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The 5'-AMP-activated protein kinase (AMPK) regulates the function and expression of human Organic Anion Transporting Polypeptide 1A2 (OATP1A2)

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Running Title: AMPK signalling regulates the function/expression of OATP1A2

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Abbreviations:

E3S: estrone-3-sulfate; MTX: methotrexate; OATP: organic anion transporting polypeptide; AMPK: 5'-AMP-activated protein kinase; SLC: solute carrier; compound C: dorsomorphin dihydrochloride.

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Abstract

The Organic Anion Transporting Polypeptides (OATPs) are important membrane proteins that mediate the cellular uptake of drugs and endogenous substances. OATP1A2 is widely distributed in many human tissues that are targeted in drug therapy; defective OATP1A2 leads to altered drug disposition influencing therapeutic outcomes. 5' AMP-activated protein kinase (AMPK) signalling plays an important role in the pathogenesis of the metabolic syndrome characterized by an increased incidence of type II diabetes and non-alcoholic fatty liver disease. This study investigated the regulatory role of AMPK on OATP1A2 transport function and expression. We found that the treatment of AMPK-specific inhibitor Compound C (dorsomorphin dihydrochloride) decreased OATP1A2-mediated uptake of estrone-3-sulfate in a concentration and time dependent manner. The impaired OATP1A2 function was associated with a decreased V_{max} (154.6 ± 17.9 pmol x (μg x 4 min)$^{-1}$ in Compound C-treated cells *vs*. 413.6 ± 52.5 pmol x (μg x 4 min)$^{-1}$ in control); the K_m was unchanged. The cell surface expression of OATP1A2 was decreased by Compound C treatment, but total cellular expression was unchanged. The impaired cell surface expression of OATP1A2 was associated with accelerated internalization and impaired targeting/recycling. Silencing of the AMPKα1-subunit using specific siRNA corroborated the findings with Compound C and also revealed a role for AMPK in regulating OATP1A2 protein stability. Overall, this study implicates AMPK in the regulation of the function and expression of OATP1A2, which potentially impacts on the disposition of OATP1A2 drug substrates that may be used to treat patients with the metabolic syndrome and other diseases.

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Introduction

Solute carrier transporters (SLCs) mediate the uptake of organic ions, amino acids, nucleotides, fatty acids and drugs into cells (Hediger et al., 2004). Within the SLC family, the *SLCO* subfamily, which encodes the Organic Anion Transporting Polypeptides (OATPs) is particularly important in drug disposition (Zhou et al., 2017). Of the eleven human OATP isoforms that have been identified, OATP1A2, 1B1, 1B3 and 2B1 are the best characterized and are abundantly present in the intestine, liver, kidney and other tissues, where they facilitate drug absorption, distribution and elimination (Kim, 2003; Kullak-Ublick et al., 1995; Steckelbroeck et al., 2004; Zhou et al., 2017).

OATPs are regulated at the transcriptional and post-translational levels. In human breast cancer cells, OATP1A2 gene transcription is activated by PXR agonists such as rifampin (Jigorel et al., 2006; Ma et al., 2007; Meyer zu Schwabedissen et al., 2008). The subcellular trafficking, recycling and internalization of OATP1A2 is regulated at the post-translational level; these processes play a major role in the expression of active transporter at the cell surface. Several protein kinases regulate OATP1A2 expression at the plasma membrane. For example, protein kinase C (PKC) modulates the plasma membrane expression and function of OATP1A2, as well as OATP1B1 and OATP2B1, by regulating protein trafficking (Hong et al., 2015; Kock et al., 2010; Zhou et al., 2011). In addition, casein kinase 2 (CKII) emerged recently as an important kinase that regulates the protein subcellular trafficking of OATP1A2 (Chan et al., 2016).

The 5'-adenosine monophosphate activated kinase (AMPK) is a cellular energy sensor in eukaryotic cells (Hardie et al., 2012). AMPK is a heterotrimeric complex that is composed of a catalytic α subunit (either the α1 or α2 isoform), a β subunit (either β1 or β2) and a γ subunit (either γ1, γ2 or γ3); the subunit composition of AMPK is distinct in different tissues, which is also associated with diverse kinase activity and stability (Russo et al., 2013). AMPK regulates genes such as acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA reductase that mediate the biosynthesis of nutrient fatty acids and cholesterol, respectively (Beg et al., 1973; Carlson and Kim, 1973). AMPK also regulates metabolic homeostasis by activating energy production and increasing insulin sensitivity (Coughlan et al., 2014; Hardie, 2011b). AMPK dysregulation occurs in obesity, type II diabetes and the metabolic syndrome (Coughlan et al., 2013; Coughlan et al., 2014; O'Neill et al., 2013; Steinberg and Kemp, 2009); while activating AMPK is the current therapeutic approach in treating such diseases (Coughlan et al., 2014). Drug transporters are known to be impaired in

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non-alcoholic fatty liver disease, which is a component of the metabolic syndrome, due in part to dysregulated N-glycosylation (Clarke et al., 2017). In this study, we found that OATP1A2 is regulated by AMPK and a series of experiments were undertaken to evaluate in greater detail the regulatory role of AMPK in the function and expression of major OATP drug transporters because of its potential significance to drug therapy in patients with the metabolic syndrome.

Material and Methods

Materials

[^{3}H]-Estrone-3-sulfate (E3S; specific activity 57.3 Ci/mmol) was purchased from PerkinElmer (Melbourne, VIC, Australia). Cell culture media was obtained from Thermo Scientific (Lidcombe, NSW, Australia). Unless otherwise stated, Compound C (dorsomorphin dihydrochloride) and all other chemicals and biochemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

Generation of HEK-293T cells that over-express OATP isoforms

The C-terminal Flag (DYKDDDDK)-tagged OATP1A2, OATP1B1, OATP1B3 and OATP2B1 containing plasmids were purchased or constructed in-house as described previously (Shams et al., 2018; Xu et al., 2013; Zheng et al., 2014; Zhou et al., 2011; Zhou et al., 2013a). Human embryonic kidney (HEK)-293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% L-glutamine at 37°C in a 5% CO_2 atmosphere. Cells were transfected using Lipofectamine 2000 (Thermo Scientific) following the manufacturer's instructions. In some experiments HEK-293T cells were transfected with AMPK α1 subunit specific siRNAs (Cat. No: sc-29673, Bio-strategy Laboratory Products, Tingalpa, QLD, Australia) or scrambled siRNAs (Cat. No: sc-37007, Bio-strategy Laboratory Products) using Lipofectamine 2000. Twenty-four hours after transfection, transporter function and protein expression were assessed.

