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## AG490, a JAK2-specific inhibitor, downregulates the expression and activity of organic anion transporter-3

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### Abstract

Human organic anion transporter-3 (hOAT3) is richly expressed in the kidney, where it plays critical roles in the secretion of clinically important drugs, including anti-viral therapeutics, anti-cancer drugs, antibiotics, antihypertensives, and anti-inflammatories. In the current study, we examined the role of AG490, a specific inhibitor of the Janus tyrosine kinase 2 (JAK2), in hOAT3 transport activity in the kidney COS-7 cells. AG490 induced a time- and concentration-dependent inhibition of hOAT3-mediated uptake of estrone sulfate, a prototypical substrate for the transporter. The inhibitory effect of AG490 correlated with a reduced expression of hOAT3 at the cell surface. Our lab previously demonstrated that Nedd4-2, a ubiquitin ligase, down regulates OAT expression and transport activity by enhancing OAT ubiquitination, which leads to an internalization of OAT from cell surface to intracellular compartments and subsequent degradation. In the current study, we showed that treatment of hOAT3-expressing cells with AG490 resulted in an enhanced hOAT3 ubiquitination and degradation, which was accompanied by a strengthened association of Nedd4-2 with hOAT3 and a reduction in Nedd4-2 phosphorylation. SiRNA knockdown of endogenous Nedd4-2 abrogated the effects of AG490 on hOAT3. In summary, our study demonstrated that AG490 regulates hOAT3 expression and transport activity through the modulation of Nedd4-2.

### Keywords

Organic anion transporter; Drug transport; Regulation; Janus kinase 2 inhibitor; Ubiquitin ligase Nedd4-2

### Introduction

Organic anion transporter 3 (OAT3) belongs to a class of organic anion transporters (OATs) consisting of over 10 membrane proteins. OAT3 is expressed at the basolateral membrane of the kidney proximal tubule cells and plays a critical role in the renal secretion of numerous

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Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

clinical drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, antihypertension drugs, and anti-inflammatories.<sup>1-7</sup>

We previously established that OATs are subjected to the modulation by a ubiquitin ligase Nedd4-2. Nedd4-2 catalyzes the conjugation of ubiquitin to cell surface OATs, which leads to OAT internalization from the cell surface to intracellular compartment and subsequent degradation.<sup>8-10</sup> We further demonstrated that Nedd4-2 serves as an important mediator for OAT regulation by several protein kinases. For example, activation of protein kinase C promotes the binding of Nedd4-2 to OATs, which results in an enhanced OAT ubiquitination and internalization from cell surface. Consequently, the amount of OATs at the cell surface is reduced and OAT transport activity is decreased. On the other hand, activation of the serum-, and glucocorticoid-inducible kinases demotes the binding of Nedd4-2 to OATs, which results in a reduced OAT ubiquitination and internalization from cell surface. Consequently, the amount of OATs at the cell surface is increased and OAT transport activity is augmented.

Cytokines and growth factors activate Janus tyrosine kinase 2 (JAK2) signaling physiologically, thus JAK2 signaling is involved in a variety of cellular processes including cell differentiation, growth and survival.<sup>11</sup> In addition, JAK2 signaling is significantly and pathologically activated in various human cancers including Breast cancer, ovarian cancer, pancreatic cancer, renal carcinoma and so on.<sup>12-16</sup> Other than human cancers, activation of JAK2 is also indicated in other disease conditions including diabetic vascular disease and myeloproliferative Diseases.<sup>17,18</sup>

Furthermore, JAK2 has been shown to regulate many cellular processes at renal proximal tubules<sup>16,19-21</sup> where OATs are abundantly expressed. It was also shown that one of the pathways through which JAK2 exerts its effect is through Nedd4-2<sup>22,23</sup>. However, the effects of JAK2 on OATs have never been investigated. In the current study, we examined the role of a JAK2-specific inhibitor AG490 in OAT expression and transport activity.

## Materials and methods

### Materials

The monkey kidney COS-7 cells were purchased from ATCC (Manassas, VA). [<sup>3</sup>H]-labeled estrone sulfate ([<sup>3</sup>H]-ES) was purchased from PerkinElmer (Waltham, MA). Membrane-impermeable biotinylation reagent NHS-SS-biotin, streptavidin-agarose beads and protein G-agarose beads were purchased from Pierce (Rockford, IL). cDNA for human Nedd4-2 was generously provided by Dr. Peter M. Snyder of the College of Medicine, University of Iowa (Iowa City, IA). Mouse anti-myc antibody (9E10) was purchased from Roche (Indianapolis, IN). Rabbit anti-Nedd4-2 and mouse anti-E-cadherin antibodies were purchased from Abcam (Cambridge, MA). Mouse anti-flag antibody was purchased from Sigma-Aldrich (St. Louis, MO). Mouse anti-ubiquitin, mouse anti- $\beta$ -actin, and mouse anti-phospho-tyrosine (anti-p-tyr) antibodies were purchased from Santa Cruz (Santa Cruz, CA). Nedd4-2 siRNA oligonucleotides (Silencer<sup>®</sup> Select, identification number S23570) were purchased from Ambion (Grand Island, NY). AG490 and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

### Cell culture and transient transfection

Parental COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Corning, Corning, NY) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) at 37 °C in 5% CO<sub>2</sub>. Cells stably expressing hOAT3 were established in our lab as previously described.<sup>24,25</sup> Cells stably expressing hOAT3 were maintained in DMEM medium supplemented with 0.2 mg/ml G418 (Invitrogen, Carlsbad, CA), 10% fetal bovine serum. Transfection with plasmids or siRNA was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

### Transport measurement

The transport activity of hOAT3 was determined by measuring [<sup>3</sup>H]-ES uptake into hOAT3-expressing cells. The uptake solution consists of phosphate-buffered saline (PBS) with 1 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> (PBS/CM) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>, pH 7.4) and [<sup>3</sup>H]-ES (300 nM). At the time points indicated, uptake was terminated by removing the uptake solution, followed by washing with ice-cold PBS twice. The cells were then lysed in 0.2 N NaOH, neutralized with 0.2 N HCl and transferred into scintillation vials for liquid scintillation counting.

