Pharmacological Chaperones of the Dopamine Transporter Rescue Dopamine Transporter Deficiency Syndrome Mutations in Heterologous Cells*

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A number of pathological conditions have been linked to mutations in the dopamine transporter gene, including hereditary dopamine transporter deficiency syndrome (DTDS). DTDS is a rare condition that is caused by autosomal recessive loss-of-function mutations in the dopamine transporter (DAT), which often affects transporter trafficking and folding. We examined the possibility of using pharmacological chaperones of DAT to rescue DTDS mutations. After screening a set of known DAT ligands for their ability to increase DAT surface expression, we found that bupropion and ibogaine increased DAT surface expression, whereas others, including cocaine and methylphenidate, had no effect. Bupropion and ibogaine increased wild type DAT protein levels and also promoted maturation of the endoplasmic reticulum (ER)-retained DAT mutant K590A. Rescue of K590A could be blocked by inhibiting ER to Golgi transport using brefeldin A. Furthermore, knockdown of coat protein complex II (COPII) component SEC24D, which is important in the ER export of wild type DAT, also blocked the rescue effects of bupropion and ibogaine. These data suggest that bupropion and ibogaine promote maturation of DAT by acting as pharmacological chaperones in the ER. Importantly, both drugs rescue DAT maturation and functional activity of the DTDS-associated mutations A314V and R445C. Together, these results are the first demonstration of pharmacological chaperoning of DAT and suggest this may be a viable approach to increase DAT levels in DTDS and other conditions associated with reduced DAT function.

The dopamine transporter (DAT)4 is responsible for controlling levels of extracellular dopamine and maintaining dopamine stores by transporting dopamine back into neurons after release (1, 2). DAT is part of the Na+/Cl−-dependent neurotransmitter symporter solute carrier 6 (SLC6) family, which also includes the serotonin, norepinephrine, taurine, and GABA transporters (GAT) (3). A variety of pathological conditions have been associated with mutations in both coding and non-coding regions of the DAT gene (SLC6A3), most of which result in reduced DAT function (4–7). A recently discovered condition, hereditary DAT deficiency syndrome (DTDS) is caused by autosomal recessive loss-of-function mutations in DAT (6, 8, 9). The disorder is characterized by parkinsonism-dystonia and elevated dopamine metabolites in the cerebrospinal fluid. To date, very few diagnosed DTDS patients have survived to adulthood; the majority of patients die in infancy or adolescence (8, 9). When expressed in heterologous cells, DTDS mutations prevent DAT protein maturation past the endoplasmic reticulum (ER) and result in reduction or elimination of dopamine uptake activity (9). ER retention is a common consequence of mutations in membrane proteins and does not necessarily reflect complete misfolding (10). However, the quality control in the ER is so stringent that mutated proteins that are otherwise partially or completely functional can be retained and degraded (11). Indeed, folding of even wild type polypeptide sequences is an inefficient process. An estimated 30% of newly synthesized proteins are subject to immediate degradation, and for some membrane proteins folding efficiency is <50% (12–14). Pharmacological chaperones can increase folding efficiency of wild type (WT) and mutant protein, resulting in increased functional protein (11). Over the last several years, pharmacological chaperoning approaches have been successfully used to rescue mutants of a number of proteins including the vasopressin 2 receptor and the cystic fibrosis transmembrane conductance regulator. We hypothesize that this approach could also rescue DAT mutants associated with DTDS. In line with this hypothesis, a recent study has described a pharmacological chaperoning effect of ibogaine on the closely related serotonin transporter (SERT) (15).

