-PAH by 41% (95% confidence interval (CI): 36% to 46%), whereas bortezomib and carfilzomib were without any effect, suggesting that bortezomib and carfilzomib are not inhibitors for OAT1. Therefore, bortezomib and carfilzomib don't regulate OAT1 through its ability to interfere with the transporter.

### **3.4. Effects of bortezomib and carfilzomib on OAT1-mediated uptake of p-aminohippuric acid**

OAT1-expressing cells were treated with bortezomib and carfilzomib for 12 h, then cytotoxicity and OAT1-mediated uptake of p-aminohippuric acid (PAH) were measured. Both bortezomib and carfilzomib induced a stimulation of PAH uptake at 25~100 nM for bortezomib (Fig. 4a) and 0.1~1 μM for carfilzomib (Fig. 4b) without cytotoxicity at corresponding concentration (Fig. 5). The transport activity of OAT1 was increased by 11% (95% CI: -7% to 30%) and 49% (95% CI: 31% to 67%) respectively at 5 nM and 25 nM bortezomib. In contrast, 12 h-treatment with lysosome inhibitors leupeptin and pepstatin A did not affect the uptake of

PAH (Fig. 4c). Further study showed that like MG132, bortezomib and carfilzomib inhibited the 20S proteasome activity after 2 h of treatment (Fig. 6a). For bortezomib at 1 nM, 5 nM and 25 nM with treatment for 12 h, the proteasome activity was inhibited by 8% (95% CI: 5% to 12%), 29% (95% CI: 26% to 33%) and 87% (95% CI: 84% to 91%) respectively, which showed a concentration-dependent inhibition of proteasome activity at 1~25 nM bortezomib (Fig. 6b).

### **3.5. Effect of bortezomib and carfilzomib on OAT1 expression**

OAT1-expressing cells were treated with bortezomib or carfilzomib, OAT1 expression both at the cell surface and in the total cell lysates were examined. We showed that treatment with bortezomib or carfilzomib led to an increase of OAT1 expression at the cell surface (Fig. 7a, top panel and Fig. 7b), and in total cell lysate (Fig. 7c, top panel, Fig. 7d). Such a change in OAT1 expression was not due to the general perturbation of cellular proteins as the expression of cell surface membrane protein marker E-Cadherin (Fig. 7a, bottom panel) and cellular protein marker GAPDH (Fig. 7c, bottom panel) was not affected under these conditions.

### **3.6. Effect of bortezomib and carfilzomib on OAT1 stability**

The stability of cell surface OAT1 was subsequently assessed based on a biotinylation approach. OAT1-expressing cells were biotinylated with membrane-impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were treated with or without bortezomib or carfilzomib for 12 h. Treated cells were then lysed and cell surface proteins were isolated using streptavidin agarose resin, followed by immunoblotting with anti-Myc antibody. Our results (Fig. 8) showed that the rate of OAT1 degradation decreased substantially after 8 and 12 h treatment with bortezomib (Fig. 8a and Fig 8b) or carfilzomib (Fig. 8c and Fig. 8d) as compared to that of control. These results indicate that bortezomib and carfilzomib substantially increases OAT1 stability.

#### 4. Discussion

The transport activity of OAT1 is critically dependent on its expression level at the cell surface. We previously demonstrated that post-translational modification of OAT1 by ubiquitination leads to OAT1 internalization from the cell surface and subsequent degradation in proteolytic systems (Xu et al., 2017; Zhang et al., 2008; Zhang et al., 2013). In the current study, we identified the proteolytic system in which the ubiquitinated OAT1 is degraded and revealed a new strategy of reversing ubiquitination-dependent OAT1 degradation.

HEK293 cells, a heterologous cell system was used in our current studies, as they have been widely used for research in the regulation of the cloned organic anion transporters and other renal transporters (El-Sheikh et al., 2013; Rodiger et al., 2010; Xue et al., 2011; Zeng et al., 2012). The fact that these cells do not express endogenous OATs is particularly advantageous, because expression of OAT1 in these cells will permit us to analyze the transport properties of OAT1 without being disturbed by other organic anion transporters. Our studies in HEK293 cells will pave the path for the upcoming work focusing on validating whether the same mechanisms exist in primary epithelia.

Our current study unveiled a key role for proteasomes in the regulation of OAT1 expression and transport activity. Treatment of OAT1-expressing cells with proteasomal inhibitors MG132 and ALLN led to an accumulation of ubiquitinated OAT1, whereas treatment of cells with lysosomal inhibitors leupeptin and pepstatin A were without any effect (Fig. 1). The degradation of cell surface OAT1 through proteasomes contrasted with most of the plasma membrane proteins, for which the canonical degradation pathway is through lysosomes (Piper and Luzio, 2007; Varghese et al., 2008). This finding opens the door for new strategies in modulating OAT1 function through controlling proteasomal activity.

Proteasomal inhibition has been developed for antitumor activities. The proteasomal inhibitors used in our current studies, bortezomib and carfilzomib are two FDA-approved antitumor drugs with the ability to inhibit proteasomes. Bortezomib administration caused a decrease of 20S proteasome activity in the white blood cells, liver, colon, muscle and prostate (Adams et al., 1999; Bross et al., 2004). Inhibition of the 20S proteasome activity after carfilzomib administration was observed in blood, adrenal, heart, lung, spleen, bone marrow and kidney (Nooka et al., 2013). Both drugs have also been shown to affect kidney function. Bortezomib can attenuate renal impairment in patients with multiple myeloma, renal fibrosis and lupus nephritis (Cohen et al., 2015; Dimopoulos et al., 2016; Hainz et al., 2012; Ward et al., 2012; Zeniya et al., 2017; Zhang et al., 2017). Carfilzomib inhibited the chymotrypsin-like activity of the 20S proteasome in the kidney by 50~60% in rats (FDA, 2012). Recent studies showed that bortezomib can affect the activities of copper transporter 1, ATP-binding cassette transporter A1 (ABCA1) and ABCG1, metal transporter ZIP14 and organic anion transporting polypeptide (OATP) 1B3 (Alam et al., 2017; Jandial et al., 2009; Ogura et al., 2011; Zhao et al., 2014). However, the potential of these antitumor drugs to modulate OAT1 activity has not been explored. By inhibiting the proteasomal activity, these drugs gained new role in regulation of OAT1 activity.

We showed that treatment of OAT1-expressing cells with bortezomib and carfilzomib lead to a substantial accumulation of the ubiquitinated OAT1 (Fig. 2), which correlated well with an enhanced OAT1 transport activity and an enhanced OAT1 expression at the cell surface (Fig. 4 and Fig. 7). The molecular weight of ubiquitinated OAT1 was more than 180 kDa, ~100 kDa larger than unubiquitinated OAT1 (~80 kDa). Since ubiquitin is an 8-kDa polypeptide, OAT1 may be poly- or multi- ubiquitinated (Fig. 1 and Fig. 2).

The increase of surface OAT1 can be attributed to reduced internalization, increased recycling, or decreased degradation. As internalization and recycling are rapid processes, the alteration of internalization or recycling may be the mechanisms in acute regulation of OAT1 during short term (<30 min); while the alteration of degradation may be the mechanism in chronic regulation of OAT1 during long term (several hours) (Wang et al., 2019; Xu et al., 2017; Zhang et al., 2008; Zhang et al., 2013; Zhang et al., 2012). Bortezomib and carfilzomib enhanced the surface expression and transport activity of OAT1 with 12-h treatment and didn't stimulate the transport activity in short-time treatment, suggesting that reduced internalization or increased recycling weren't involved in OAT1 regulation. Further exploring the underlying mechanism, we found that the degradation rate of OAT1 was decelerated after treatment with bortezomib and carfilzomib (Fig. 8). 20S proteasome is the drug target of bortezomib and carfilzomib. Our results showed bortezomib and carfilzomib inhibited the 20S proteasome activity (Fig. 6a), and there was a correlation between the degree of proteasomal inhibition and increase of OAT1 transporter function at 5 and 25 nM bortezomib (Fig. 4a and Fig. 6b). Therefore, bortezomib- and carfilzomib-stimulated OAT1 expression and transport activity was mainly due to proteasome inhibition and subsequent deceleration rate of OAT1 degradation.