Transport uptake assays

Uptake of [^{3}H]-E3S (300 nM; 57.3 nCi/well) by OATP1A2-, OATP1B1-, OATP2B1-expressing cells and uptake of [^{3}H]-cholecystokinin-8 (CCK-8, 2 nM; 23.4 nCi/well) by OATP1B3-expressing cells was conducted at 37ºC in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) containing 5mM

glucose. Eight min was selected for transporter uptake assays and 4 min for kinetic experiments, as described previously (Chan et al., 2015a; Johnston et al., 2014; Lu et al., 2018; Shams et al., 2018). Uptake was terminated by rapidly washing cells in ice-cold PBS. Cells were then incubated with 0.2 M NaOH at room temperature for 20 min, followed by 0.2 M HCl for 20 min, before aliquots were taken for liquid scintillation counting. Control uptake rates by vector-transfected cells were subtracted from all data. In kinetic studies, the E3S concentration range was 0.05−50 μM and apparent K_m and V_{max} values for uptake were calculated using GraphPad Prism 5.0 (GraphPad Inc., LaJolla, CA, USA). Data were derived in three independent experiments with triplicate repeats for each treatment.

Cell surface biotinylation

The membrane-impermeable biotinylation reagent NHS-SS-biotin (Thermo Scientific) was used to determine the cell surface expression of OATP1A2, as described before (Chan et al., 2016; Zheng et al., 2014; Zhou et al., 2011; Zhou et al., 2013a; Zhou et al., 2010). After three washes with ice-cold PBS (pH 8.0), cells were incubated on ice with freshly prepared NHS-SS-biotin (0.5 mg/mL) for 30 min, then washed three times with PBS containing 100 mM glycine and twice with cold PBS. Cells were lysed with RIPA buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, containing protease inhibitor cocktail; Cat. No: R-1101, Sapphire Biosciences, Redfern, NSW, Australia). Following centrifugation at 14,000g (4°C), streptavidin-conjugated agarose beads (Thermo Scientific) were added to lysates to capture membrane-bound proteins. Proteins were eluted from beads in Laemmli buffer and subjected to SDS-polyacrylamide gel electrophoresis.

Internalization assay

As shown in Supplemental Figure 1A, the internalization of biotinylated OATP1A2 was evaluated in untreated HEK-293T cells, or cells that were pre-treated with Compound C (10 μM, 1 h, 37 °C) (Chan et al., 2016; Zheng et al., 2014; Zhou et al., 2011). As described above, cells were warmed to 37°C to initiate internalization for varying times following NHS-SS-biotin pre-labeling. Sodium 2-mercaptoethanesulfanate (100 mM) was then added in NT buffer (150 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin, 20 mM Tris, pH 8.6) to remove residual biotinylated, but non-internalized, proteins that remained at the cell surface. After washing with cold PBS (5 times), cells were lysed and streptavidin-agarose beads were added to capture biotinylated proteins that had been internalized.

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Targeting assay

We assessed the plasma membrane targeting of newly synthesized OATP1A2 protein in combination with OATP1A2 protein that had been internalized and recycled back to the plasma membrane (Supplemental Figure 1B). OATP1A2 over-expressing HEK-293T cells were incubated in the presence and absence of Compound C (10 μM, 1 h, 37°C) (Chan et al., 2016; Zheng et al., 2014; Zhou et al., 2011) and then labelled with NHS-SS-biotin. Cells were then warmed to 37°C to initialize recycling/targeting for varying times and an additional aliquot of NHS-SS-biotin (0.5 mg/mL, 30 min) was added to further label the proteins newly recycled/targeted to cell surface. Residual NHS-SS-biotin was quenched with glycine and cells were lysed as described above. Following centrifugation equivalent quantities (300 μg protein/sample) of lysates from individual cell incubations were loaded onto streptavidin-agarose beads and subjected to electrophoresis and immunoblotting for OATP1A2. The quantity of recycled/targeted OATP1A2 at multiple time points was determined relative to initially biotinylated OATP1A2.

Recycling assay

HEK-293T cells that overexpressed OATP1A2 were pre-treated with Compound C (10 μM, 1 h, 37°C). After an initial biotinylation, the residual NHS-SS-biotin was quenched on ice with glycine and the cells were then warmed to 37°C for 1 min to initialize internalization (Supplemental Figure 1C). Sodium 2-mercaptoethanesulfonate (100 mM) in NT buffer was added to remove biotinylated, but non-internalized, proteins that remained at the cell surface. The cells were again warmed to 37°C to initiate recycling for varying times. At intervals the cells were treated with sodium 2-mercaptoethanesulfonate for a second time to remove biotinylated and proteins that had been recycled to the cell surface. Cell lysates were prepared and streptavidin-agarose beads were added to isolate biotinylated proteins. The relative quantity of recycled OATP1A2 was calculated as the difference between the biotinylation signals after the first and second treatments with sodium 2-mercaptoethanesulfonate (total internalized protein and internalized, but not recycled, protein, respectively).

Electrophoresis and immunoblotting

Protein samples were denatured at 50°C for 30 min, loaded onto 7.5% polyacrylamide minigels and electrophoresed (Bio-Rad, Gladesville, NSW, Australia). After transfer to polyvinylidene fluoride membranes in an electroelution cell (Bio-Rad) the membranes were blocked with 5% non-fat dry milk in PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4,

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1.4 mM KH_2PO_4 and 0.05% Tween 20, pH 7.5) for 30 min at room temperature. Membranes were subsequently washed with PBST and incubated with anti-Flag primary antibody (Cat. No: 2368S, 1 μg/mL; Genesearch Pty Ltd, Arundel, QLD, Australia) at 4°C overnight. After washing, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cat. No: sc-2004, 1:5,000; Bio-Strategy Laboratory Products) for 1 h at room temperature. Signals were then detected using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Densitometry analysis was conducted using ImageJ software (NIH, USA).

Statistics

The Student's *t*-test was used to compare two groups of normally distributed data. Differences in transport activity with or without compound C (multiple groups) were detected by one-way analysis of variance and Dunnett's test. Data are presented as mean ± SD with a *P* value of <0.05 considered as statistically significant.

Results

Selective modulation of OATP1A2-dependent transporter activity by AMPK inhibition

In initial experiments HEK-293T cells that over-expressed OATP1A2, OATP1B1, OATP1B3 or OATP2B1 were treated with the AMPK inhibitor Compound C (10 μM) for 1 h and then transport uptake was estimated. Compound C is the widely used commercially available cell-permeable AMPK specific inhibitor with K*i* of 109 nM (Handa et al., 2011). As shown in Fig. 1A, Compound C selectively impaired OATP1A2 transport activity, but not that of the other three OATPs. It is known that compound C can also inhibit other signaling factors, but it has no significant inhibitory effect on kinases that structurally related to AMPK (Liu et al., 2014). We pre-treated the cells with AMPK activator AICAR prior to the Compound C incubation (Fig. 1B). We found that AICAR can pronouncedly reverse the effect of Compound C, which suggested the specific regulatory role of AMPK on OATP1A2.

In further studies the time- and concentration-dependence of compound C treatment on OATP1A2 transporter function was assessed (Fig. 1C, 1D). Pronounced decreases in E3S uptake were produced by 15-90 min of treatment with 10 μM Compound C. Kinetic studies showed that AMPK inhibition decreased the V_{max} for E3S uptake relative to control (154.6 ± 17.9 pmol· (μg x 4min)$^{-1}$ *versus* 413.6 ± 52.5 pmol·(μg x 4min)$^{-1}$), whereas the K_m after

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Compound C treatment was unchanged from control (44.1 ± 9.2 μM *versus* 63.9±12.9 μM; Fig 2).