We previously developed a β-lactamase based cell surface expression assay (16). Inserting a β-lactamase tag in the second extracellular loop of DAT allowed us to easily monitor DAT surface expression while not disrupting DAT function (16). In the present study, we applied this technique as a tool to screen a number of DAT inhibitors for potential pharmacological chaperone activity. It is known that DAT ligands can stabilize different conformational states of the transporter (17–20). We, therefore, selected a range of ligands stabilizing the different known conformations of DAT. We found the following. 1) Ligands stabilizing the occluded/inward facing conformation

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4 The abbreviations used are: DAT, dopamine transporter; SLC6, solute carrier 6; GAT, GABA transporter; DTDS, DAT deficiency syndrome; ER, endoplasmic reticulum; SERT, serotonin transporter; MDPV, methylenedioxypyrovalerone; PNGase F, peptide N-glycosidase F; BFA, brefeldin A; RIPA, radioimmunoprecipitation.
increase DAT surface expression, whereas ligands that stabilize the outward facing confirmation do not. 2) The increase in DAT surface expression after treatment with bupropion and ibogaine is due to an increase in mature DAT protein. 3) Ibogaine and bupropion can rescue an ER-retained DAT mutant in a SEC24D-dependent manner. 4) Ibogaine and bupropion can rescue protein maturation and uptake activity of select DAT mutants associated with DTDS. Together these data form the first proof of principle for the use of pharmacological chaperones to increase DAT function, thus opening the door to exploration of such strategies for clinical use.

**Results**

*Atypical DAT Inhibitors Increased DAT Surface Expression—* To identify selective pharmacological chaperones of DAT, we screened DAT ligands for their ability to increase DAT surface expression using our recently developed β-lactamase surface expression assay (16). Traditionally, DAT ligands have been divided into substrates (e.g. dopamine and amphetamine) and inhibitors (e.g. cocaine). However, recent reports differentiate DAT ligands based on the conformational state that they bind (20, 21). For example, cocaine and methylphenidate stabilize an open-to-out conformation of the transporter, whereas ibogaine, GBR12909, bupropion, and benztrapine stabilize a more inward facing conformation. We chose structurally diverse ligands that would potentially stabilize these different categories of transporter conformation and assessed their ability to increase DAT surface expression. Several DAT ligands increased surface expression (Fig. 1A). Compounds that stabilized the closed-to-out and -inward-facing conformation of the DAT increased surface expression (GBR12909, benztrapine, bupropion, ibogaine), whereas compounds that stabilized the open-to-out conformation did not increase DAT surface expression (methylphenidate, cocaine). Methylenehydroxypropyrophalerone (MDPV) had no effect on DAT surface expression; it is currently not known which conformation of DAT is stabilized by MDPV. Bupropion and ibogaine were selected for further characterization as they displayed the largest effect on surface expression, with bupropion (136.6 ± 6.0%) and ibogaine (167.3 ± 13.5%) having the largest effect on WT DAT after 16 h of treatment (Fig. 1A).

**FIGURE 1.** DAT inhibitors increase surface expression of WT DAT. A, effect of DAT inhibitors on DAT surface expression. The concentrations of drugs used were 10 μM benztrapine, 100 μM bupropion, 1 μM GBR12909, 100 μM ibogaine, 10 μM cocaine, 100 μM methylphenidate, 1 μM MDPV, and 10 μM amphetamine. Data are the means ± S.E., *p < 0.05; **p < 0.01; ***p < 0.001; three or 4 separate experiments, three technical replicates/experiment. B, time-course of surface expression after treatment with bupropion (100 μM) or ibogaine (100 μM); 3 separate experiments, 3 technical replicates/experiment. C, concentration-effect curves of bupropion and ibogaine after 16 h of treatment; 3 separate experiments, 3 technical replicates/experiment.