Following intravenous administration of the first 1.3 mg/m<sup>2</sup> dose to multiple myeloma patients, the mean maximum plasma concentration ( $C_{max}$ ) of bortezomib was 291 nM (112 ng/ml) (Reece et al., 2011). For carfilzomib, the mean  $C_{max}$  following a 2- to 10-min intravenous infusion of 27 mg/m<sup>2</sup> dose or a 30-min infusion of 56 mg/m<sup>2</sup> dose were 5.9  $\mu$ M (4232 ng/ml) and 2.9  $\mu$ M (2079 ng/ml), respectively (FDA, 2018). The concentration of bortezomib (5~100 nM) and carfilzomib (0.1~1  $\mu$ M) we selected in cell model has a clinical relevance. Besides, other proteasome inhibitors ixazomib (approved), marizomib, oprozomib, and delanzomib's (in clinical trials) influence on the kidney OAT1 should be paid attention. In this study, we reported



the cellular mechanisms, and *in vivo* study is undergoing in our lab to validate the roles of bortezomib or carfilzomib in OAT1 ubiquitination, expression, and renal clearance of drugs.

Like proteasomes, several deubiquitinases may also regulate the OAT function. These deubiquitinases are associated with the 19S regulatory particles of proteasome (D'Arcy et al., 2015). Recently, it has been revealed that inhibition of proteasome-associated deubiquitinases activity is an alternative strategy to 20S proteasome inhibitors for cancer treatment (Chen et al., 2017; Mofers et al., 2017). Therefore, it is interesting to explore whether proteasome-associated deubiquitinases is a novel target for OAT1 regulation.

Our studies that proteasome inhibitors, e.g. bortezomib and carfilzomib can stimulate the transport activity of OAT1 has physiological implication. When drugs are overdose or endogenous/exogenous metabolites, uremic/environmental toxins are increased in blood, we can use this method to accelerate their clearance to avoid systemic toxicity and maintain the body homeostasis. It also be used to reverse the decreased expression under disease conditions.

In conclusion, our study demonstrated for the first time that ubiquitinated cell surface OAT1 degrades through proteasome instead of lysosome, and anticancer drugs bortezomib and carfilzomib have a novel role in regulating OAT1 expression and transport activity, indicating their potential influence on the OAT1-mediated renal excretion of drugs during cancer and comorbidity therapies.

## **Acknowledgement**

We thank Dr. Haoxun Wang for his preliminary work, which paved the way for this study.

## **Authorship Contributions**

*Participated in research design:* Fan, You.

Conducted experiments: Fan

Performed data analysis: Fan, You.

Wrote or contributed to the writing of the manuscript: Fan, You

## References

- Adams J, Palombella VJ, Sausville EA, Johnson J, Destree A, Lazarus DD, Maas J, Pien CS, Prakash S and Elliott PJ (1999) Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer research* **59**(11): 2615-2622.
- Alam K, Farasyn T, Crowe A, Ding K and Yue W (2017) Treatment with proteasome inhibitor bortezomib decreases organic anion transporting polypeptide (OATP) 1B3-mediated transport in a substrate-dependent manner. *PLoS one* **12**(11): e0186924.
- Brandoni A, Anzai N, Kanai Y, Endou H and Torres AM (2006a) Renal elimination of p-aminohippurate (PAH) in response to three days of biliary obstruction in the rat. The role of OAT1 and OAT3. *Biochimica et biophysica acta* **1762**(7): 673-682.
- Brandoni A, Villar SR, Picena JC, Anzai N, Endou H and Torres AM (2006b) Expression of rat renal cortical OAT1 and OAT3 in response to acute biliary obstruction. *Hepatology* **43**(5): 1092-1100.
- Bross PF, Kane R, Farrell AT, Abraham S, Benson K, Brower ME, Bradley S, Gobburu JV, Goheer A, Lee SL, Leighton J, Liang CY, Lostritto RT, McGuinn WD, Morse DE, Rahman A, Rosario LA, Verbois SL, Williams G, Wang YC and Pazdur R (2004) Approval summary for bortezomib for injection in the treatment of multiple myeloma. *Clinical cancer research : an official journal of the American Association for Cancer Research* **10**(12 Pt 1): 3954-3964.
- Burckhardt G (2012) Drug transport by Organic Anion Transporters (OATs). *Pharmacology & therapeutics* **136**(1): 106-130.
- Chen X, Yang Q, Xiao L, Tang D, Dou QP and Liu J (2017) Metal-based proteasomal deubiquitinase inhibitors as potential anticancer agents. *Cancer metastasis reviews* **36**(4): 655-668.
- Clague MJ and Urbe S (2010) Ubiquitin: same molecule, different degradation pathways. *Cell* **143**(5): 682-685.
- Cohen C, Royer B, Javaugue V, Szalat R, El Karoui K, Caulier A, Knebelmann B, Jaccard A, Chevret S, Touchard G, Femand JP, Arnulf B and Bridoux F (2015) Bortezomib produces high hematological response rates with prolonged renal survival in monoclonal immunoglobulin deposition disease. *Kidney international* **88**(5): 1135-1143.
- D'Arcy P, Wang X and Linder S (2015) Deubiquitinase inhibition as a cancer therapeutic strategy. *Pharmacology & therapeutics* **147**: 32-54.
- Dimopoulos MA, Sonneveld P, Leung N, Merlini G, Ludwig H, Kastritis E, Goldschmidt H, Joshua D, Orlowski RZ, Powles R, Vesole DH, Garderet L, Einsele H, Palumbo A, Cavo M, Richardson PG, Moreau P, San Miguel J, Rajkumar SV, Durie BG and Terpos E (2016) International Myeloma Working Group Recommendations for the Diagnosis and Management of Myeloma-Related Renal Impairment. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **34**(13): 1544-1557.
- El-Sheikh AA, Greupink R, Wortelboer HM, van den Heuvel JJ, Schreurs M, Koenderink JB, Masereeuw R and Russel FG (2013) Interaction of immunosuppressive drugs with human organic anion transporter (OAT) 1 and OAT3, and multidrug resistance-associated protein (MRP) 2 and MRP4. *Translational research : the journal of laboratory and clinical medicine* **162**(6): 398-409.
- FDA (2012) Pharmacology Review(s) for KYPROLIS® (carfilzomib). [https://www.accessdata.fda.gov/drugsatfda\\_docs/nda/2012/202714Orig1s000PharmR.pdf](https://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/202714Orig1s000PharmR.pdf).
- FDA (2018) Label revision for KYPROLIS® (carfilzomib) [https://www.accessdata.fda.gov/drugsatfda\\_docs/label/2018/202714s019lbl.pdf](https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/202714s019lbl.pdf).
- Goldberg AL and Rock K (2002) Not just research tools--proteasome inhibitors offer therapeutic promise. *Nature medicine* **8**(4): 338-340.
- Hainz N, Thomas S, Neubert K, Meister S, Benz K, Rauh M, Daniel C, Wiesener M, Voll RE and Amann K (2012) The proteasome inhibitor bortezomib prevents lupus nephritis in the NZB/W F1 mouse