### Kinetics of ES transport

hOAT3-expressing cells were pretreated with or without AG490 (20 μM) for 1 h, and 4-min uptake of [<sup>3</sup>H]-ES was measured at 0.05–20 μM ES. The uptake procedure was detailed in the section "Transport measurement". The kinetic parameters of estrone sulfate transport by hOAT3 were calculated using non-linear least-squares regression analysis from the following Michaelis–Menten equation:  $V = V_{max} \times [S] / (K_m + [S])$ . Transport kinetic values were calculated using the Eadie–Hofstee transformation.

### Cell surface biotinylation

The expression level of hOAT3 at the cell surface was examined using a biotinylation strategy. The cells in monolayer culture were washed with ice-cold PBS and then incubated with 1 ml of membrane-impermeable NHS-SS-biotin (0.5 mg/ml in PBS pH8.0/CM) on ice for two consecutive 20 min periods under gentle shaking. Biotinylation was stopped by rinsing with 100 mM glycine in PBS/CM. Afterwards, the cell extracts were prepared in lysis buffer (10 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 with 1/100 protease inhibitor cocktail) for 30 min at 4 °C and cleared by centrifugation at 16,000 × g at 4 °C. The supernatant was mixed with streptavidin-agarose beads (Pierce, Rockford, IL) to isolate cell surface proteins. Membrane-expressed hOAT3 was detected by SDS-PAGE and immunoblotting with an anti-myc antibody (epitope myc was tagged to hOAT3 for immune-detection).

### Degradation of cell surface hOAT3

hOAT3 expressing cells were plated in 35 mm dishes. Each dish of cells was incubated with 1 ml of membrane-impermeable biotinylation reagent sulfo-NHS-SS-biotin (0.5 mg/ml in PBS pH8.0/CM) in two successive 20 min incubations under trafficking impermeable condition (4 °C) with very gentle shaking. The reagent was freshly prepared for each

incubation. After biotinylation, each dish was rinsed with 2 ml PBS pH8.0/CM containing 100 mM glycine and then incubated with the same solution for 20 min on ice, to ensure complete quenching of the unreacted NHS-SS-biotin. The biotin-labeled cells were incubated in DMEM containing with or without 20  $\mu$ M AG490 at 37 °C. Treated cells were collected at 0, 2, and 4 h and lysed in lysis buffer with protease inhibitor cocktail. The cell lysates were cleared by centrifugation at 16,000  $\times$  g at 4 °C. 40  $\mu$ l of streptavidin-agarose beads 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.

### Electrophoresis and immunoblotting

The protein samples were separated on 7.5% SDS-PAGE minigels (Bio-Rad, Hercules, CA) and electroblotted on to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The blots were blocked with 5% nonfat dry milk for 1–2 h in PBS-Tween 20 (PBST; 0.05% Tween-20 in PBS) at room temperature, washed and incubated overnight at 4 °C with appropriate primary antibodies. The primary antibodies included rabbit anti-Nedd4-2, mouse anti-E-cadherin (Abcam, Cambridge, MA), mouse anti-myc (Roche, Indianapolis, IN), mouse anti-flag (Sigma–Aldrich, St. Louis, MO), mouse anti- $\beta$ -actin, mouse anti-ubiquitin, and mouse anti-phospho-tyrosine (anti-p-tyr) (Santa Cruz, Santa Cruz, CA). The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies, followed by detection with a SuperSignal West Dura Extended Duration Substrate kit (Pierce, Rockford, IL). The FluorChem 8000 imaging system (Alpha Innotech Corp., San Leandro, CA) was applied to quantify the nonsaturating, immune-reactive protein bands.

### Data analysis

Each experiment was repeated at least three times. Student's paired t-tests were used to perform statistical analysis. A value of  $p < 0.05$  was considered significant.

## Results

### AG490 inhibits hOAT3 transport activity

We pretreated hOAT3-expressing cells with AG490 for 0–4 h, followed by measuring hOAT3-mediated [ $^3$ H]-ES uptake. Our results showed that AG490 inhibited hOAT3-mediated ES uptake with ~40% inhibition after 4 h pretreatment (Fig. 1a). AG490 also induced a dose-dependent inhibition of hOAT3-mediated transport with ~50% inhibition at 40  $\mu$ M of AG490 (Fig. 1b). To examine the mechanism of AG490-induced inhibition of hOAT3 transport activity, we determined hOAT3-mediated [ $^3$ H]-ES uptake at different substrate concentrations. An Eadie-Hofstee analysis of the derived data (Fig. 2) showed that treatment of hOAT3-expressing cells with AG490 resulted in a decreased maximal transport velocity  $V_{\max}$  of hOAT3 ( $245.9 \pm 13.7$  pmol·mg $^{-1}$ ·4 min $^{-1}$  with control cells and  $102.6 \pm 7.2$  pmol·mg $^{-1}$ ·4 min $^{-1}$  with cells treated with AG490) with no significant change in the substrate-binding affinity  $K_m$  of the transporter ( $8.85 \pm 0.65$   $\mu$ M with control cells and  $8.96 \pm 0.67$   $\mu$ M with cells treated with AG490). The value of the  $K_m$  in this study is similar to those obtained in other systems previously published by us and other labs.<sup>26,27</sup>

### **AG490 decreases hOAT3 expression**

A decreased maximal transport velocity  $V_{\max}$  (Fig. 2) could be a result from either a decreased amount of the transporter at the cell surface or a decreased transporter turnover number. We conducted experiments that differentiate between these possibilities by measuring transporter expression both at the cell surface and in the total cell lysates. We showed that treatment of hOAT3-expressing cells with AG490 for 1 h (20  $\mu$ M) resulted in a decreased cell surface expression of hOAT3 without affecting the total cell expression of the transporter (Fig. 3).

### **AG490 enhances hOAT3 ubiquitination**

We previously demonstrated that ubiquitination of cell surface OATs leads to OAT internalization from cell surface to intracellular compartment. As a result, the amount of OATs at the cell surface is reduced and OAT transport activity is decreased.<sup>8,28</sup> In this experiment, we examined whether AG490-induced decrease in OAT transport activity was due to the ubiquitination of this transporter. hOAT3-expressing cells were treated with AG490 for 1 h (20  $\mu$ M). Treated cells were then lysed, and hOAT3 was immunoprecipitated, followed by immunoblotting with anti-ubiquitin antibody. As shown in Fig. 4, AG490 enhanced hOAT3 ubiquitination as compared to that of the control.