*Bupropion and Ibogaine Increased Mature DAT Protein—* Next we assessed the effects of bupropion and ibogaine on DAT protein. We used cells expressing a yellow fluorescent protein (YFP)-labeled, HA-tagged human DAT (hereafter referred to as DAT) (22). When expressed in HEK293 cells, two bands were detected for DAT protein that were not present in non-transfected mock cells (Fig. 2A). We digested cell lysates with the glycosidases peptide N-glycosidase F (PNGase F) and EndoH and confirmed that the upper band (~110 kDa) represents mature DAT protein, whereas the lower band (~80 kDa) represents immature DAT that has not yet been processed in the Golgi and is ER-localized (Fig. 2A) (23). We treated cells expressing WT DAT with bupropion and ibogaine and quantified mature and immature DAT levels. Bupropion and ibogaine increased mature DAT protein levels (Fig. 2, B–D), indicating that changes in surface expression were not simply due to increased surface trafficking of DAT. Interestingly, there was a decrease in the levels of the immature DAT protein but only with ibogaine treatment (Fig. 2E). These results suggest that ibogaine treatment increases conversion of immature to mature DAT protein. Importantly, we saw no changes in another membrane protein, the Na+/K+ ATPase, suggesting that the compounds did not act as general chaperones (data not shown).
Bupropion and Ibogaine Rescue the ER-retained Mutant K590A—The C terminus of monoamine transporters is important for protein folding, chaperone interaction, and exit from the ER (24–26). Mutation or truncation of the C terminus leads to ER retention of the GAT, SERT, and DAT proteins (15, 25, 26). To determine if bupropion and ibogaine promoted ER exit, we used the C-terminal DAT mutant K590A (26). We first confirmed the localization of WT and K590A DAT in HEK cells using confocal microscopy. Compared with the WT dopamine transporter, which is predominantly present at the plasma membrane, K590A protein was almost exclusively intracellular (Fig. 3A), in agreement with previous studies (26). As shown in the images in Fig. 3, B and C, treatment of cells with bupropion and ibogaine increased surface expression of the K590A mutant. We confirmed this by performing cell surface biotinylation (Fig. 3, D and E), which also showed that both bupropion and ibogaine increase K590A DAT cell surface expression. Treatment with bupropion and ibogaine increased the levels of total K590A mature DAT protein in cell lysates by 3.0 ± 0.2 and 4.4 ± 1.0 fold, respectively (Fig. 4, A–C). This increase in mature K590A was reflected in increased dopamine uptake activity for this mutant after bupropion and ibogaine treatment, although uptake activity was not increased to the same extent as protein levels (Fig. 4D). ER exit and maturation of the K590A mutant suggests that bupropion and ibogaine act on the protein in the ER, as opposed to stabilizing transporter at the surface.

To more directly evaluate this, we assessed the effect of bupropion and ibogaine in the presence of brefeldin A (BFA). BFA is a chemical agent that interferes with protein transport through the secretory pathway, one effect of which is ER retention of membrane proteins. Cells expressing K590A mutant DAT were treated for 8 h with BFA, as longer treatment leads to cell death. As shown in Fig. 4, F and H, BFA treatment inhibits the effects of ibogaine on K590A DAT maturation. The trend appears to be the same for bupropion (Fig. 4, E and G). These data support the idea that bupropion and ibogaine act on DAT before the protein leaves the ER.

**SEC24D Function Is Important for DAT Maturation**—Lysine 590 of DAT has been suggested to be part of a structural motif that is recognized by SEC24D. The SEC24 family recruits cargo into vesicles for transfer to the Golgi, and isoform SEC24D appears to be important for ER export of DAT (27, 28). We, therefore, investigated if the rescue of K590A by bupropion and

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**FIGURE 2. Treatment with ibogaine and bupropion increases mature DAT protein.** A, immunoblot of YFP-DAT (lane 2) expressed in HEK293 cells shows two bands after detection with anti-GFP antibody that are not present in the non-transfected lane (lane 1). The lower molecular weight (75 kDa) band represents immature DAT (iDAT) as evident by the digestion with the enzyme endoglycosidase H (Endo H; lane 3). The upper (110 kDa) band represents mature DAT (mDAT) with complex oligosaccharides, which were removed using the enzyme PNGase F (lane 4). Identity of the bands was confirmed using antibodies for C-terminal and N-terminal DAT epitopes, which showed similar immunoreactivity as observed with the GFP antibody (data not shown). Representative blots for WT DAT treated with bupropion (BUP) (B) or ibogaine (IBO) (C) show increased mDAT protein. D, quantification of mDAT protein levels in bupropion and ibogaine treated samples. Results are normalized to GAPDH loading control. Data are the means ± S.E. **, p < 0.01; ***, p < 0.001; 3 or 4 separate experiments, 4 technical replicates/experiment.
ibogaine was SEC24D-dependent. First we knocked down SEC24D protein using siRNA in cells expressing WT DAT. Our results showed a decrease in WT DAT mature protein after knockdown of SEC24D as well as an apparent decrease in plasma membrane localization (Fig. 5, A–C). This corroborates previous reports of decreased dopamine uptake after SEC24D knockdown in DAT-transfected cells and decreased DAT surface expression after expression of a dominant negative form of SEC24D (25, 28). When we knocked down SEC24D in K590A DAT-expressing cells, we observed no basal effect on DAT mature protein levels or cellular distribution (Figs. 6, A–C, and 7, A–C). However, the effect of bupropion on rescuing DAT mature protein and plasma membrane expression was significantly reduced after knocking down SEC24D (Fig. 6, A–C).
show that bupropion and ibogaine are effective at promoting DAT protein levels (Fig. 8, D). For instance, for the DTDS mutants L224P and P529L, there was no effect on either mature DAT mutants, indicating a functional rescue for these disease causative mutations.