- model by preservation of glomerular and tubulointerstitial architecture. *Nephron Experimental nephrology* **120**(2): e47-58.
- Jandial DD, Farshchi-Heydari S, Larson CA, Elliott GI, Wrasidlo WJ and Howell SB (2009) Enhanced delivery of cisplatin to intraperitoneal ovarian carcinomas mediated by the effects of bortezomib on the human copper transporter 1. *Clinical cancer research : an official journal of the American Association for Cancer Research* **15**(2): 553-560.
- Kim GH, Na KY, Kim SY, Joo KW, Oh YK, Chae SW, Endou H and Han JS (2003) Up-regulation of organic anion transporter 1 protein is induced by chronic furosemide or hydrochlorothiazide infusion in rat kidney. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* **18**(8): 1505-1511.
- Kisselev AF and Goldberg AL (2001) Proteasome inhibitors: from research tools to drug candidates. *Chemistry & biology* **8**(8): 739-758.
- Kwon O, Hong SM and Blouch K (2007) Alteration in renal organic anion transporter 1 after ischemia/reperfusion in cadaveric renal allografts. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **55**(6): 575-584.
- Kwon O, Wang WW and Miller S (2008) Renal organic anion transporter 1 is maldistributed and diminishes in proximal tubule cells but increases in vasculature after ischemia and reperfusion. *American journal of physiology Renal physiology* **295**(6): F1807-1816.
- Lee S, Sato Y and Nixon RA (2011) Lysosomal proteolysis inhibition selectively disrupts axonal transport of degradative organelles and causes an Alzheimer's-like axonal dystrophy. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31**(21): 7817-7830.
- Liang Y, Li S and Chen L (2015) The physiological role of drug transporters. *Protein & cell* **6**(5): 334-350.
- Miao Q, Liu Q, Wang C, Meng Q, Guo X, Sun H, Peng J, Ma X, Kaku T and Liu K (2013) Inhibitory effect of 1 $\alpha$ ,25-dihydroxyvitamin D(3) on excretion of JBP485 via organic anion transporters in rats. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences* **48**(1-2): 351-359.
- Mofers A, Pellegrini P, Linder S and D'Arcy P (2017) Proteasome-associated deubiquitinases and cancer. *Cancer metastasis reviews* **36**(4): 635-653.
- Monica Torres A, Mac Laughlin M, Muller A, Brandoni A, Anzai N and Endou H (2005) Altered renal elimination of organic anions in rats with chronic renal failure. *Biochimica et biophysica acta* **1740**(1): 29-37.
- Nigam SK (2015) What do drug transporters really do? *Nature reviews Drug discovery* **14**(1): 29-44.
- Nigam SK, Bush KT, Martovetsky G, Ahn SY, Liu HC, Richard E, Bhatnagar V and Wu W (2015) The organic anion transporter (OAT) family: a systems biology perspective. *Physiological reviews* **95**(1): 83-123.
- Nooka A, Gleason C, Casbourne D and Lonial S (2013) Relapsed and refractory lymphoid neoplasms and multiple myeloma with a focus on carfilzomib. *Biologics : targets & therapy* **7**: 13-32.
- Ogura M, Ayaori M, Terao Y, Hisada T, Iizuka M, Takiguchi S, Uto-Kondo H, Yakushiji E, Nakaya K, Sasaki M, Komatsu T, Ozasa H, Ohsuzu F and Ikewaki K (2011) Proteasomal inhibition promotes ATP-binding cassette transporter A1 (ABCA1) and ABCG1 expression and cholesterol efflux from macrophages in vitro and in vivo. *Arteriosclerosis, thrombosis, and vascular biology* **31**(9): 1980-1987.
- Piper RC and Luzio JP (2007) Ubiquitin-dependent sorting of integral membrane proteins for degradation in lysosomes. *Current opinion in cell biology* **19**(4): 459-465.
- Reece DE, Sullivan D, Lonial S, Mohrbacher AF, Chatta G, Shustik C, Burris H, 3rd, Venkatakrishnan K, Neuwirth R, Riordan WJ, Karol M, von Moltke LL, Acharya M, Zannikos P and Keith Stewart A (2011) Pharmacokinetic and pharmacodynamic study of two doses of bortezomib in patients with relapsed multiple myeloma. *Cancer chemotherapy and pharmacology* **67**(1): 57-67.

- Rodiger M, Zhang X, Ugele B, Gersdorff N, Wright SH, Burckhardt G and Bahn A (2010) Organic anion transporter 3 (OAT3) and renal transport of the metal chelator 2,3-dimercapto-1-propanesulfonic acid (DMPS). *Canadian journal of physiology and pharmacology* **88**(2): 141-146.
- Vallon V, Eraly SA, Rao SR, Gerasimova M, Rose M, Nagle M, Anzai N, Smith T, Sharma K, Nigam SK and Rieg T (2012) A role for the organic anion transporter OAT3 in renal creatinine secretion in mice. *American journal of physiology Renal physiology* **302**(10): F1293-1299.
- Varghese B, Barriere H, Carbone CJ, Banerjee A, Swaminathan G, Plotnikov A, Xu P, Peng J, Goffin V, Lukacs GL and Fuchs SY (2008) Polyubiquitination of prolactin receptor stimulates its internalization, postinternalization sorting, and degradation via the lysosomal pathway. *Molecular and cellular biology* **28**(17): 5275-5287.
- Villar SR, Brandoni A, Anzai N, Endou H and Torres AM (2005) Altered expression of rat renal cortical OAT1 and OAT3 in response to bilateral ureteral obstruction. *Kidney international* **68**(6): 2704-2713.
- Wang C, Wang C, Liu Q, Meng Q, Cang J, Sun H, Peng J, Ma X, Huo X and Liu K (2014) Aspirin and probenecid inhibit organic anion transporter 3-mediated renal uptake of cilostazol and probenecid induces metabolism of cilostazol in the rat. *Drug metabolism and disposition: the biological fate of chemicals* **42**(6): 996-1007.
- Wang H, Xu D, Toh MF, Pao AC and You G (2016) Serum- and glucocorticoid-inducible kinase SGK2 regulates human organic anion transporters 4 via ubiquitin ligase Nedd4-2. *Biochemical pharmacology* **102**: 120-129.
- Wang H, Zhang J and You G (2019) Activation of Protein Kinase A Stimulates SUMOylation, Expression, and Transport Activity of Organic Anion Transporter 3. *The AAPS journal* **21**(2): 30.
- Wang L and Sweet DH (2013) Renal organic anion transporters (SLC22 family): expression, regulation, roles in toxicity, and impact on injury and disease. *The AAPS journal* **15**(1): 53-69.
- Ward F, Dunne O, Crotty TB, Fennelly D, Watson A and Holian J (2012) Successful use of combined high cut-off haemodialysis and bortezomib for acute kidney injury associated with myeloma cast nephropathy. *Irish medical journal* **105**(5): 148-149.
- Xu D, Wang H and You G (2016a) Posttranslational Regulation of Organic Anion Transporters by Ubiquitination: Known and Novel. *Medicinal research reviews* **36**(5): 964-979.
- Xu D, Wang H, Zhang Q and You G (2016b) Nedd4-2 but not Nedd4-1 is critical for protein kinase C-regulated ubiquitination, expression, and transport activity of human organic anion transporter 1. *American journal of physiology Renal physiology* **310**(9): F821-831.
- Xu D and You G (2017) Loops and layers of post-translational modifications of drug transporters. *Advanced drug delivery reviews* **116**: 37-44.
- Xu D, Zhang J, Zhang Q, Fan Y, Liu C and You G (2017) PKC/Nedd4-2 Signaling Pathway Regulates the Cell Surface Expression of Drug Transporter hOAT1. *Drug metabolism and disposition: the biological fate of chemicals* **45**(8): 887-895.
- Xue X, Gong LK, Maeda K, Luan Y, Qi XM, Sugiyama Y and Ren J (2011) Critical role of organic anion transporters 1 and 3 in kidney accumulation and toxicity of aristolochic acid I. *Molecular pharmaceutics* **8**(6): 2183-2192.
- Yang YP, Hu LF, Zheng HF, Mao CJ, Hu WD, Xiong KP, Wang F and Liu CF (2013) Application and interpretation of current autophagy inhibitors and activators. *Acta pharmacologica Sinica* **34**(5): 625-635.
- You G (2002) Structure, function, and regulation of renal organic anion transporters. *Medicinal research reviews* **22**(6): 602-616.
- You G (2004) Towards an understanding of organic anion transporters: structure-function relationships. *Medicinal research reviews* **24**(6): 762-774.