AMPK inhibition impacts on the expression of OATP1A2 at the plasma membrane

The decreased V_{max} of OATP1A2-mediated E3S uptake after treatment with Compound C could be due to impaired transporter expression at the cell surface; this was tested directly in further studies. At the plasma membrane, OATP1A2 immunoreactive protein is present as the fully N-glycosylated mature isoform (~95 kDa);at intracellular locations, a partially N-glycosylated immature isoform (~60kDa) is also present in addition to the ~95kDa isoform. (Chan et al., 2016; Zheng et al., 2014; Zhou et al., 2011; Zhou et al., 2013b). It was found that AMPK inhibition by Compound C impaired the cell surface expression of OATP1A2 (Fig. 3A and 3B) but had no effect on total OATP1A2 expression (Fig. 3C and 3D).

AMPK regulates subcellular trafficking of OATP1A2

Previous studies have shown that OATP1A2 is trafficked continuously between the cell surface and intracellular compartments (Chan et al., 2016; Zheng et al., 2014; Zhou et al., 2011). Trafficking processes include internalization from the cell surface to intracellular compartments, recycling from intracellular compartments back to the cell surface, and targeting of newly synthesized protein to the cell surface. We conducted biotinylation-based assays to investigate the effect of AMPK inhibition on OATP1A2 trafficking, in particular internalization, recycling and targeting.

As shown in Fig. 4A, AMPK inhibition accelerated the internalization of OATP1A2 from the cell surface to intracellular compartments. In addition, the rate at which OATP1A2 protein was trafficked from intracellular compartments back to the cell surface and the recycling of OATP1A2 were impaired by AMPK inhibition (Fig. 4B, 4C).

AMPK silencing modulates OATP1A2 activity and expression

As mentioned, heterotrimeric AMPK consists of a catalytic α-subunit and regulatory β- and γ-subunits (Mihaylova and Shaw, 2011). We also adopted an independent approach from chemical inhibition to verify the specific regulatory role of AMPK on OATP1A2. Knockdown of the AMPK α-subunit has been shown to disrupt AMPK signalling in HEK-293T cells (Tangeman et al., 2012; Tzatsos and Tsichlis, 2007). In the present study we utilised siRNA silencing of the AMPKα1 subunit to corroborate and extend the findings with compound C on OATP1A2 regulation. Following AMPKα1 silencing E3S uptake was decreased in HEK-293T cells to ~6.1% of control scrambled siRNA-transfected cells;

P<0.001, Fig. 5B). Like compound C treatment, such AMPKα1 silencing decreased the expression of OATP1A2 at the plasma membrane (Fig. 5C and 5D). Such observation was also validated with another independent siRNA obtained from Thermo Scientific (Supplemental Figure 2). However, unlike AMPK inhibition, AMPKα1 silencing also appeared to decrease protein stability (Fig. 5C and 5D). The additional effect of siRNA silencing on OATP1A2 protein stability is possibly due to the long-term effect of AMPK inhibition (48 hr after co-transfection of OATP1A2 and AMPKα1 siRNA) as compared to the acute effect induced by Compound C (60 min treatment).

We further treated HEK-293T cells with cycloheximide (100 μg/ml) in a time-course experiment to inhibit protein synthesis. As shown in Fig. 5E, AMPKα1 silencing impaired OATP1A2 stability by accelerating the degradation of the transporter protein.

Discussion

OATP1A2 is abundantly located in the epithelium of many tissues and influences the disposition of numerous drugs, xenobiotics and endobiotics (Kullak-Ublick et al., 1995; Liu and Li, 2014; Steckelbroeck et al., 2004). At the apical membrane of renal tubular cells OATP1A2 mediates the reabsorption of xenobiotics (Lee et al., 2005), in the biliary epithelium OATP1A2 facilitates the excretion of xenobiotics into bile and at the luminal membrane of the blood-brain barrier OATP1A2 modulates the uptake of opioid analgesic peptides and other CNS-active agents into the brain (Liu et al., 2015). More recently, OATP1A2 has been detected on the apical membrane of the retinal pigment epithelium, where it facilitates the uptake of retinoids (Chan et al., 2015b; Gao et al., 2015). Therefore, OATP1A2 dysfunction may impair drug pharmacokinetics and disposition that could influence the outcomes of drug therapy and may also impair endobiotic uptake into target tissues.

AMPK is an established regulator of metabolic homeostasis because it modulates the expression and function of proteins that control energy production and utilization (Ronnebaum et al., 2014). There is evidence that AMPK regulates epithelial ion transporters of physiological importance. For instance, AMPK phosphorylates the renal-specific Na^+-K^+-$2Cl^-$-co-transporter (SLC12A1) and the cystic fibrosis transmembrane conductance regulator (ABCC7), as well as the proximal tubule vacuolar H^+-ATPase (Al-bataineh et al., 2014;

Fraser et al., 2007; Hallows et al., 2003). Through two independent approaches (chemical inhibition and siRNA knockdown), the present findings now indicate that the SLC transporter OATP1A2, which is important in drug disposition, is also potentially regulated by AMPK signalling. Dysregulation was selective for OATP1A2 because the activity of three other major drug-transporting OATPs was unchanged by treatment of cells with the AMPK inhibitor Compound C (Fig. 1A). This also indicated that the regulatory effect of AMPK on OATP1A2 is not likely due to the changes in cellular energy hemostasis.

Kinetic analysis indicated that the maximal rate of E3S uptake by OATP1A2 was decreased by AMPK inhibition, while the apparent affinity of the transporter for E3S was unchanged (Fig. 2). Expression of OATP1A2 at the plasma membrane, but not its total cellular expression, was decreased by Compound C treatment, which is consistent with altered trafficking of the transporter (Fig. 3). Using a series of trafficking protocols, we found that AMPK inhibition and silencing increased the internalization and decreased the recycling of OATP1A2 (Fig. 4). In addition to the decrease in OATP1A2 transport function and cell surface expression, the stability of the transporter protein was also impaired by AMPKα1 silencing (Fig. 5). Taken together, the present findings indicate that AMPK signalling plays an important role in regulating the internalization, targeting, recycling and stability of OATP1A2. We also tested the potential significance of the present findings in *ex vivo* and *in vivo* settings. As we reported before, OATP1A2 is known to express in human Retinal Pigmented Epithelial (RPE) cells (Chan et al., 2015b; Xu et al., 2016); therefore, we evaluated the response of OATP1A2 to AMPK inhibition in human primary RPE cells (Supplementary Figure 3A). Consistently, OATP1A2 mediated ^{3}H-E3S uptake was statistically significantly reduced upon Compound C pre-treatment (p<0.05), which suggested that OATP1A2 response to AMPK inhibition is not restricted in renal cells. Although there are no direct rodent orthologues of human OATP1A2, mouse Oatp1 (Oatp1a1) has ~70% similarity to OATP1A2 at the amino acid level. We found that the transport function in HEK-293T cells that over-expressed mouse Oatp1 was decreased by treatment with the AMPK inhibitor compound C. Further, the expression of immunoreactive Oatp1 protein was decreased in the liver of male AMPK β1-null mice (Dzamko et al., 2010; Mount et al., 2012) (Supplemental Figure 3). AMPK dysregulation has been widely indicated in obesity, diabetes and the metabolic syndrome (Coughlan et al., 2014; Steinberg and Kemp, 2009) and decreased mouse Oatp1 expression was reported in diabetic rodent models by us and others (Li et al., 2017; Xu et al., 2015), so our study is clinically relevant to elucidate the

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altered drug response in these diseases. Moreover, we acknowledge that under the physiological condition, the regulatory machinery of AMPK may react differently from that observed in the overexpressing *in vitro* system, so future studies may verify our findings in *in vivo* models.