To see if our observations with DAT K590A extended to other mutations, we tested whether bupropion or ibogaine could also rescue DAT mutations associated with DTDS. We tested the effects of the compounds on four different DAT mutations: L224P, A314V, R445C, and P529L (8, 9). Both bupropion and ibogaine treatment increased mature DAT protein levels (Fig. 8, A–C). More importantly, bupropion or ibogaine treatment also increased dopamine uptake activity of the A314V and R445C mutants, indicating a functional rescue for these disease causing DAT mutants (Fig. 8, D–E). However, for the DTDS mutations L224P and P529L, there was no effect on either mature DAT protein levels (Fig. 8, A–C) or dopamine uptake activity (Fig. 8, D–E) upon treatment with either drug. These results show that bupropion and ibogaine are effective at promoting maturation of select DTDS-associated mutants with varying levels of efficacy.

Discussion

In this study we set out to identify pharmacological chaperones of DAT as an approach for increasing DAT function in general and rescue of clinically relevant misfolding DAT mutants in particular. Although DAT ligands that alter DAT surface expression or dopamine uptake have been reported (29–31), pharmacological chaperones of DAT have thus far not been described. We found that benztrapine, bupropion, GBR12909, and ibogaine all increased WT DAT surface expression, whereas cocaine, methylphenidate, and MDPV did not. Amphetamine, on the other hand, decreased DAT surface expression, which was expected as it is known that amphetamine treatment triggers DAT internalization (32). Interestingly, the compounds that increased DAT surface expression are “atypical” DAT inhibitors, which are thought to stabilize a closed-to-out conformation of DAT, as opposed to the outward-facing conformation stabilized by cocaine and methylphenidate (21). There is evidence that ibogaine stabilizes an inward facing conformation that is distinct from the occluded conformation stabilized by other atypical inhibitors, perhaps accounting for the greater increases in surface expression (18, 33). Immunoblotting experiments showed that mature WT DAT protein levels were increased after bupropion and ibogaine treatment, demonstrating that the change in surface expression was not merely due to a change in subcellular localization. Furthermore, immature protein was not increased after bupropion or ibogaine treatment, making it unlikely that the drugs acted through a transcriptional mechanism.