### **AG490 promotes the association between hOAT3 and Nedd4-2**

Our lab previously established that OAT ubiquitination is catalyzed by a specific ubiquitin ligase Nedd4-2.<sup>8</sup> In this experiment, we examined whether AG490 affected the association of Nedd4-2 with hOAT3 (Fig. 5). We treated hOAT3-expressing cells with AG490 for 1 h (20  $\mu$ M). hOAT3 in treated cells was then immunoprecipitated, followed by immunoblotting with anti-Nedd4-2 antibody. We showed that in hOAT3 immunoprecipitates, there were more Nedd4-2 in AG490-treated sample than that in control sample, suggesting that more Nedd4-2 associated with hOAT3 after AG490 treatment.

### **AG490 decreases Nedd4-2 phosphorylation**

Protein kinases exert the effects by phosphorylating their substrates. JAK2 is a tyrosine kinase. We therefore examined whether Nedd4-2 was a direct substrate for JAK2-induced tyrosine phosphorylation. hOAT3-expressing cells were transfected with Nedd4-2. Nedd4-2 was then immunoprecipitated, followed by immunoblotting with anti-phospho-tyrosine antibody. As shown in Fig. 6a, top panel, the anti-phospho-tyrosine antibody detected a band at ~120 kDa, the molecular size of Nedd4-2, suggesting that Nedd4-2 was tyrosine-phosphorylated under normal condition. Treatment of cells with AG490 resulted in a decrease in Nedd4-2 phosphorylation. The difference in Nedd4-2 phosphorylation was not due to the difference in the amount of Nedd4-2 immunoprecipitated because the amount of Nedd4-2 pulled down was similar between control and AG490-treated cells (Fig. 6a, bottom panel).

### **Nedd4-2 siRNA abrogates the effects of AG490 on OAT3**

As an independent approach, we used a siRNA strategy to knockdown the endogenous Nedd4-2 and evaluated the role of Nedd4-2 in the effects of AG490 on hOAT3 function and

expression. Our lab has well-established that transfection of 50 pmol Nedd4-2- specific siRNA into COS-7 cells for 48 h knocked down the endogenous Nedd4-2 by ~80%.<sup>8,29</sup> As shown in Fig. 7, AG490 significantly reduced hOAT3-mediated transport in control cells, whereas in Nedd4-2-specific siRNA-transfected cells, the effect of AG490 on hOAT3 transport activity was abrogated (Fig. 7a). Similarly, AG490 significantly reduced hOAT3 expression at the cell surface in control cells (Fig. 7b and c), whereas in Nedd4-2-specific siRNA-transfected cells, the effect of AG490 on hOAT3 expression was abrogated (Fig. 7d and e).

### AG490 accelerates the rate of hOAT3 degradation

We previously established that ubiquitination of OAT leads to OAT internalization from cell surface to intracellular compartments and subsequent degradation.<sup>28</sup> Since AG490 enhanced hOAT3 ubiquitination (Fig. 4), we reasoned that AG490 may be involved in hOAT3 stability. In this experiment, we examined such possibility. The biotin-labeled cells were incubated in DMEM medium with or without 20  $\mu$ M AG490 at 37 °C. Treated cells were collected at 0, 2, and 4 h. The degradation rates of plasma membrane hOAT3 in these cells were then determined using a biotinylation approach. As shown in Fig. 8, the degradation rate of hOAT3 was much faster in AG490 treated cells than that in control cells, suggesting that AG490 plays an important role in hOAT3 degradation.

## Discussion

Organic anion transporters (OATs) are critical players in the efficacy and toxicity of therapeutic agents. Therefore, uncovering how OATs are regulated at the molecular and cellular levels is clinically and pharmacologically significant. The current investigation revealed that AG490, a JAK2-specific Inhibitor, has a significant role in modulating hOAT3 expression and transport activity.

JAK2 is known to affect a variety of renal functions, such as renal fibrosis, diabetic nephropathy, reperfusion injury, etc.<sup>30,31</sup> In addition, JAK2 has been shown to specifically regulate a set of transporters/exchangers such as Na<sup>+</sup>/H<sup>+</sup> exchanger, as well as Na<sup>+</sup>/glucose cotransporters SGLT1 and SGLT2 in renal proximal tubules,<sup>32,33</sup> where OATs are expressed.

We chose to carry out our investigation in monkey kidney COS-7 cells, a widely-used model cell system for mechanistic studies of many renal transport processes.<sup>33–35</sup> Importantly, COS-7 cells have been shown to express endogenous JAK2.<sup>36,37</sup> Therefore, our studies in these cells using a JAK2-specific inhibitor as a tool will pave the path for the future exploration in evaluating whether similar mechanisms are working *in vivo*.

From our investigation, we obtained several pieces of valuable information. AG490 inhibited hOAT3 transport activity by redistributing the transporter from cell surface to intracellular compartment (Fig. 3) and by enhancing the degradation of the transporter (Fig. 8). The effect of AG490 on hOAT3 seems to be mediated by a ubiquitin ligase Nedd4-2 (Figs. 5–7). Our lab previously demonstrated that serum- and glucocorticoid-inducible kinase (sgk) and protein kinase C (PKC) exert opposite effects on OAT expression and transport activity through a central switch molecule Nedd4-2. Activation of PKC inhibits OAT expression and

transport activity by strengthening the interaction of OAT with Nedd4-2, thereby, enhancing OAT ubiquitination and accelerating ubiquitination-dependent OAT internalization from the cell surface. As a result, the amount of OAT at the cell surface is reduced and OAT transport activity is decreased.<sup>9</sup> In contrast to PKC, activation of *sgk* stimulates OAT expression and transport activity by weakening the interaction of OAT with Nedd4-2, thereby, decreasing OAT ubiquitination and decelerating ubiquitination-dependent OAT internalization from the cell surface. As a result, the amount of OAT at the cell surface is enhanced and OAT transport activity is increased.<sup>27,38</sup> Both PKC and *sgk* are serine/threonine kinases and phosphorylate their substrates at serine or threonine residues. For example, we previously demonstrated that *sgk* phosphorylates Nedd4-2 at serine 327.<sup>27</sup> JAK2 is a tyrosine kinase, and in the current study we provide first evidence that the inhibitory effect of AG490 on hOAT3 expression and transport activity was mediated by Nedd4-2 through a decrease in the tyrosine phosphorylation of Nedd4-2, and an enhanced interaction of hOAT3 with Nedd4-2, and an enhanced hOAT3 ubiquitination, which led to an accelerated ubiquitination-dependent hOAT3 degradation. Consequently, hOAT3 expression at the cell surface is reduced and hOAT3 transport activity is decreased.