The consensus AMPK phosphorylation motif spans ~11 amino acid residues and consists of a central Ser/Thr/Tyr residue with one or more basic residues (Arg/Lys/His) at the N-terminal end, and one or more hydrophobic residues (*eg*. Leu/Met/Ile/Val/Phe) (Hardie, 2011a). Inspection of the primary amino acid sequence of OATP1A2 identified Ser-36 and Ser-313 as potential AMPK phosphorylation sites; the other three drug-transporting OATP isoforms that were evaluated in the present study lacked analogous serine residues (Supplemental Figure 4A). We tested the functional roles of Ser-36 and Ser-313 using site-directed mutagenesis. However, E3S uptake by the mutagenized OATP1A2 transporters was unchanged from wild-type, which suggests either that AMPK does not mediate OATP1A2 phosphorylation directly or that atypical AMPK regulatory sites might be involved (Supplemental Figure 4B). As to the previous reports, the protein trafficking of OATPs is also facilitated by other chaperon proteins like PDZ-domain containing proteins (Ferreira et al., 2018; Murray and Zhou, 2017; Zheng et al., 2014); therefore, it is plausible that AMPK inhibition impacts on chaperon proteins and indirectly influences the expression and function of OATP1A2. Further studies to explore the impact of AMPK inhibition on these chaperon proteins and the potential atypical AMPK motifs in OATP1A2 are now warranted.

The interplay between multiple kinases modulates the dynamic trafficking of a number of transport proteins. The internalization of Glucose Transporter 4 (GLUT4; SLC2A4) was impaired and its expression at the plasma membrane of skeletal muscle cells was increased by activation of AMPK and PKC (Antonescu et al., 2008; Klip et al., 2009). In contrast, expression of the Creatine Transporter (SLC6A8) was decreased by AMPK and PKC activation (Li et al., 2010) and we found previously that OATP1A2 trafficking was regulated by PKC and CKII (Fig. 6) (Chan et al., 2016; Murray and Zhou, 2017; Zhou et al., 2011). Like AMPK inhibition, PKC activation accelerated the internalisation of OATP1A2. Although PKC activation did not influence OATP1A2 recycling/targeting, AMPK inhibition also decreased the recycling and membrane targeting of the transporter. Thus it is feasible that the regulatory actions of PKC on OATP1A2 could be related in part to AMPK signalling. Similarities between AMPK and CKII inhibition were noted because both decreased OATP1A2 recycling and targeting (Fig. 6) (Chan et al., 2016). However, unlike AMPK

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inhibition, CKII inhibition impaired the internalisation of OATP1A2. Overall, the regulatory effects of CK2 and PKC only partially overlapped with that of AMPK. Thus OATP1A2 protein trafficking is subject to complex regulations by a group of kinases including AMPK, PKC and CKII, but AMPK was unlikely the "master" regulatory signalling upstream of PKC or CK2. Since the main focus of our current study is to explore how OATP1A2 was regulated by AMPK that influences on drug performance in disease treatments; therefore, a detailed AMPK signalling pathway mapping involved in this regulation or to explore the potential atypical AMPK motifs involved in such regulation is beyond the scope of this study and also is with less relevance to drug treatments, which will be included in our future studies.

In summary, the present study is the first to demonstrate a regulatory role for AMPK on human drug-transporting OATPs. AMPK regulated the expression and function of OATP1A2 by modulating its subcellular trafficking and stability. The dysregulation of AMPK has been associated with obesity, type II diabetes and other manifestations of the metabolic syndrome, such as non-alcoholic fatty liver disease. Indeed, the present findings are compatible with those of Clarke *et al*. (Clarke et al., 2017), who reported that transporter N-glycosylation and trafficking were impaired in non-alcoholic fatty liver disease. Drug therapy may be complicated in patients with obesity or the metabolic syndrome. For example, obesity is associated with a higher incidence of chronic myelogenous leukemia, for which the OATP1A2 substrate imatinib is a front-line treatment (Strom et al., 2009). The present information provides the basis for future studies on how OATP transport activity is regulated in disease. This information could be taken into consideration in drug selection for optimal therapy in patients with the metabolic syndrome.

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Authorship contributions:

Participated in research design: Lu, Zhou

Conducted experiments: Lu, Chan, Cheng, Shames, Zhou

Contributed new reagents or analytic tools: Murray

Performed data analysis: Lu, Zhu, Murray, Zhou

Wrote or contributed to the writing of the manuscript: Lu, Zhu, Murray, Zhou

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Conflict of interest:

The authors state no conflict of interest.

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Figure Legend

Figure 1. Effect of AMPK inhibition on [^{3}H]-E3S uptake by HEK-293T cells that over-expressed OATP isoforms. (A) Substrate uptake by OATP1A2, OATP1B1, OATP2B1 and OATP1B3 in over-expressing HEK-293T cells in the presence or absence of Compound C (10 μM, 37°C, 60 min). Uptake of [^{3}H]-E3S (300 nM) was used for OATP1A2, OATP1B1 and OATP2B1; uptake of [^{3}H]-CCK-8 (2 nM) was used for OATP1B3, as described in Methods. (B) AMPK activator AICAR reverses the inhibitory effect of Compound C on OATP1A2-mediated [^{3}H]-E3S uptake. OATP1A2 over-expressing HEK-293T cells were pre-treated with or without AICAR (2 mM, 37°C, 30 min) and followed with the treatment of Compound C (10 μM, 37°C, 60 min). Uptake of [^{3}H]-E3S (300 nM) was used for OATP1A2 was measured as described in the Methods. (C) Effect of duration of treatment of OATP1A2-over-expressing HEK-293T cells with Compound C (10 μM, 37°C) on [^{3}H]-E3S uptake (300 nM). (D) Effect of Compound C concentration on [^{3}H]-E3S (300 nM) uptake by OATP1A2-over-expressing HEK-293T cells (60 min, 37°C). Values are mean ± SD (n=3). *P < 0.05, **P < 0.01, ***P < 0.005; different from DMSO-control.

Figure 2. Kinetic analysis of [^{3}H]-E3S uptake by HEK-293T cells that over-expressed OATP1A2 in the presence or absence of Compound C. [^{3}H]-E3S concentrations ranged from 0.05 to 50 μM (4 min uptake); cells were treated with 10 μM compound C or DMSO for 60 min at 37 °C. Transporter kinetic parameters (K_m and V_{max}) were calculated using GraphPad Prism 5. Values are mean ± SD (n = 3). (A) Michaelis-Menten plot. (B) Calculated K_m and V_{max} values of OATP1A2. **P < 0.01, different from DMSO-control.