- Zeng Y, Zhang R, Wu J, Liu M, Peng W, Yu X and Yang X (2012) Organic anion transporter 1 (OAT1) involved in renal cell transport of aristolochic acid I. *Human & experimental toxicology* **31**(8): 759-770.
- Zeniya M, Mori T, Yui N, Nomura N, Mandai S, Isobe K, Chiga M, Sohara E, Rai T and Uchida S (2017) The proteasome inhibitor bortezomib attenuates renal fibrosis in mice via the suppression of TGF-beta1. *Scientific reports* **7**(1): 13086.
- Zhang H, Liu Z, Huang L, Hou J, Zhou M, Huang X, Hu W and Liu Z (2017) The short-term efficacy of bortezomib combined with glucocorticoids for the treatment of refractory lupus nephritis. *Lupus* **26**(9): 952-958.
- Zhang Q, Hong M, Duan P, Pan Z, Ma J and You G (2008) Organic anion transporter OAT1 undergoes constitutive and protein kinase C-regulated trafficking through a dynamin- and clathrin-dependent pathway. *The Journal of biological chemistry* **283**(47): 32570-32579.
- Zhang Q, Li S, Patterson C and You G (2013) Lysine 48-linked polyubiquitination of organic anion transporter-1 is essential for its protein kinase C-regulated endocytosis. *Molecular pharmacology* **83**(1): 217-224.
- Zhang Q, Suh W, Pan Z and You G (2012) Short-term and long-term effects of protein kinase C on the trafficking and stability of human organic anion transporter 3. *International journal of biochemistry and molecular biology* **3**(2): 242-249.
- Zhao N, Zhang AS, Worthen C, Knutson MD and Enns CA (2014) An iron-regulated and glycosylation-dependent proteasomal degradation pathway for the plasma membrane metal transporter ZIP14. *Proceedings of the National Academy of Sciences of the United States of America* **111**(25): 9175-9180.

## Footnotes

This work was supported by grants (to Dr. Guofeng You) from National Institute of General Medical Sciences (R01-GM079123 and R01-GM127788).

## Figure Legends

**Fig. 1. Effects of protease inhibitors on the accumulation of ubiquitinated OAT1.** Top panel: OAT1-expressing HEK293 cells were treated proteasome inhibitor MG132 (10  $\mu$ M), ALLN (100  $\mu$ M), or lysosome inhibitor leupeptin (10  $\mu$ M), pepstatin A (10  $\mu$ M) for 2 h. Treated cells were then lysed, and OAT1 was immunoprecipitated with anti-Myc antibody or with mouse IgG (as negative control, lane 6), followed by immunoblotting (IB) with anti-ubiquitin antibody (anti-Ub). Bottom panel: The same immunoblot from top panel was reprobed with anti-Myc antibody to determine the amount of OAT1 immunoprecipitated.

**Fig. 2. Effects of proteasomal inhibitors bortezomib and carfilzomib on the accumulation of ubiquitinated OAT1.** Top panel: OAT1-expressing HEK293 cells were treated with bortezomib (25 nM) or carfilzomib (0.5  $\mu$ M) for 12 h. Treated cells were then lysed, and OAT1 was immunoprecipitated with anti-Myc antibody or with mouse IgG (as negative control, lane 3 and 6), followed by immunoblotting (IB) with anti-ubiquitin antibody (anti-Ub). Bottom panel: The same immunoblot from top panel was reprobed with anti-Myc antibody to determine the amount of OAT1 immunoprecipitated.

**Fig. 3. Cis-effect of bortezomib or carfilzomib on OAT1-mediated uptake of [<sup>3</sup>H]p-aminohippuric acid.** The uptake of [<sup>3</sup>H]-p-aminohippuric acid (PAH, 20  $\mu$ M) in the presence of bortezomib (3  $\mu$ M), carfilzomib (3  $\mu$ M), or probenecid (20  $\mu$ M) for 3 min were measured in OAT1-expressing HEK293 cells. Each data point represented only carrier mediated transport after subtraction of values from parental cells. Uptake activity was expressed as percentage of uptake measured in control cells from three independent experiments. Values are mean  $\pm$  SD (n=3). \*P < 0.05. ns: not statistically significant.



**Fig. 4. Effect of bortezomib, carfilzomib, and lysosome inhibitors on OAT1 activity.** OAT1-expressing HEK293 cells were treated with bortezomib (Fig. 4a) or with carfilzomib (Fig. 4b) at indicated concentrations, or lysosome inhibitor 10  $\mu$ M leupeptin, 10  $\mu$ M pepstatin A (Fig. 4c) for 12 h. The uptake of [ $^3$ H]-PAH (20  $\mu$ M) for 3 min was then performed. Each data point represented only carrier mediated transport after subtraction of values from parental cells. Uptake activity was expressed as percentage of uptake measured in control cells from three independent experiments. Values are mean  $\pm$  SD (n=3). \*P < 0.05. ns: not statistically significant.

**Fig. 5 Cytotoxicity of bortezomib and carfilzomib on OAT1-expressing HEK293 cells.** OAT1-expressing HEK293 cells were treated with bortezomib (Fig. 5a) or with carfilzomib (Fig. 5b) at indicated concentrations for 12 h. The lactate dehydrogenase (LDH) released in supernatant was then performed. 10% Triton X-100-treated and untreated cells were used as positive control and negative control, respectively.

**Fig. 6. Effect of bortezomib and carfilzomib on the 20S proteasome activity.** (a) OAT1-expressing HEK293 cells were treated with MG132 (10  $\mu$ M), bortezomib (25 nM) or carfilzomib (0.5  $\mu$ M) for 2 h. (b) OAT1-expressing HEK293 cells were treated with bortezomib (1 nM, 5 nM and 25 nM) for 12 h. The 20S proteasome activity of cells was then performed. The 20S proteasome activity was expressed as percentage of control cells from three independent experiments. Values are mean  $\pm$  SD (n=3). \*P < 0.05.

**Fig. 7. Effect of bortezomib and carfilzomib on OAT1 expression.** (a) Top panel: OAT1-expressing HEK293 cells were treated with bortezomib (25 nM) or carfilzomib (0.5  $\mu$ M) for 12 h. Cell surface biotinylation was performed. Biotinylated (cell surface) proteins were separated with using streptavidin agarose resin and analyzed by immunoblotting (IB) with an anti-Myc antibody. Bottom panel: The same blot from the top panel was reprobed with an anti-E-Cadherin antibody. E-Cadherin is an integral membrane protein marker. (b) Densitometry plot of results from Fig. 7a, top panel as well as from other experiments. Values are means  $\pm$  SD (n=3). \*P < 0.05. (c) Top panel: OAT1-expressing HEK293 cells were treated with bortezomib (25 nM) or carfilzomib (0.5  $\mu$ M) for 12 h. Cells were then lysed, followed by immunoblotting (IB) with anti-Myc antibody. Bottom panel: The same blot from the top panel was reprobed with an anti-GAPDH antibody. GAPDH is a cellular protein marker. (d) Densitometry plot of results from Fig. 7c, top panel as well as from other experiments. Values are means  $\pm$  SD (n=3). \*P < 0.05.

**Fig. 8. Effect of bortezomib or carfilzomib on OAT1 stability.** (a) OAT1-expressing HEK293 cells were biotinylated with membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were then treated with bortezomib (25 nM) at 37°C for 4, 8, 12 h, respectively. Treated cells were lysed and cell surface proteins were isolated using streptavidin agarose resin, followed by immunoblotting (IB) with anti-Myc antibody. (b) Densitometry plot of results from Fig. 8a as well as from other experiments. Values are mean  $\pm$  SD (n=3). \*P < 0.05. ns: not statistically significant. (c) OAT1-expressing HEK293 cells were biotinylated with membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were then treated with carfilzomib (0.5  $\mu$ M) at 37°C for 4, 8, 12 h, respectively. Treated cells were lysed and cell surface proteins were isolated using streptavidin agarose resin, followed by immunoblotting (IB) with anti-Myc antibody. (d) Densitometry plot of results from Fig. 8c as well as from other experiments. Values are mean  $\pm$  SD (n=3). \*P < 0.05. ns: not statistically significant.