In addition to modulating the rate of hOAT3 degradation, AG490 may also affect other steps in hOAT3 trafficking process. We previously demonstrated<sup>10,28</sup> that members of OAT family constitutively internalize from and recycle back to cell surface. Nedd4-2-catalyzed ubiquitination leads to a reduced OAT3 expression and activity in three distinct steps. First, ubiquitination increases the rate of OAT3 internalization from cell surface to intracellular endosomes. Second, once in the endosomes, OAT3 either recycles back to cell surface or targets to proteolytic system. This endosomal sorting decision is governed by ubiquitination. With prolonged Nedd4-2 binding, OAT3 may attain sufficient ubiquitination and targets to proteolytic system. Third, the proteases in the proteolytic system degrade OAT3. Since AG490 regulates the interaction between Nedd4-2 and hOAT3, it will likely affect multiple steps in hOAT3 trafficking.

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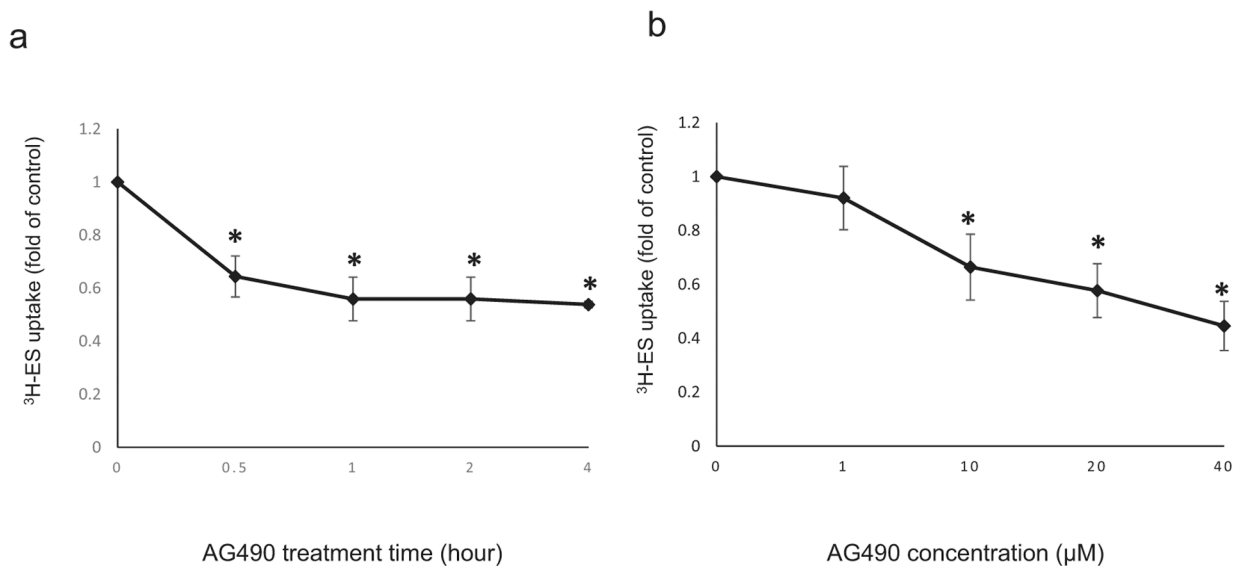
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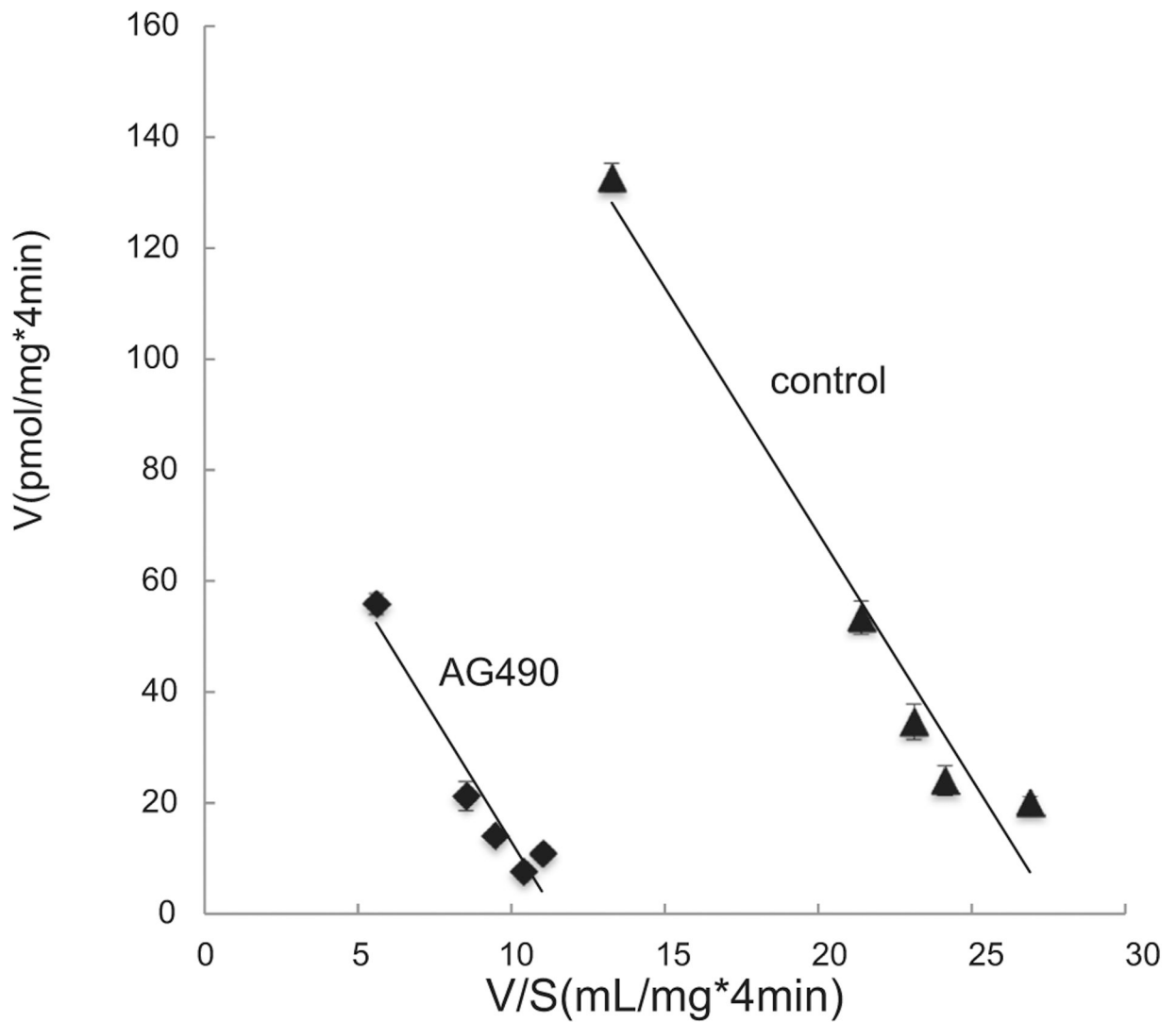