Figure 3. Effect of AMPK inhibition on OATP1A2 protein expression. (A) Cell surface expression of OATP1A2 with or without Compound C pre-treatment assessed with biotinylation assay. Over-expressing HEK-293T were pre-treated with Compound C (10 μM) or DMSO for 60 min at 37 °C. Top panel: protein lysate probed with anti-Flag antibody. Bottom panel: after stripping, the blot was re-probed with anti-Na^+-K^+-ATPase antibody. (B) Densitometric analysis of OATP1A2 cell surface expression. Data were derived in three independent experiments. Values are mean ± SD (n=3). **P < 0.01, different from DMSO-control. (C) Total cellular expression of immunoreactive OATP1A2 with or without Compound C pre-treatment. Over-expressing HEK-293T were pre-treated with Compound C (10 μM) or DMSO for 60 min at 37°C. Top panel: protein lysate probed with anti-Flag antibody. Bottom panel: after stripping, the blot was re-probed with anti-Na^+-K^+-ATPase

antibody. (D) Densitometry analysis of protein total cell expression. Data were derived in three independent experiments. Values are mean ± SD (n=3). NC: vector-transfected HEK-293T cells.

Figure 4. Trafficking assays of OATP1A2 in over-expressing HEK-293T cells with or without Compound C treatment. (A) OATP1A2 internalisation process. Left panel: OATP1A2 internalization assay was conducted as described in Material and Methods. Cells were pre-treated with Compound C or DMSO (10 μM, 60 min, 37 °C). Cells were allowed to internalize for 0.5, 1.5, 3 min at 37°C. Right panel: Densitometric analysis of the internalized transporter protein as a percentage of the total initially biotinylated OATP1A2 at the cell surface. (B) OATP1A2 membrane targeting process. Left panel: OATP1A2 targeting was evaluated as described in Material and Methods. Cells were pre-treated with Compound C or DMSO (10 μM, 60 min, 37 °C). Additional biotin labelling was allowed for 5, 10 and 15 min at 37 °C. Right panel: Densitometric analysis of transporter protein that was newly targeted to the cell surface (newly synthesised and previously internalised protein combined) as a percentage of the total initially biotinylated OATP1A2 at cell surface. (C) OATP1A2 recycling process. Left panel: OATP1A2 recycling was assessed as described in Material and Methods. Cells were pre-treated with Compound C or DMSO (10 μM, 60 min, 37 °C). Cells were allowed to internalize for 1 min at 37°C and after the first stripping step, cells were allowed to internalise for 1, 2 or 3 min at 37°C. Right panel: Densitometric analysis of recycled transporter protein as a percentage of the total initially biotinylated OATP1A2 at the cell surface before initiation of recycling (the protein signal at t=0 min was subtracted from each time point). A representative blot is presented; each experiment was repeated on three occasions. Values are mean ± SD (n=3). ** $P < 0.01$, different from DMSO-control. SC: stripping control; NC: vector-transfected HEK-293T cells.

Figure 5. The effect of AMPK α1 subunit silencing on the uptake function and protein expression of OATP1A2. (A) Total cellular expression of AMPKα1 subunit in HEK-293T cells that over-expressed OATP1A2 after transfection with an AMPKα1-specific or scrambled siRNAs. Top panel: Immunoblot was probed with anti-AMPK α1 antibody. Bottom panel: After stripping, the blot was re-probed with anti-β-actin antibody. (B) [^{3}H]-E3S uptake was performed in HEK-293T cells that over-expressed OATP1A2 and were transfected with either AMPKα1-specific or scrambled siRNAs. (C) Total cellular and plasma membrane expression of OATP1A2 in HEK-293T cells that over-expressed