# Fig. 1

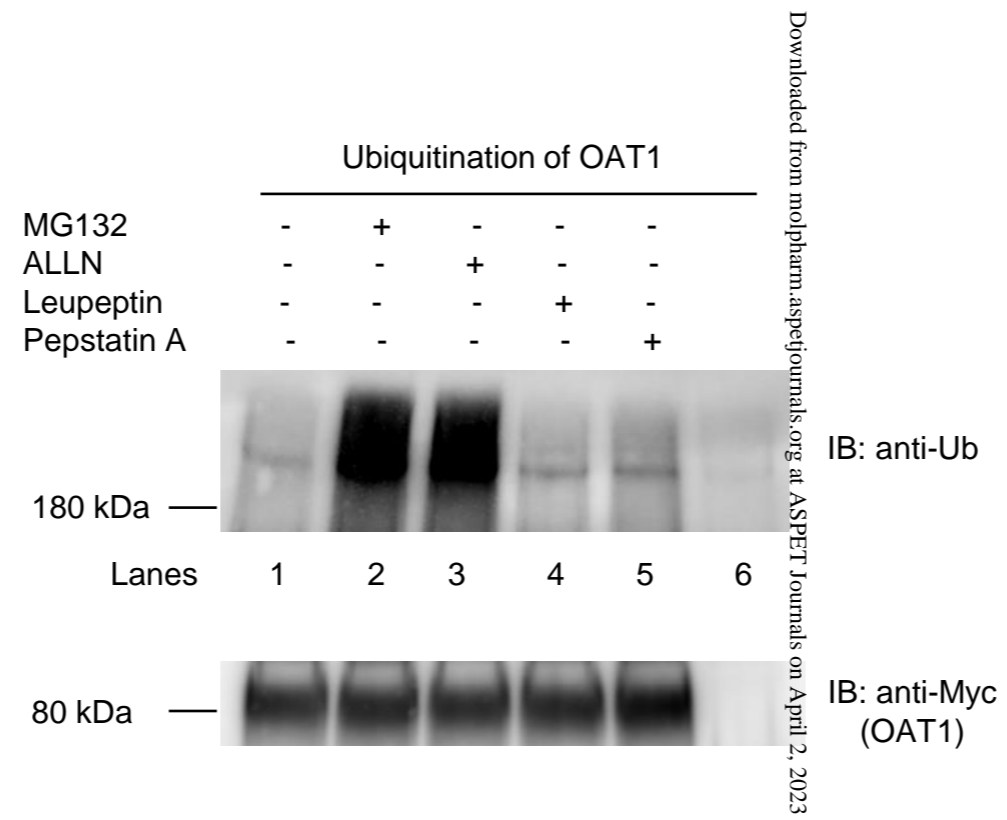


Fig. 2

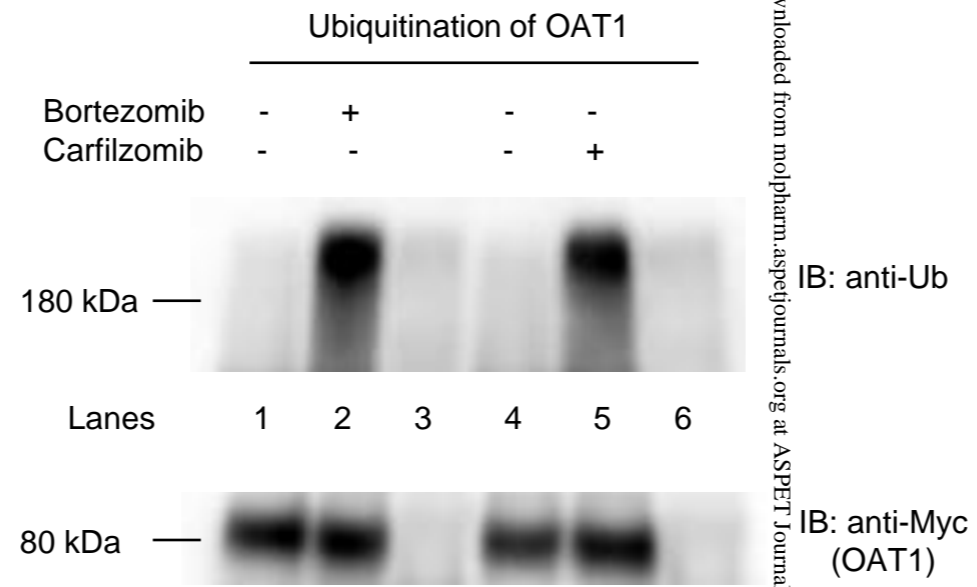


Fig. 3

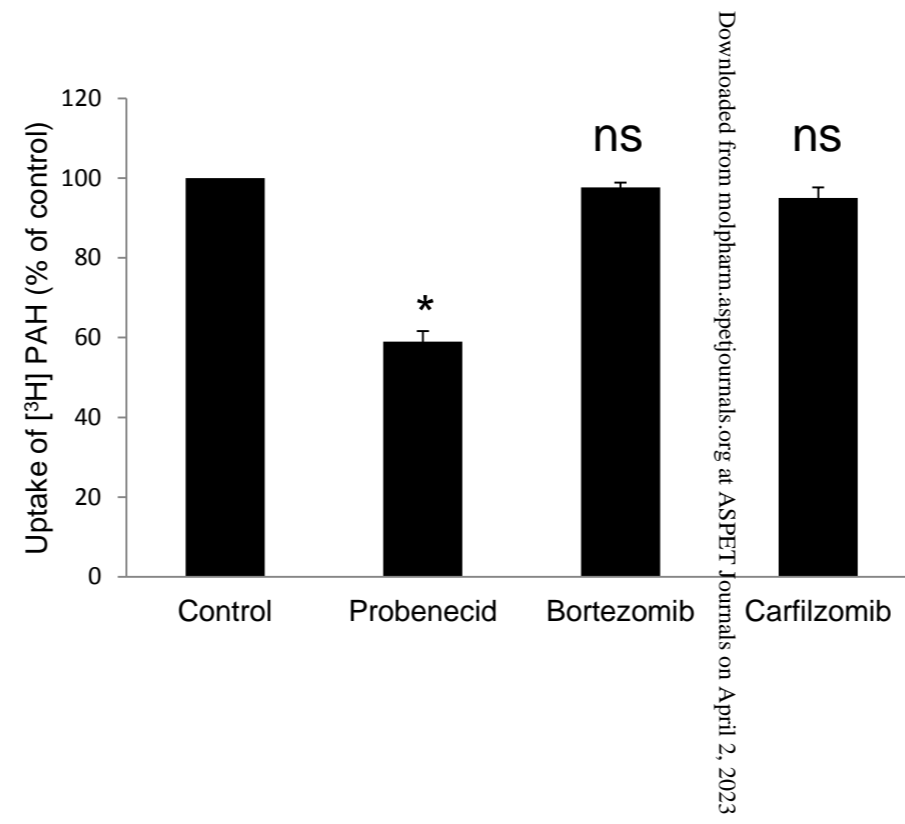
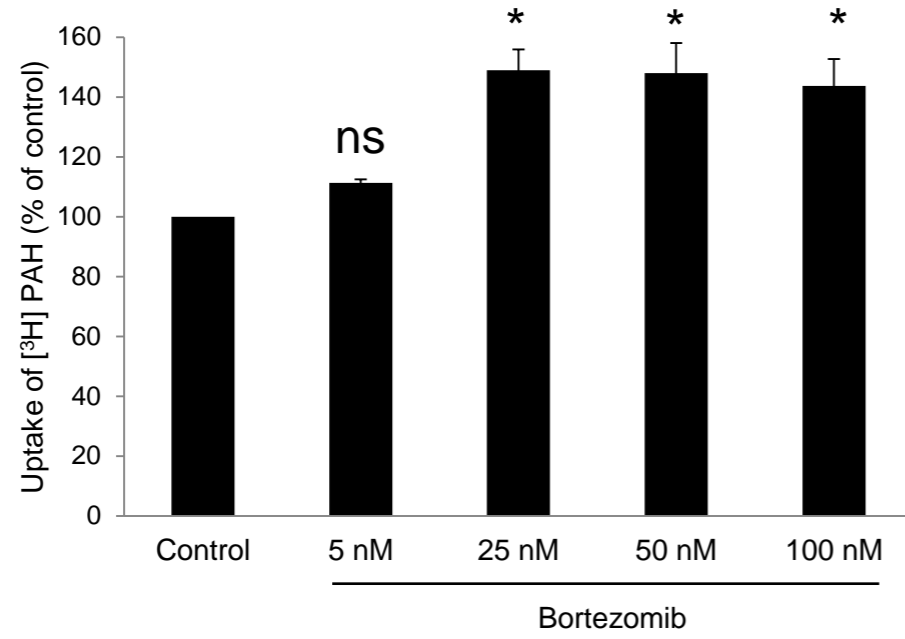
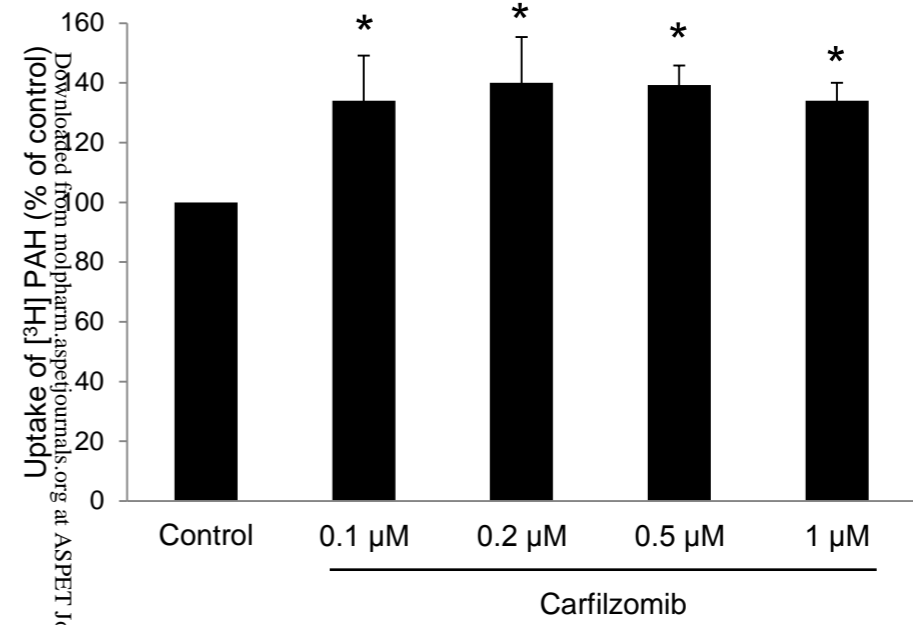