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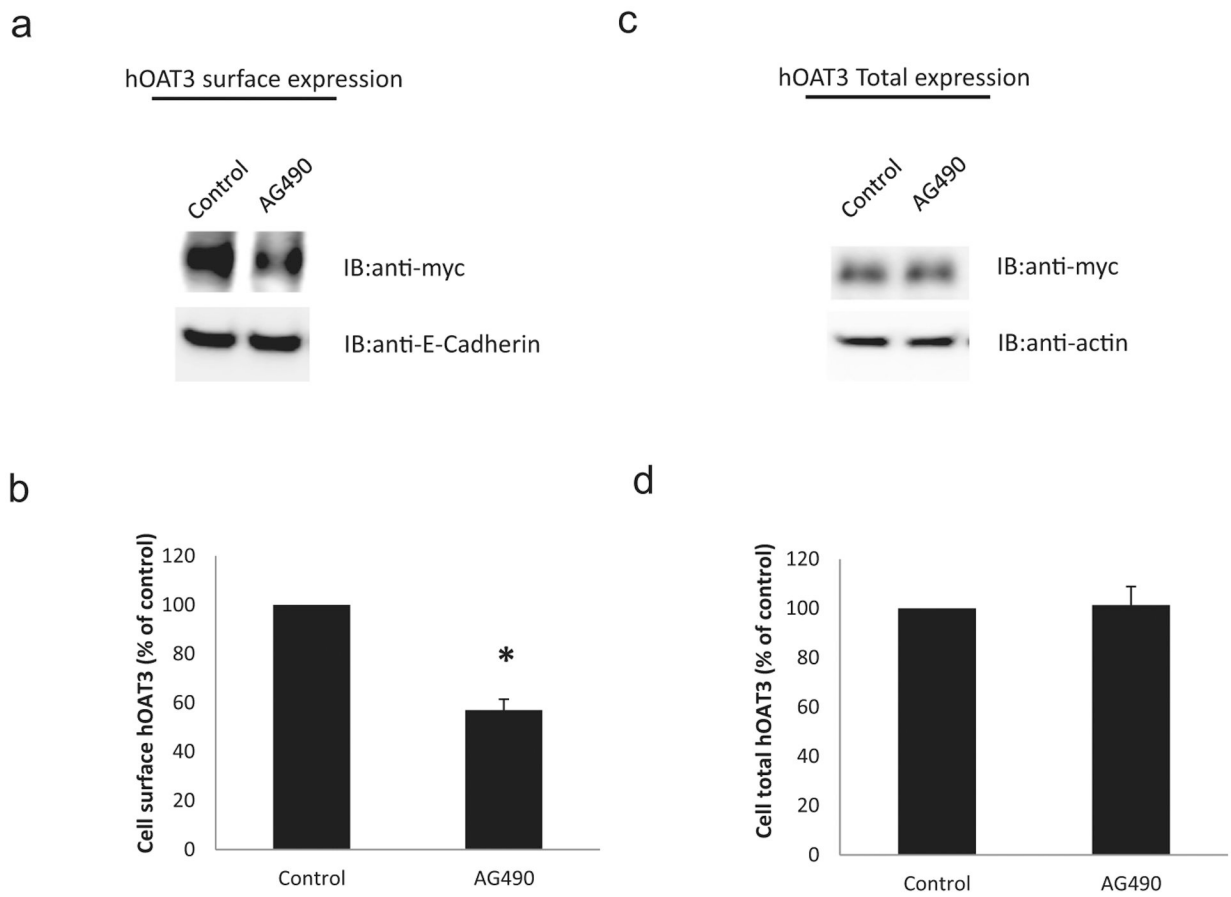
**Fig. 1. AG490 inhibits hOAT3 transport activity.**

(a). Time dependence. hOAT3-expressing cells were pretreated with AG490 (20  $\mu\text{M}$ ) for 0–4 h. 4-min uptake of [ $^3\text{H}$ ]-estrone sulfate (ES, 300 nM) was then measured. Uptake activity was expressed as folds of the uptake measured in control cells. The data represent uptake into hOAT3-expressing cells minus uptake into mock cells (parental cells). Uptake value of mock cells (parental cells) is ~20% of that of hOAT3-expressing cells without treatment (mock cells: ~700 CPM and hOAT3-expressing cells without treatment: ~3500 CPM). Values are mean  $\pm$  S.E. ( $n = 3$ ). \* $P < 0.05$ . (b). Dose dependence. hOAT3-expressing cells were pretreated for 1 h with AG490 at various doses (0–40  $\mu\text{M}$ ). 4-min uptake of [ $^3\text{H}$ ]-ES (300 nM) was then measured. Uptake activity was expressed as folds of the uptake measured in control cells. The data represent uptake into hOAT3-expressing cells minus uptake into mock cells (parental cells). Values are mean  $\pm$  S.E. ( $n = 3$ ). \* $P < 0.05$ .