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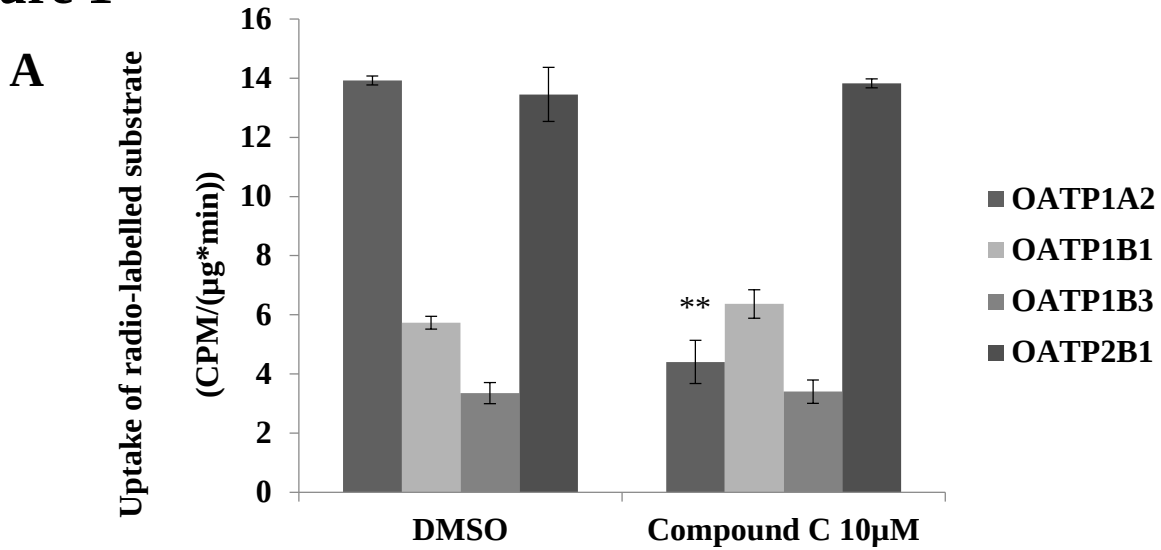
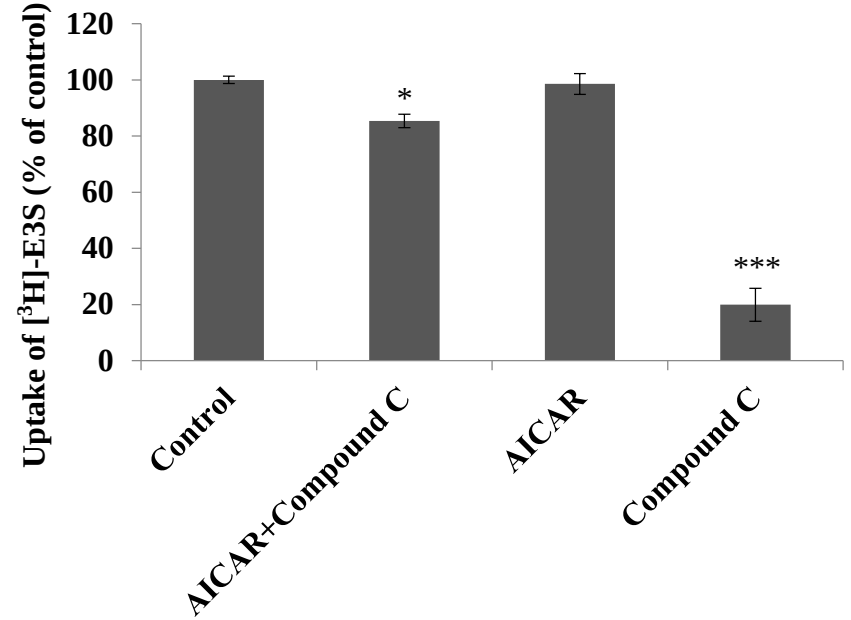
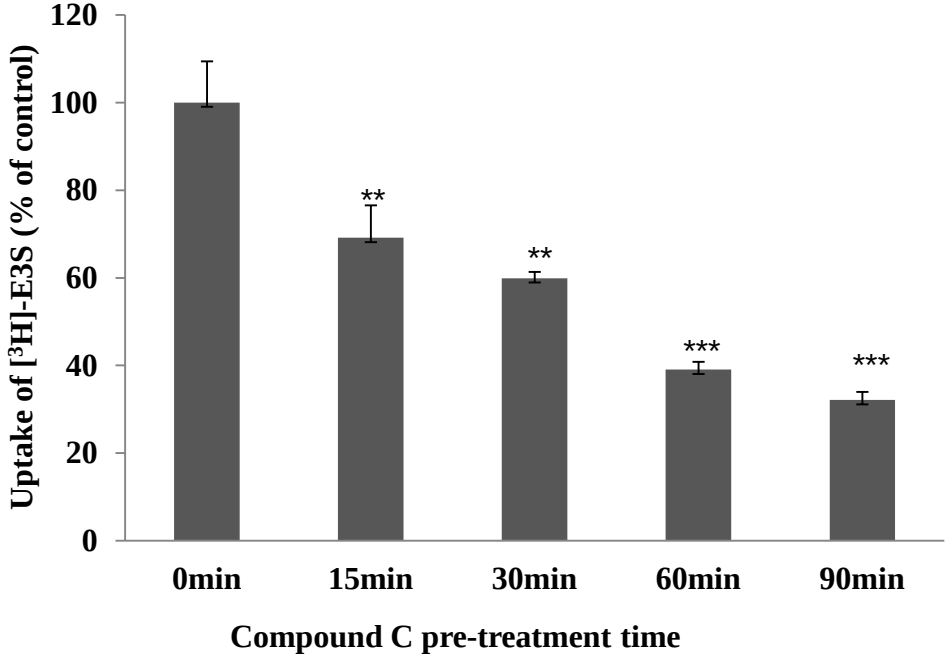
OATP1A2 and were transfected with either AMPKα1-specific or scrambled siRNAs. Top panel: OATP1A2 cell surface expression evaluated *via* biotinylation analysis. Middle panel: Total cellular expression of OATP1A2 assessed by immunoblotting. Bottom panel: After stripping, the blot from the middle panel was re-probed with an anti-β-actin antibody. (D) Densitometric analysis of OATP1A2 cell surface and total cell expression. Data were derived in three independent experiments. Values are mean ± SD (n=3). ** $P < 0.01$; *$P < 0.05$, different from scrambled siRNA transfected control. (E) Stability of OATP1A2 protein with or without AMPK silencing. HEK-293T cells that over-expressed OATP1A2 were transfected with either AMPKα1-specific or scrambled siRNAs, and were treated with the protein synthesis inhibitor cycloheximide (100 μg/mL) for varying times as shown. Immunoblotting analysis was conducted to analyse expression of OATP1A2. Top panel: Total cellular expression of OATP1A2 after CHX treatment. Middle panel: After stripping, the blot was re-probed with anti-β-actin antibody. Bottom panel: Densitometric analysis of the ratio of OATP1A2 to β-actin as a percentage of that at the 8 hr time point. A representative blot is presented; each experiment was repeated on three occasions. Values are mean ± SD (n=3). ** $P < 0.01$, different from scrambled siRNA transfected control.

Figure 6. Multiple signaling kinases regulate the membrane targeting, internalization, recycling and degradation of OATP1A2.

Figure 1







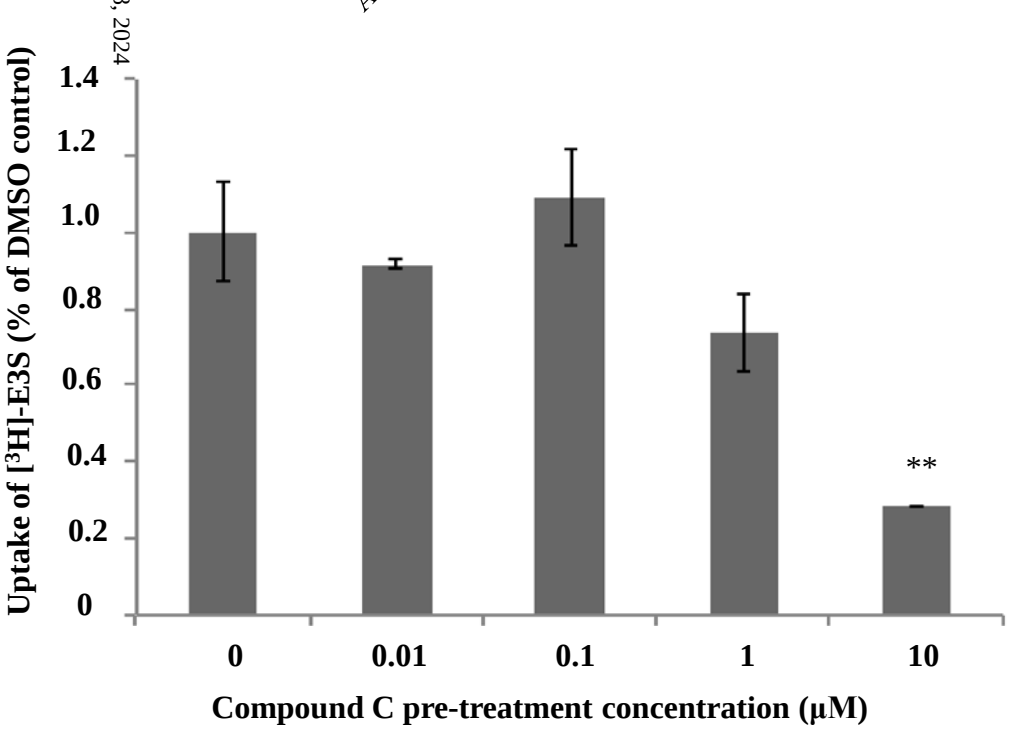
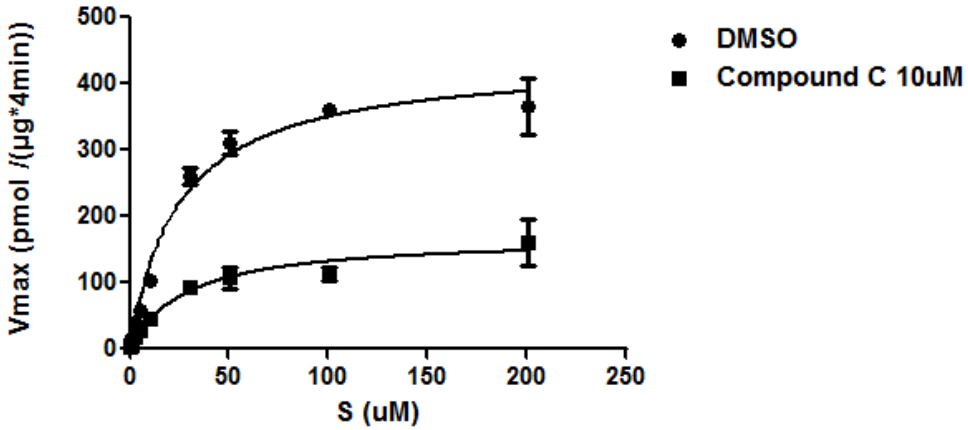