Fig. 4

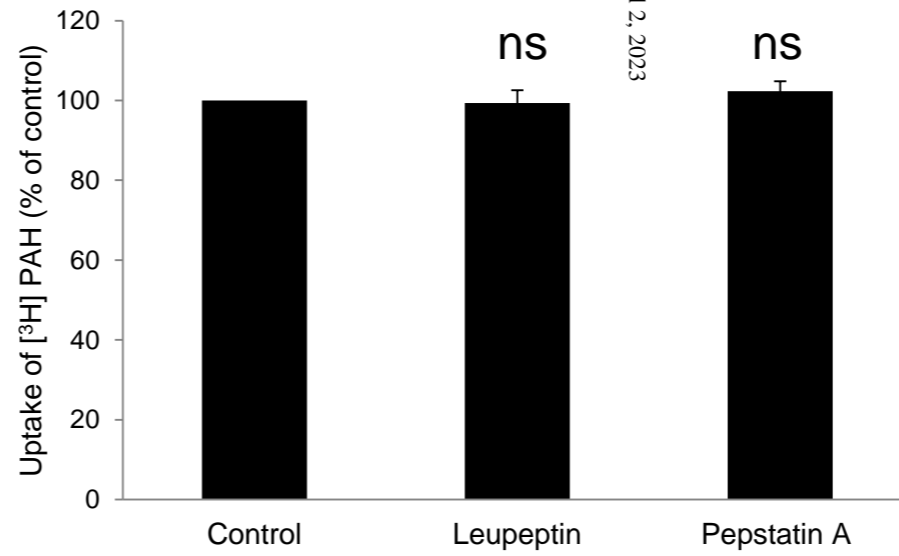
**a**



**b**



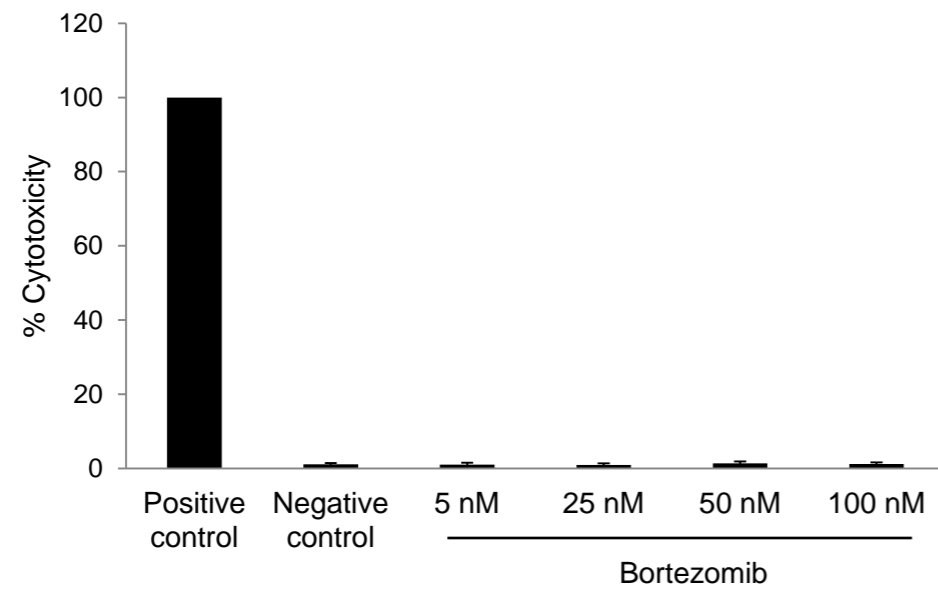
**c**



Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 2, 2023

Fig. 5

**a**



**b**

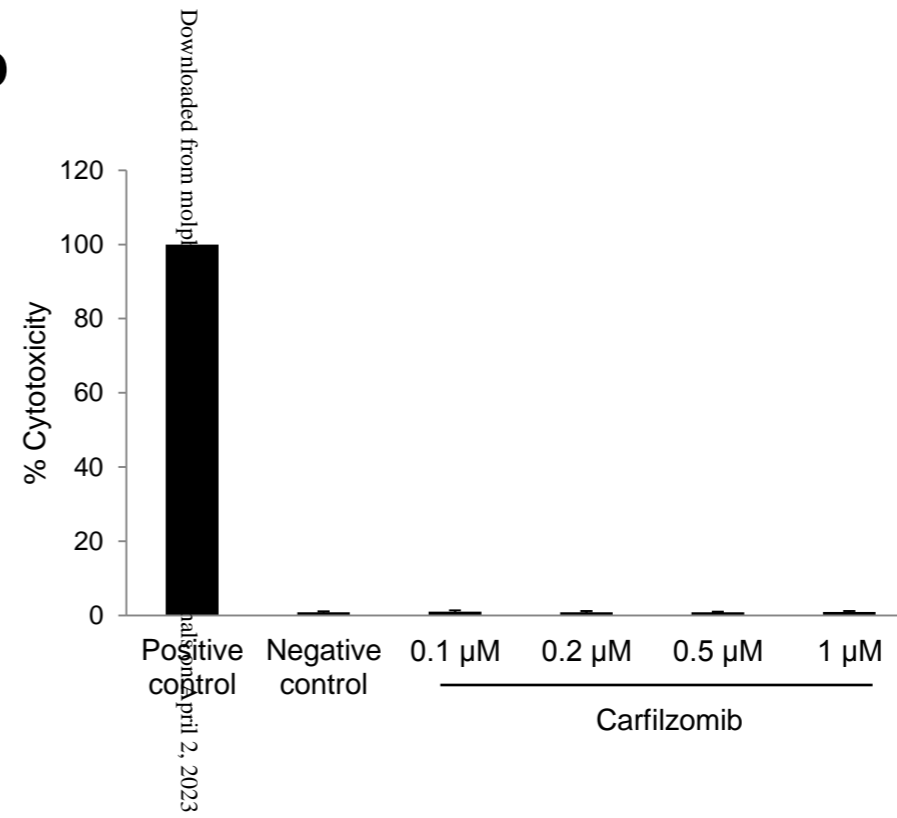
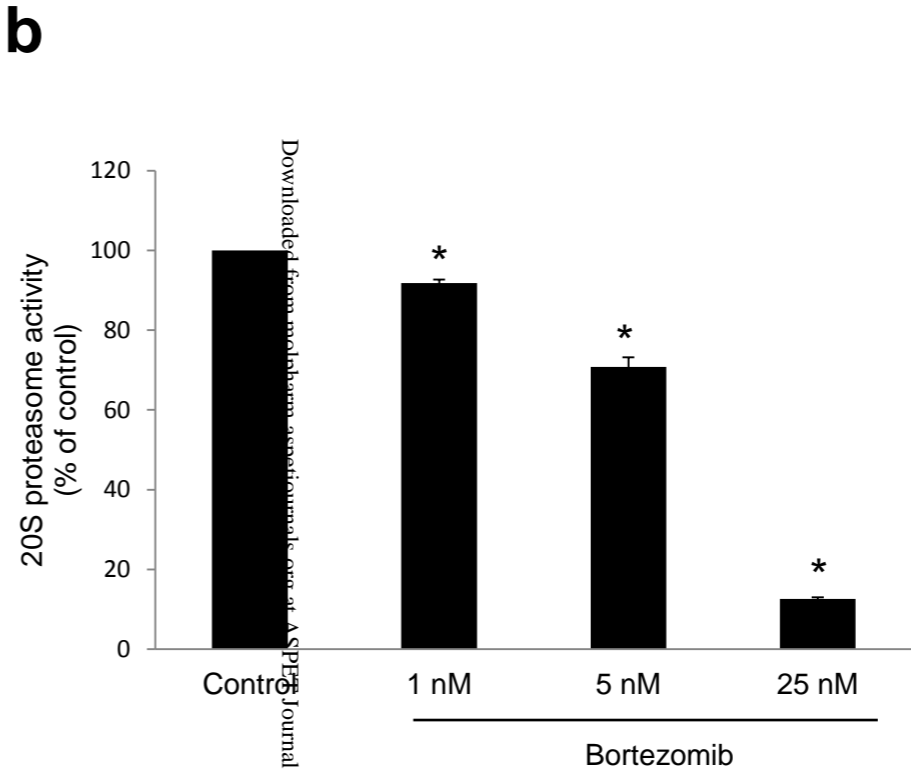
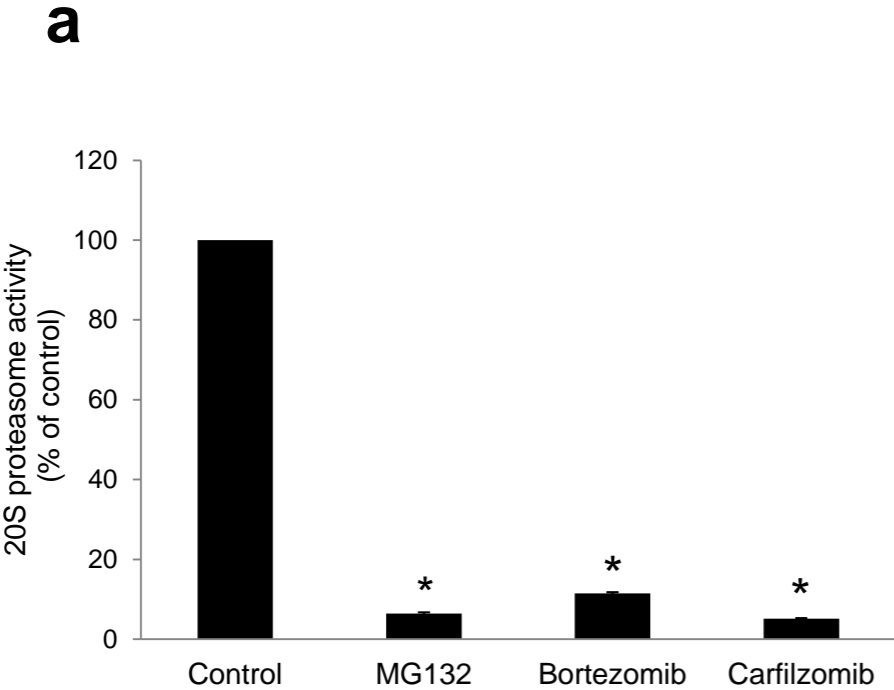