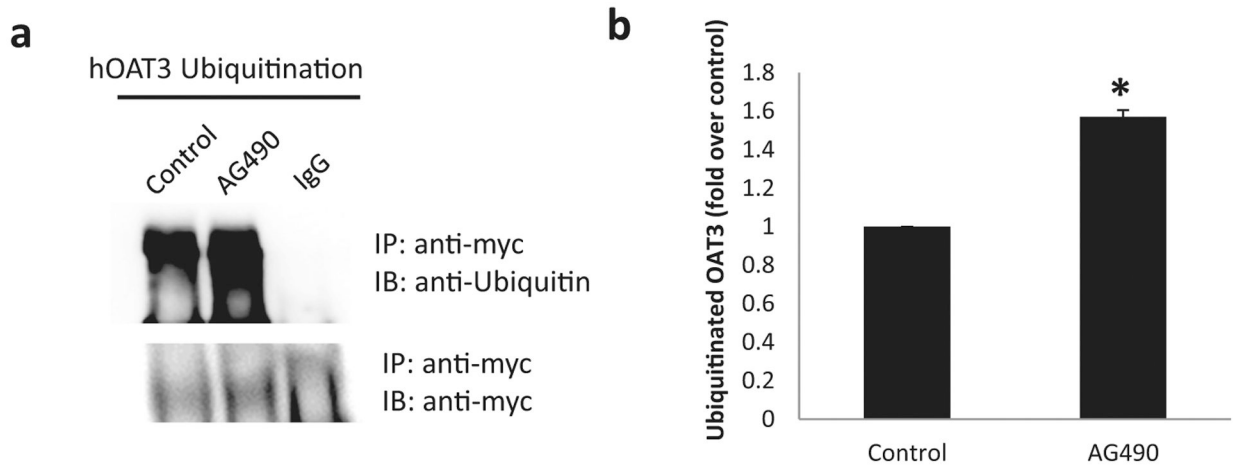
**Fig. 2. AG490 altered the kinetics of hOAT3-mediated ES transport.**

hOAT3-expressing cells were pretreated with or without AG490 (20  $\mu$ M) for 1 h, and 4-min uptake of [ $^3$ H]-ES was measured at 0.05–20  $\mu$ M ES. The data represent uptake into hOAT3-transfected cells minus uptake into mock cells (parental COS-7 cells). Values are means  $\pm$  S.D. (n = 3). V, velocity; S, substrate concentration. Transport kinetic values were calculated using the Eadie–Hofstee transformation.



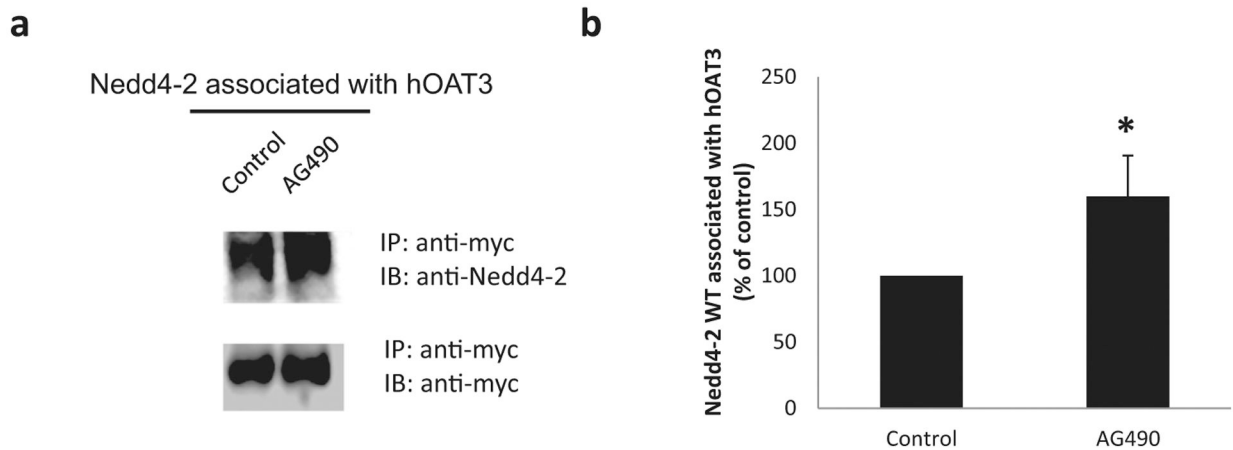
**Fig. 3. AG490 decreases hOAT3 expression.**

(a). *Top panel*: Cell surface expression of hOAT3. hOAT3-expressing cells were pretreated with AG490 (1 h, 20  $\mu$ M). Cells were labeled with membrane impermeable biotin. Biotinylated cell surface proteins were separated with streptavidin beads, followed by immunoblotting (IB) with an anti-myc antibody (hOAT3 was tagged with epitope myc for immunodetection). *Bottom panel*: The expression of cell surface protein marker E-cadherin. The same blot from the top panel was re-probed with anti-E-cadherin antibody. (b). Densitometry plot of results from Fig. 3a, top panel as well as from other experiments. The values are mean  $\pm$  S.E. (n = 3). \*P < 0.05. (c). *Top panel*: Total cell expression of hOAT3. hOAT3-expressing cells were pretreated with AG490 (1 h, 20  $\mu$ M). Cells were lysed, followed by immunoblotting (IB) with an anti-myc antibody. *Bottom panel*: Total cell expression of cellular protein marker  $\beta$ -actin. The same blot from top panel was re-probed with anti- $\beta$ -actin antibody. (d). Densitometry plot of results from Fig. 3c, top panel as well as from other experiments. The values are mean  $\pm$  S.E. (n = 3). \*P < 0.05.