Figure 2

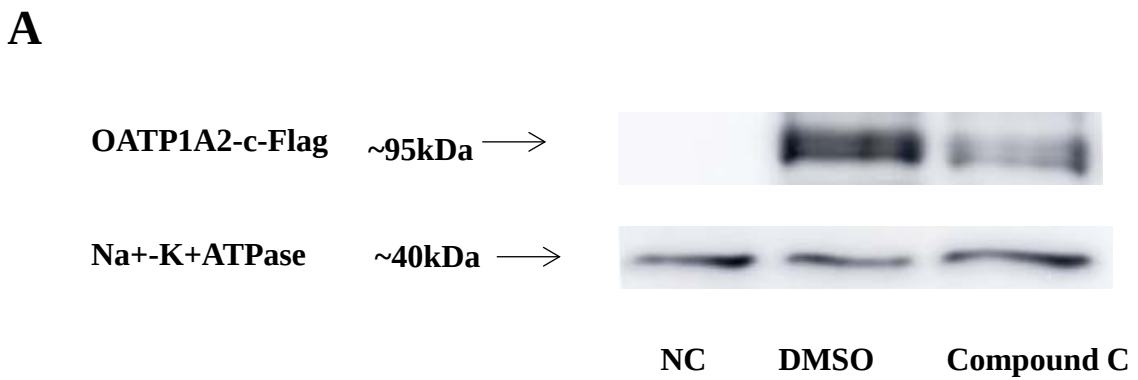
A

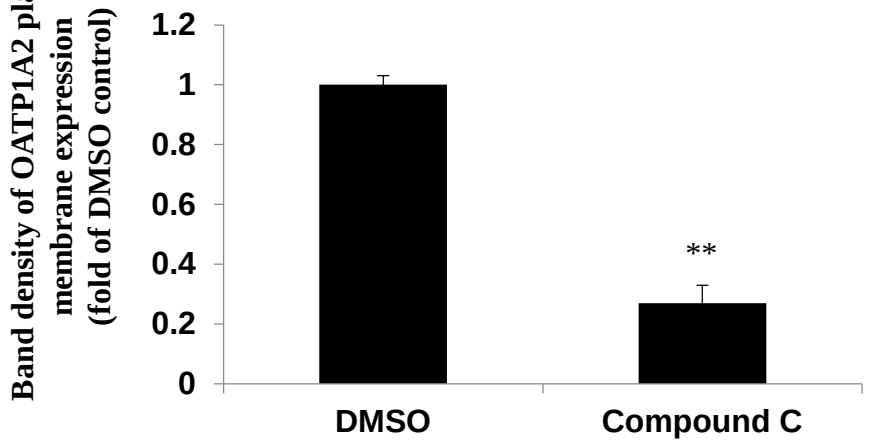


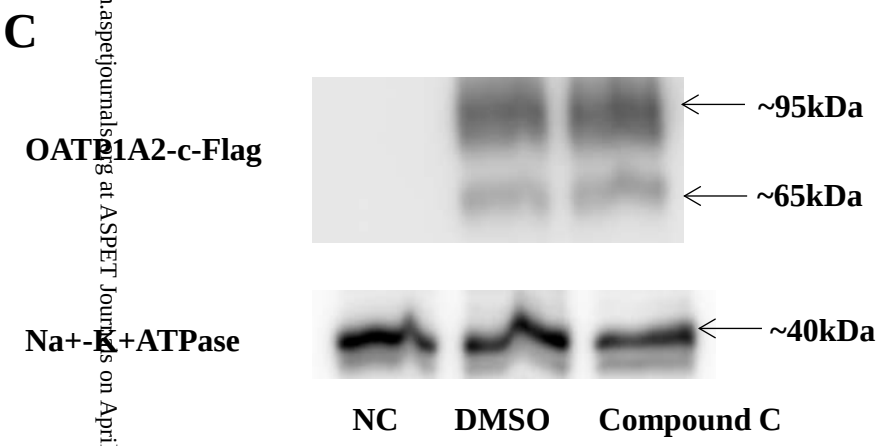
B

	K_m (µM)	V_{max} [pmol x (µg x 4 min)$^{-1}$]
DMSO	63.9 ± 12.9	413.6 ± 52.5
Compound C 10 µM	44.1 ± 9.1	154.6 ± 17.9 **