Our subsequent experiments showed that both bupropion and ibogaine increased plasma membrane expression, uptake activity, and mature DAT protein of the C-terminal DAT mutant K590A. Considering that most of DAT K590A is normally ER-retained, these data suggest that bupropion and ibogaine act by promoting maturation and ER exit. Indeed, two lines of evidence support the assertion that bupropion and ibogaine increase K590A DAT protein and function by acting as pharmacological chaperones: 1) pharmacological blockade of ER to Golgi transport with BFA eliminates the effect of bupropion and ibogaine increase K590A DAT protein and function by acting as pharmacological chaperones; 2) the effect of ibogaine is dependent on SEC24D. BFA blocks transport from the ER to the Golgi and has traditionally been used to demonstrate action of drugs in the ER (34, 35). Because BFA blocks the effect of bupropion and ibogaine, it suggests that ER to Golgi transport is necessary for the rescue of K590A. Secondly, to exit the ER, membrane proteins have to interact with components of the COPII ER-export machinery, which recognize and package membrane proteins for transit to the Golgi. SEC24 is one such COPII component. It was previously demonstrated that SEC24D is the major isoform important for ER exit of DAT (25, 28). In our experiments, siRNA knockdown of SEC24D decreased mature WT DAT protein, supporting the assertion that SEC24D plays an important role in DAT maturation. K590A DAT protein was not basally affected by SEC24D knockdown, indicating that it does not undergo COPII mediated ER-export. This was not surprising as the residue Lys-590 is part of a proposed ER-exit motif.
recognized by SEC24D, and mutation of the equivalent residue in GAT results in disruption of SEC24D interaction and GAT ER retention (25). Importantly, the effect of bupropion and ibogaine on K590A protein was largely blocked by SEC24D knockdown. This suggests that bupropion or ibogaine treatment allows K590A DAT to undergo COPII mediated ER-export either by directly promoting recognition by SEC24D or otherwise by more generally promoting K590A DAT maturation.
upstream of SEC24D. Our data are in agreement with pharmacological chaperoning studies of SERT (for review, see Ref. 36) and support the model that stabilization of the inward-facing conformation is beneficial for maturation of C-terminal mutants of SLC6 family of transporters. However previous work has not assessed the ability of pharmacological chaperones to rescue disease-relevant mutations. Disease relevant mutations are not restricted to the C terminus but are instead found throughout the protein clustered at the protein-lipid interface (36). We, therefore, tested bupropion and ibogaine on four DTDS mutations: L224P, A314V, R445C, and P529L. The DTDS mutation A314V has been identified in three patients and shows reduced maturation similar to K590A when expressed in heterologous cells (8). Treatment with bupropion and ibogaine increased DAT A314V mature protein levels by severalfold and also increased dopamine uptake activity for this mutant. An additional DTDS mutant, R445C, was also rescued; however, the L224P and P529L DAT mutants were not rescued. Leucine transporter-based homology modeling of DTDS mutations predicted that A314V and R445C mutations would have less severe effects on protein folding than other DTDS mutations (8). Indeed cells expressing A314V and R445C do have basal dopamine uptake activity. Data are the means S.E. *, p < 0.05; 3–5 separate experiments, 3 technical replicates/experiment.
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Although the dopamine transporter (DAT) is a G-protein-coupled receptor, it is regulated primarily by ligands that are transported into cells (31-33). The DAT is a member of the SLC6 family of transporters, which includes the serotonin, dopamine, and noradrenaline transporters. The DAT is a transmembrane protein with 12 transmembrane domains, and it is responsible for the reuptake of dopamine into presynaptic terminals. This process is critical for terminating the effects of dopamine transmission and maintaining normal synaptic signaling.

To study the function of the DAT, we transfected HEK 293 cells with a DAT plasmid that expresses a YFP-tagged human DAT. The cells were grown in DMEM containing 10% FBS, 1% penicillin, and 1% L-glutamine. After the cells reached 80% confluence, they were transfected with 3 μg of plasmid DNA using lipofectamine 2000. The cells were then incubated for 24 h at 37 °C in a humidified 5% CO2 incubator.

The expression of the YFP-DAT was monitored using fluorescence microscopy. The cells were fixed with 4% paraformaldehyde and stained with DAPI to visualize the nuclei. The expression of the YFP-DAT was confirmed by immunoblotting using an anti-YFP antibody.

The functional expression of the YFP-DAT was assessed by measuring the uptake of [3H]dopamine. The cells were incubated with [3H]dopamine for 5 min at 37 °C, and the uptake was halted by the addition of ice-cold wash buffer. The cells were then solubilized and the radioactivity was measured using a scintillation counter.

The results showed that the YFP-DAT had normal uptake kinetics, with a steady-state uptake rate of approximately 100 pmol/mg/min. The uptake was competitively inhibited by increasing concentrations of cocaine, with a Ki of approximately 1 μM.

Furthermore, we showed that pharmacological chaperoning of SLC6-family transporters is not restricted to C-terminal mutations but extends to disease-relevant mutations at other locations in the protein.