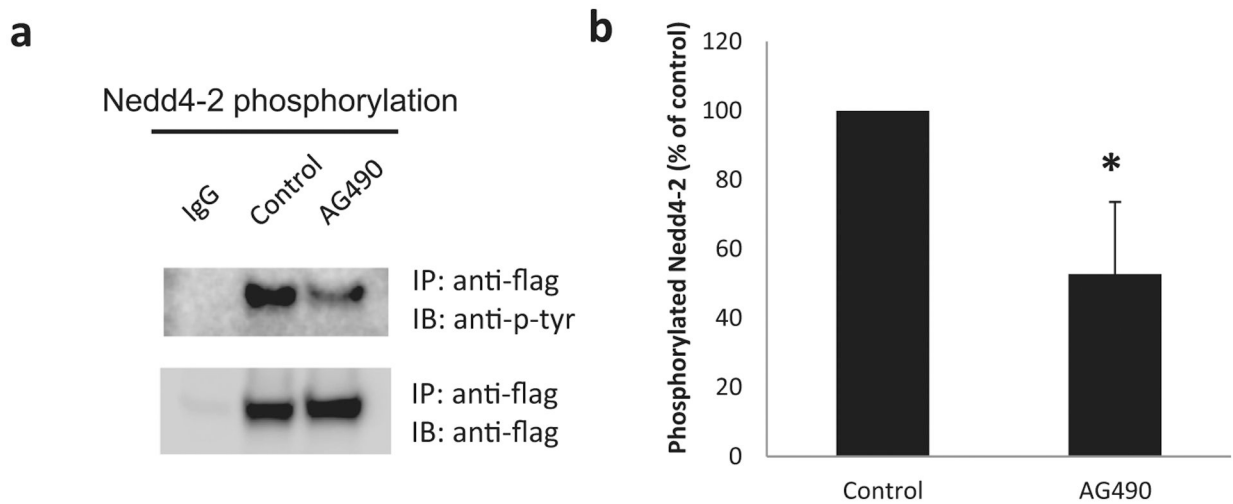
**Fig. 4. AG490 enhances hOAT3 ubiquitination.**

(a) Top panel: hOAT3-expressing cells were pretreated with AG490 (1 h, 20  $\mu$ M) in the presence of proteasomal inhibitor cocktail and deubiquitinase inhibitor N-Ethylmaleimide. Treated cells were then lysed and hOAT3 was immunoprecipitated with anti-myc antibody (hOAT3 was tagged with epitope myc), followed by immunoblotting with anti-ubiquitin antibody. *Bottom panel*: The same blot from top panel was re-probed with anti-myc antibody. (b). Densitometry plot of results from Fig. 4a, top panel as well as from other experiments. The values are mean  $\pm$  S.E. (n = 3). \*P < 0.05.



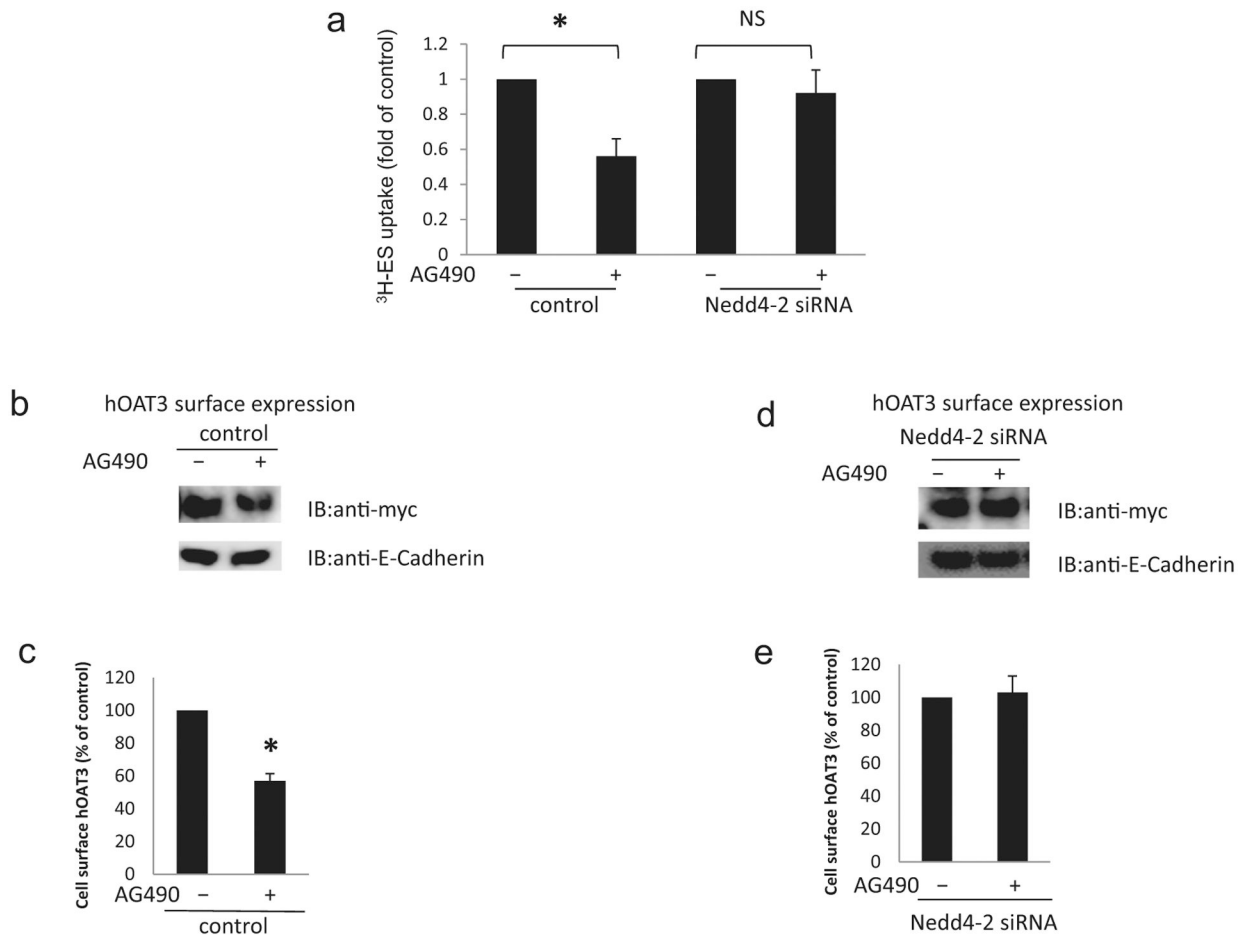
**Fig. 5. AG490 promotes the association of hOAT3 with Nedd4-2.**