Figure 3







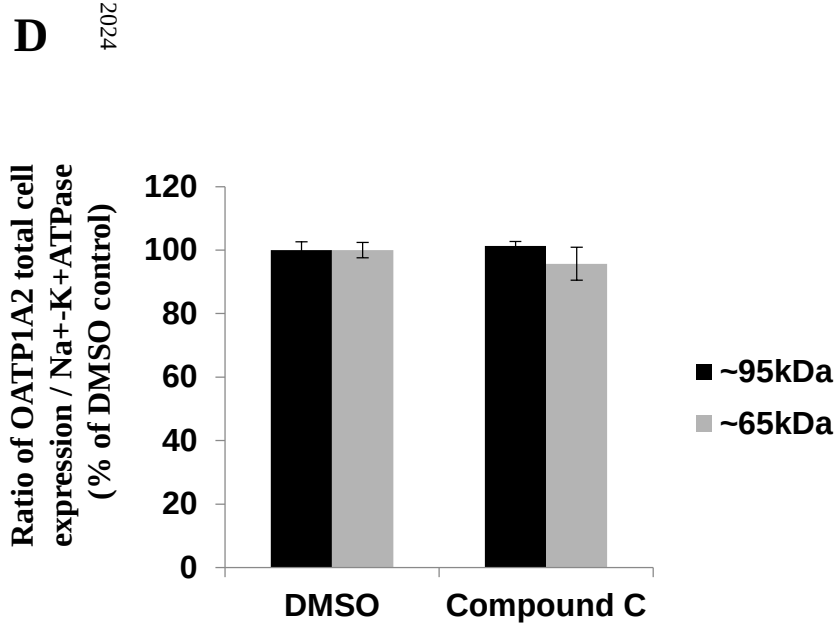


Figure 4

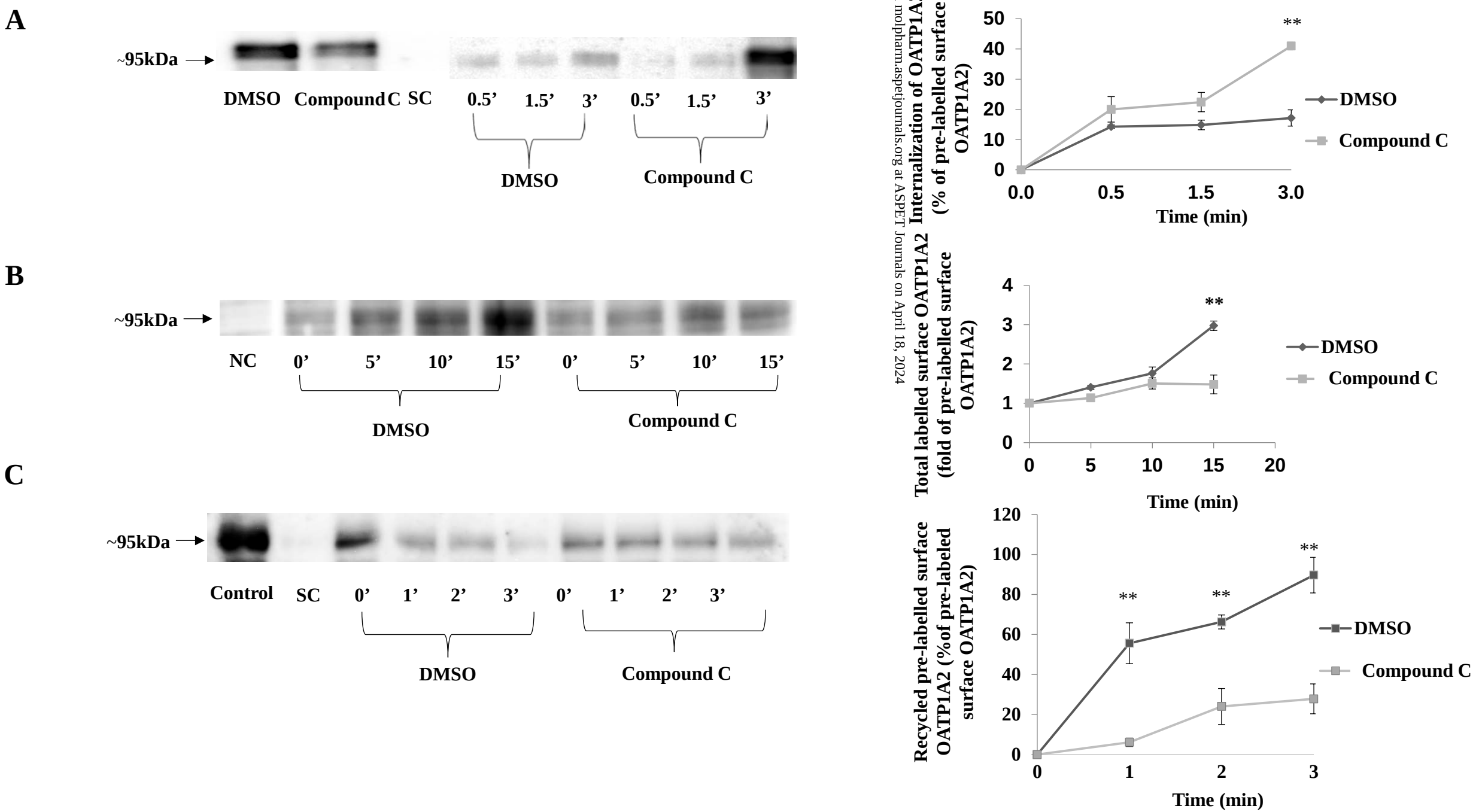


Figure 5

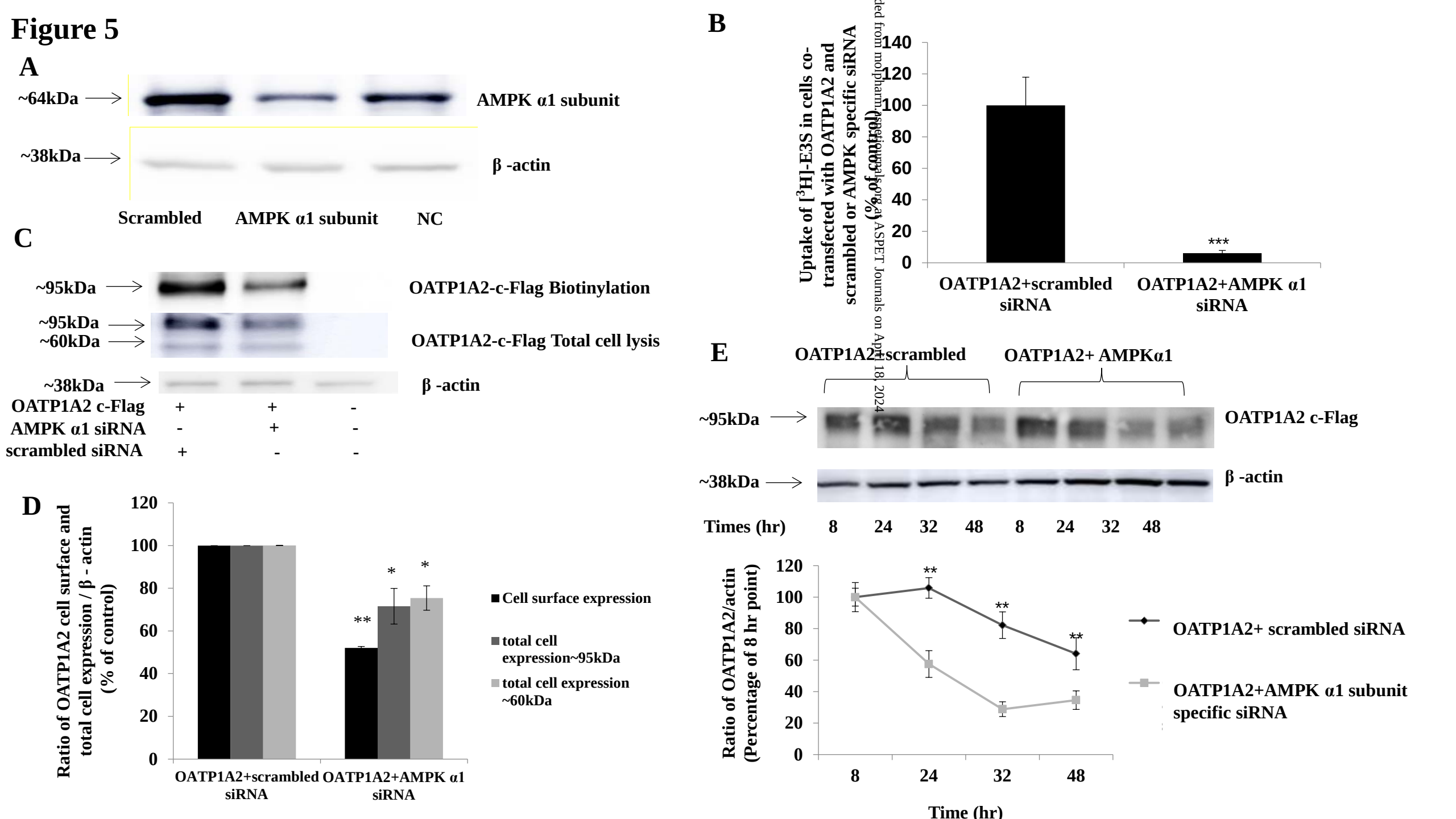


Figure 6