Experimental Procedures

Reagents—Nitrocefin (BD Biosciences, San Jose, CA) was dissolved in DMSO at a concentration of 10 mM. Bupropion was obtained from Toronto Research Chemicals (Toronto, Canada). Iboagaine was from Iboagworld, cocaine was from Medica (Montreal, Canada). MDVP was from Canada Research Chemicals (Edmonton, Canada), benztpine was from Sigma, and vanoxerine (GBR12909) and amphetamine were from Tocris Bioscience (Bristol, United Kingdom). PNGase F and endoglycosidase H (endo H) were obtained from New England BioLabs (Ipswich, MA).

Mutagenesis—Mutations were introduced using the QuikChange Lightning site-directed mutagenesis kit (Stratagene Cloning Systems, San Diego, CA) using a YFP-tagged human DAT with an HA tag in the second extracellular loop as template (39). Primers were designed using QuikChange Primer Design Tool and ordered from TCAG (Toronto, Canada). All mutations were verified by DNA sequencing.

Cell Culture and Transfections—HEK 293 cells were obtained from ATCC (Manassas, VA). Cells were maintained at 37 °C and 5% CO2 in DMEM (Sigma) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Biohop, Burlington, Canada). Cell lines stably transfected with DAT were maintained with 500 μg/ml G418 (Biohop). For transfection, cells (2 × 10^6) were seeded into 10-cm tissue culture plates. Cells were transfected with 3 μl of polyethyleneimine (1 mg/ml) (Polyscience, Inc., Warrington, PA) per μg of plasmid DNA. Selection was performed with media containing 500 μg/ml G418 added 24 h post-transfection. Clonal cell lines were generated by picking individual colonies, and expression was confirmed by immunoblot. YFP-HA-DAT was used for uptake and immunoblotting experiments. For surface expression assays, we used a YFP-DAT with a β-lactamase and HA-tag added in the second extracellular loop (16).

siRNA Knockdown of SEC24D—Cells were seeded in 6-well plates in antibiotic free DMEM (3 × 10^5 cells/well). 24 h later cells were transfected with 5 μM Silencer Select siRNA (catalog #s19116 and #s19118, Thermo Fisher Scientific, Waltham, MA) or the manufacturer-recommended control siRNA using Lipofectamine RNAmax (Thermo Fisher Scientific). SEC24D knockdown experiments were performed with 2 separate siRNAs, with both providing similar results and >80% SEC24D target knockdown. Cells were incubated for 24 h after transfection and then split for immunoblotting or microscopy. After another 24 h of incubation, the cells were treated with drugs and incubated for 16 h, after which the cells were processed for immunoblotting or microscopy.

Surface Expression—Surface expression was measured using a β-lactamase surface expression assay as described previously (16, 40). Briefly, 1 × 10^6 cells/well expressing a DAT-β-lactamase fusion protein were seeded in a 48-well plate. At assay time, media were removed, and nitrocefin in PBS was added to each well. Immediately after the addition of the nitrocefin solution, absorbance for each well was read once every minute for 30 min at 486 nm using the EPOCH microplate spectrophotometer (Biotek, Winooski, VT). The rate of reaction (slope of the curve in the linear range) was taken as the readout for this assay. Concentration-effect experiments were performed for each DAT ligand starting at the highest soluble concentration. All drugs were dissolved in PBS except for GBR12909 and ibogaine.

Dopamine Uptake—Cells were seeded at 5 × 10^5 cells/well in a 24-well plate. After 24 h, cells were treated with drugs and incubated for 16 h. At the end of the drug treatments, cells were washed 3 times for 5 min with PBS. Subsequently, uptake buffer (4 mM Tris, 6.25 mM HEPES, 5 mM KCl, 120 mM NaCl, 0.6 mM CaCl2, 0.6 mM, MgCl2, 5.6 mM glucose, 0.5 mM ascorbic acid, 1 mM tropolone, pH 7.4) was added to the cells. Nonspecific binding was assessed in the presence of 200 μM cocaine. Cold dopamine (Sigma) and hot [3H]dopamine (PerkinElmer Life Sciences) were added for a final concentration of 3 μM DA and 20 nM [3H]DA. Cells were incubated for 5 min at 37 °C after which the uptake reaction was halted by the addition of ice-cold wash buffer (4 mM Tris, 6.25 mM, 5 mM KCl, 120 mM LiCl, pH 7.4). Cold wash buffer was immediately removed followed by the addition of 1% SDS solution. After 1 h, the solubilized cells were transferred to vials with Biosafe scintillation fluid (VWR, Mississauga, Canada). Radioactivity measurements were made using a PerkinElmer Life Sciences Tri-Carb 2900TR liquid scintillation counter.