Fig. 6



Downloaded from molbank.aspenjournals.com at ASPEN Journals on April 2, 2023



Fig. 7

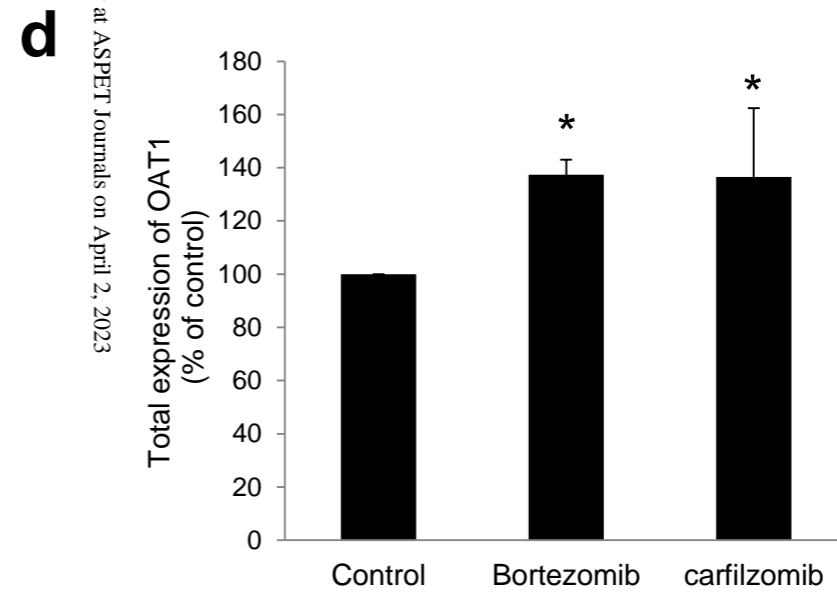
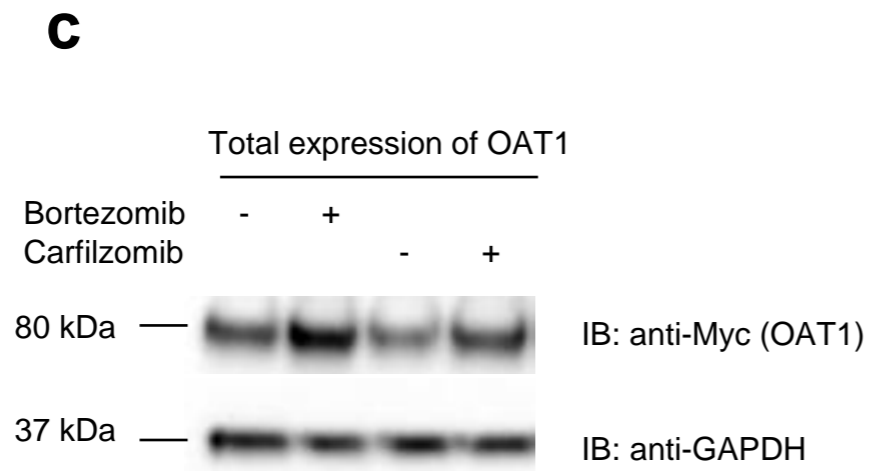
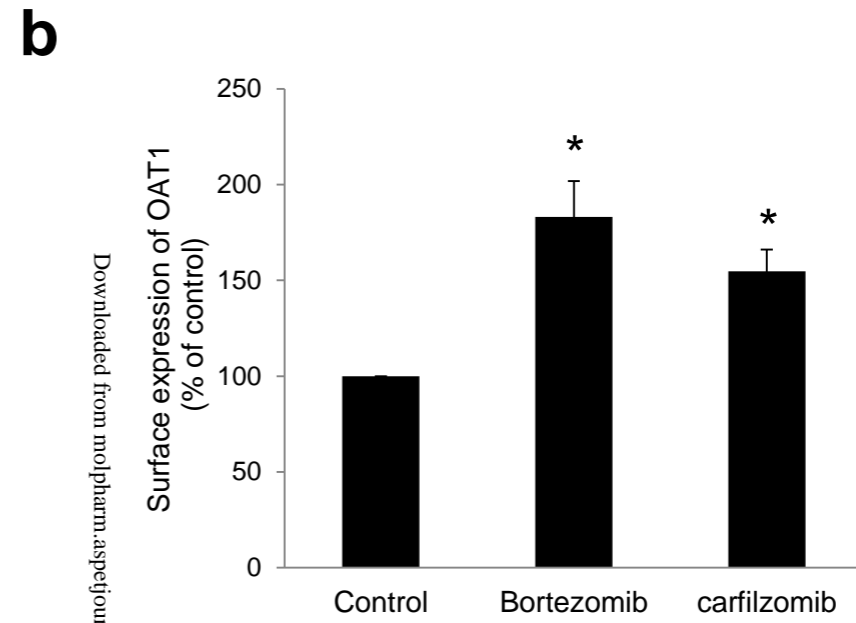
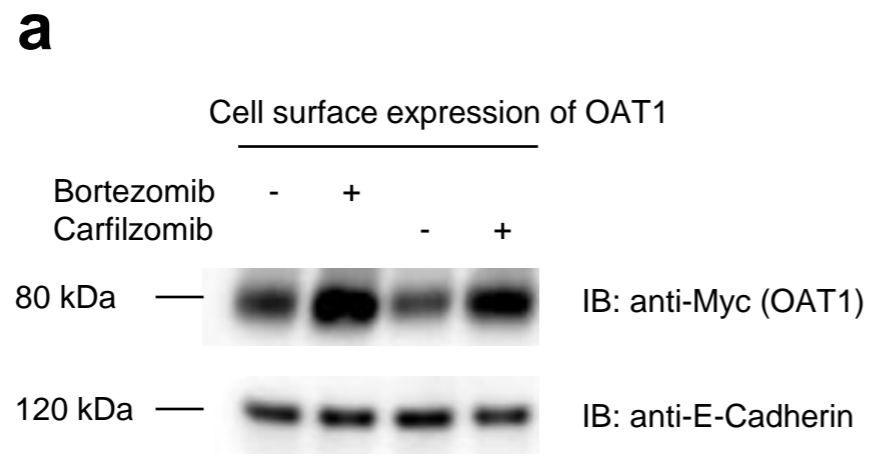
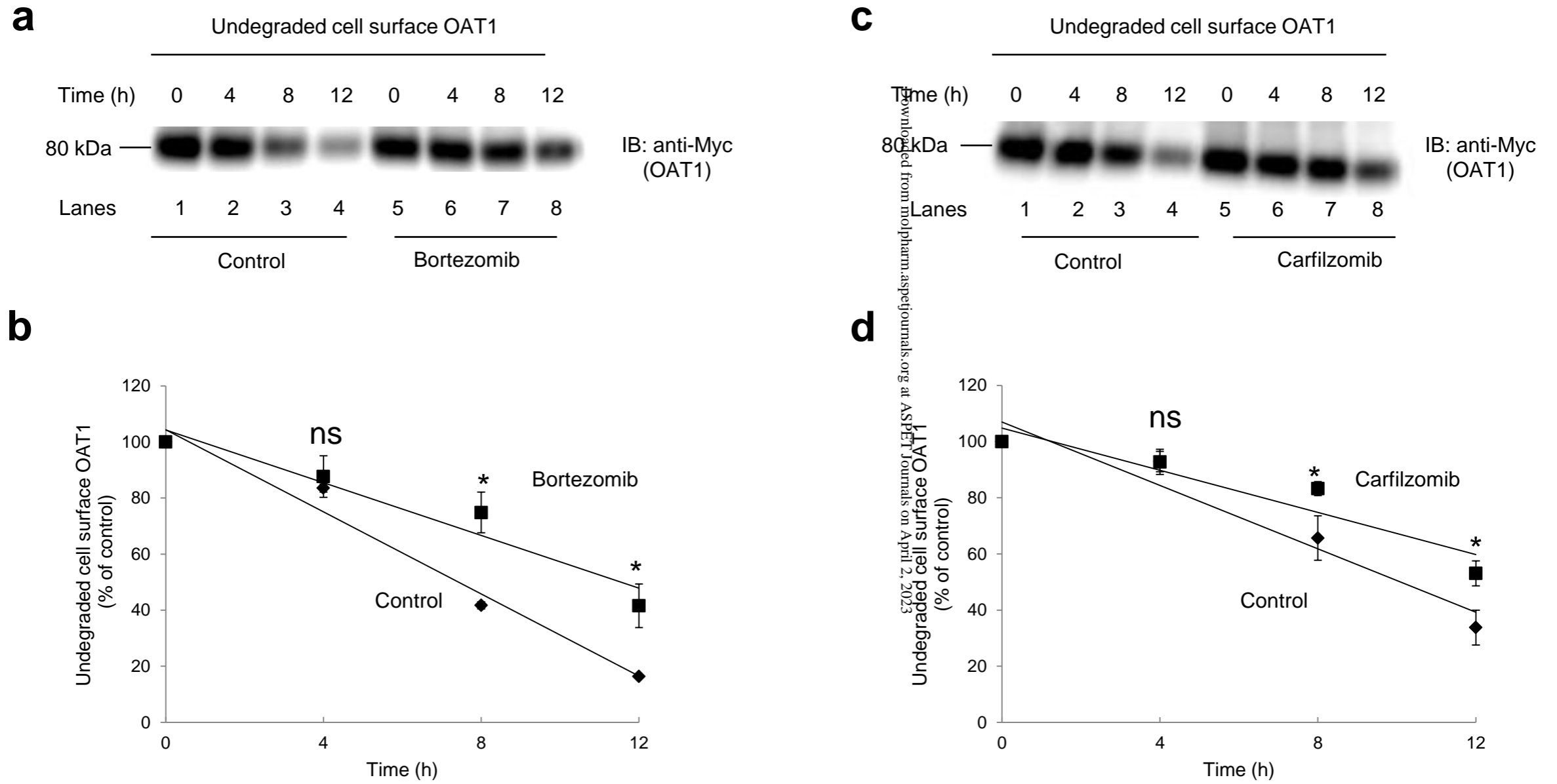


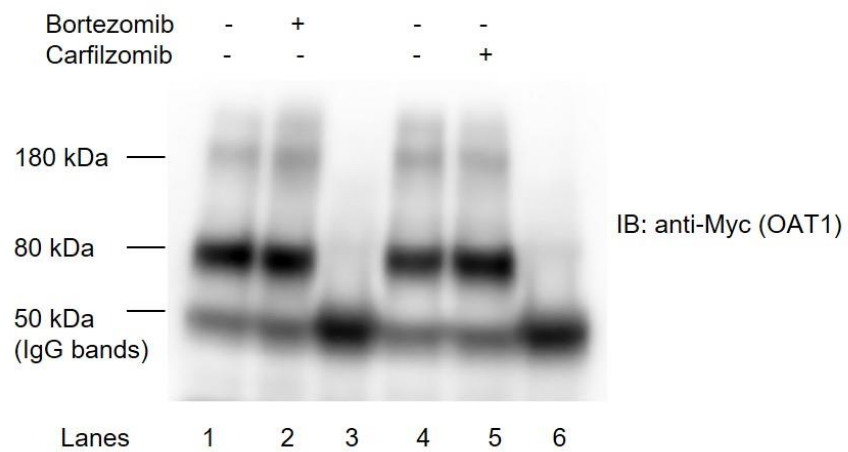
Fig. 8



# Proteasome Inhibitors Bortezomib And Carfilzomib Stimulate the Transport Activity of Human Organic Anion Transporter 1

Yunzhou Fan and Guofeng You\*

Molecular Pharmacology



**Supplemental Figure 1. Full blot for Fig 2: immunoprecipitated OAT1.** OAT1-expressing HEK293 cells were treated with bortezomib (25 nM) or carfilzomib (0.5  $\mu$ M) for 12 h. Treated cells were then lysed, and OAT1 was immunoprecipitated with anti-Myc antibody or with mouse IgG (as negative control, lane 3 and 6), followed by immunoblotting (IB) with anti-Myc antibody to determine the amount of OAT1 immunoprecipitated.