(a). *Top panel*: COS-7 cells were co-transfected with hOAT3 and Nedd4-2. Transfected cells were treated with or without AG490 (20  $\mu$ M, 1 h). Treated cells were then lysed and hOAT3 was immunoprecipitated by anti-myc antibody (epitope myc was tagged to hOAT3), followed by immunoblotting with anti-Nedd4-2 antibody. *Bottom panel*: The same immunoblot from Fig. 5a, *top panel* was reprobed by anti-myc antibody to determine the amount of hOAT3 immunoprecipitated. (b). Densitometry plot of results from Fig.5a, *Top panel* as well as from other experiments. The values are mean  $\pm$  S.E. (n = 3). \*P < 0.05.



**Fig. 6. AG490 dephosphorylates Nedd4-2.**

(a). Top panel: COS-7 cells were co-transfected with hOAT3 and Nedd4-2 (epitope flag-tagged). Transfected cells were treated with or without AG490 (20  $\mu$ M, 1 h) and then lysed. Nedd4-2 was immunoprecipitated (IP) with anti-flag M2 affinity gel or with control IgG-agarose (as negative control), followed by immunoblotting (IB) with anti-phospho-tyrosine (anti-p-tyr) antibody. Bottom panel: The blot from the top panel was reprobed by anti-flag antibody to determine the amount of Nedd4-2 immunoprecipitated. (b). Densitometry plot of results from 6a, top panel, as well as from other repeat experiments. Values are means  $\pm$  SE. (n = 3). \*P < 0.05.



**Fig. 7. Nedd4-2 siRNA abrogates the effects of AG490 on OAT3.**

(a) hOAT3 transport activity. hOAT3-expressing COS-7 cells transfected with or without Nedd4-2-specific siRNA, were treated with or without AG490 (20  $\mu$ M, 1 h). Uptake of [ $^3$ H] ES was then performed. Uptake activity was expressed as a fold of the uptake measured in cells without AG490 treatment. The data represent uptake into hOAT3-transfected cells minus uptake into mock cells (parental COS-7 cells). Values are mean  $\pm$  S.E. (n = 3). \*P < 0.05. (b top panel) hOAT3 expression at the cell surface in control group cells. hOAT3-expressing COS-7 cells in control group were treated with or without AG490 (20  $\mu$ M, 1 h). Cell surface biotinylation was performed. Biotinylated (cell surface) proteins were separated with streptavidin beads and analyzed by immunoblotting (IB) with an anti-myc antibody. (b, bottom panel) The same blot as (b, top panel) was reprobbed with antibody against cell surface protein marker E-cadherin. (c) Densitometry plot of results from (b, top panel) as well as from other experiments. The values are mean  $\pm$  S.E. (n = 3). \*P < 0.05. (d, top panel) hOAT3 expression at the cell surface in Nedd4-2-specific siRNA-transfected cells. hOAT3-expressing COS-7 cells, transfected Nedd4-2-specific siRNA, were treated with or without AG490 (20  $\mu$ M, 1 h). Cell surface biotinylation was performed. Biotinylated (cell surface) proteins were separated with streptavidin beads and analyzed by immunoblotting (IB) with an anti-myc antibody. (d, bottom panel) The same blot as (d, top panel) was reprobbed with antibody against cell surface protein marker E-cadherin. (e) Densitometry plot of results



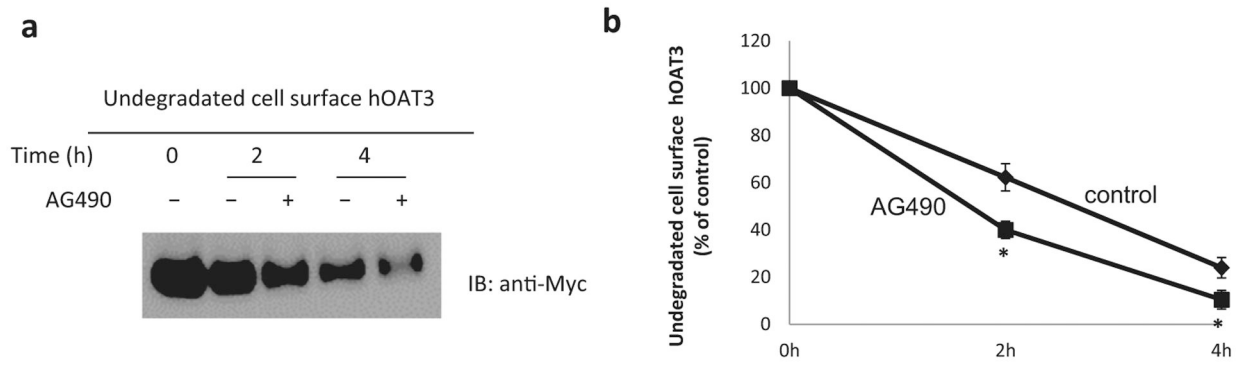
from (d, top panel) as well as from other experiments. The values are mean  $\pm$  S.E. (n = 3).  
\*P < 0.05.

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**Fig. 8. AG490 destabilizes hOAT3.**

(a). Biotinylation analysis of constitutive and JAK2-modulated degradation of cell surface hOAT3. The biotin-labeled cells were incubated in DMEM medium with or without 20 mM AG490 at 37 °C. Treated cells were collected at 0, 2, and 4 h. Treated cells were lysed in lysis buffer with protease inhibitor cocktail and then followed by immunoblotting (IB) with anti-myc antibody. (b). Densitometry plot of results from Fig. 8a as well as from other experiments. The values are mean  $\pm$  S.E. (n = 3).