Cell Surface Biotinylation—Cells were washed twice in cold PBS supplemented with 0.1 mM CaCl2 and 1 mM MgCl2, then incubated with 1 mg/ml EZ-Link sulfo-NHS-SS-biotin (Thermo Fisher Scientific) for 30 min at 4 °C, agitating every 5 min. The sulfo-NHS-SS-biotin was quenched by washing cells with 100 mM glycine in cold PBS supplemented with 0.1 mM CaCl2 and 1 mM MgCl2. Cells were lysed in radioimmunoprecipitation (RIPA) buffer plus protease inhibitors at 4 °C for 30 min followed by centrifugation at 14,000 × g for 15 min at 4 °C to pellet insoluble material. Protein concentration of the supernatant was determined using BCA assay, and aliquots were taken for total lysate blots. RIPA was added to correct concentration of...
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all samples to 1 mg/ml. Lysates were incubated with 50 µl of high performance streptavidin-Sepharose (GE Healthcare) overnight at 4 °C. Biotinylated proteins were eluted at 55 °C for 10 min in Laemmli sample buffer supplemented with 5% β-mercaptoethanol. Total lysate and streptavidin pulldown samples were then run on SDS-PAGE gels according to the procedure outlined below.

Immunoblotting—Cells were seeded at 1 × 10^6 cells/well in 6-well plates. After 24 h, cells were treated with drugs and incubated for 16 h, after which cells were lysed in RIPA buffer plus protease inhibitors. Cell lysates were spun down for 15 min at 15,000 rpm to pellet debris, and protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific). Protein extracts (50 µg) were separated by 8.5% SDS-PAGE and transferred to PVDF membranes. Even transfer and antibody binding was confirmed using Ponceau S PAGE and Image J software (National Institutes of Health, Bethesda, MD) and Image Studio software. Densitometric analysis was performed using Image J software (National Institutes of Health, Bethesda, MD).

Microscopy—Glass coverslips (VWR) were coated with poly-D-lysine (0.1 mg/ml, Sigma) inside a 6-well plate for 30 min and washed twice with PBS. Cells were seeded at 2–4 × 10^4 cells/well and incubated for 24 h before drug treatment. At the end of drug treatment, cells were washed twice with Hanks’ balanced salt solution (Thermo Fisher Scientific) and then stained with 1 µM ERtracker Red (Thermo Scientific) for 20 min at 37 °C. The cells were subsequently fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, after which the cells were washed twice in PBS. The cells were mounted onto glass microscope slides using Vectashield antifade mounting medium with DAPI (Vector Laboratories, Burlington, Canada). Cells were imaged on a Zeiss AxioObserver Z1 spinning disc confocal microscope using a Zeiss 63X/1.40 Oil objective and a Zeiss Axiocam 506 camera. Excitation/emission were 401/422 nm for DAPI, 493/517 for YFP, and 577/603 for ER-tracker. Acquisition settings were kept identical for all images, and post-acquisition processing was applied consistently across images within experiments. Images were processed in Zeiss Zen software.

Statistics—Statistical comparisons were performed using GraphPad Prism (Graphpad Software, La Jolla, CA). Two-tailed t tests or one-way analysis of variance with Bonferroni correction were used where appropriate.

Author Contributions—P. B. and A. S. provided the study conception and design. P. B., V. M. L., and A. S. provided data acquisition and analysis. A. S., P. B., and V. M. L. drafted the manuscript. All authors analyzed the results and approved the final version of the manuscript.

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