Transient Compound Treatment Induces a Multigenerational Reduction of Oxysterol-Binding Protein (OSBP) Levels and Prophylactic Antiviral Activity

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Supporting Information

ABSTRACT: Oxysterol-binding protein (OSBP) is a lipid transport and regulatory protein required for the replication of Enterovirus genus viruses, which includes many significant human pathogens. Short-term exposure (i.e., 1−6 h) to a low dose (i.e., 1 nM) of the natural product compound OSW-1 induces a reduction of cellular OSBP levels by ∼90% in multiple different cell lines with no measurable cytotoxicity, defect in cellular proliferation, or global proteome reduction. Interestingly, the reduction of OSBP levels persists multiple days after the low-dose, transient OSW-1 compound treatment is ended and the intracellular OSW-1 compound levels drop to undetectable levels. The reduction in OSBP levels is inherited in multiple generations of cells that are propagated after the OSW-1 compound treatment is stopped. The enduring multiday, multigenerational reduction of OSBP levels triggered by the OSW-1 compound is not due to proteasome degradation of OSBP or due to a reduction in OSBP mRNA levels. OSW-1 compound treatment induces transient autophagy in cells, but blocking autophagy does not rescue OSBP levels. Although the specific cellular mechanism of long-term OSBP repression is not yet identified, these results clearly show the existence of an OSBP specific cellular regulation process that is triggered upon treatment with an OSBP-binding compound. The stable reduction of OSBP levels upon short-term, transient OSW-1 compound treatment will be a powerful tool to understand OSBP regulation and cellular function. Additionally, the persistent reduction in OSBP levels triggered by the transient OSW-1 compound treatment substantially reduces viral replication in treated cells. Therefore, the long-term, compound-induced reduction of OSBP in cells presents a new route to broad spectrum anti-Enterovirus activity, including as a novel route to antiviral prophylactic treatment through small molecule targeting a human host protein.

Oxysterol-binding protein (OSBP) and the OSBP-related proteins (ORPs) are a family of lipid and sterol binding proteins conserved in all eukaryotes.1,2 The 12 OSBP/ORP human proteins share a conserved ~50 kDa, C-terminal ligand binding domain.1,2 OSBP and ORP4, the member most closely related to OSBP, share substantial sequence similarity, and both contain N-terminal pleckstrin homology (PH) and FFAT domains.1,2 Individual OSBP/ORP family members are reported to have many different cellular functions,1,2 including serving as cellular sensors for lipid membrane composition.3−6 OSBP is reported to be localized at the membrane contact site between the ER and Golgi, and from this location, OSBP is

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reported to coordinate the transfer of phosphoinositide-4-phosphate (PI(4)P) and cholesterol between the ER and Golgi.3,7−9 OSBP also indirectly regulates the synthesis of some lipids and regulates membrane lipid composition.3−5 The PH and FFAT domains alter OSBP cellular localization upon binding of ligands in the C-terminal ligand binding domain.1,2 OSBP binding partners, including several regulatory proteins, have been reported.1,2 OSBP gene expression and protein regulation are not well-defined.

ORP4 shares significant sequence and domain similarity to OSBP.2,10 OSBP is expressed in all tissues, but ORP4 is expressed in only a few select human tissues.1,2 ORP4 is highly expressed in some cancer cells and shown to be required for cancer cell line proliferation.2,10 ORP4 is selectively overex-

Figure 1. Brief exposure to nontoxic, low-nanomolar OSW-1 compound concentrations causes long-term OSBP reduction in multiple cell lines. (A) Compound structure of OSW-1 (1) and deuterated OSW-1 (d-OSW-1) (2) with in vitro inhibition binding constants (i.e., \( K_i \) values) for OSW-1 against human OSBP and ORP4. (B) OSBP and ORP4 levels in HEK-293, HCT116, and K562 cell lines treated for indicated times with 1 nM OSW-1 compound. Indicated times are after the compound has been removed. Included controls are the DMSO vehicle control and 10 nM taxol. (D) OSW-1 compound washout experiment with 6 h, 1 nM OSW-1 treatment followed by 24 h recovery in multiple cell lines, including cancerous and nontumorigenic cell lines. (E) OSBP levels in cells treated with 1 nM OSW-1 compound for the indicated times (i.e., 15 min to 6 h) followed by compound washout and a 24 h recovery. (F) Cell viability after OSW-1 compound washout experiment with 1 nM treatment for 6 h, followed by indicated recovery times.
pressed and serves as a critical driver for proliferation in T-cell acute lymphoblastic leukemia (T-ALL) cells isolated from patients, with a cellular function linked to metabolism control at the mitochondria.\(^\text{11}\) In contrast, knockdown of OSBP in cancer cells is not cytotoxic or antiproliferative.\(^\text{12}\)

In 2011, OSBP and ORP4 were revealed to be the cellular target of the antiproliferative natural product compounds OSW-1 and cephalostatin 1.\(^\text{12}\) Also, the natural product, schweinfurthin A, preferentially targets OSBP but not ORP4; schweinfurthin A is 40-fold more selective in binding OSBP over the closely related ORP4 protein.\(^\text{12}\) The identification of OSBP and ORP4 as the targets of biological relevance for these natural product compounds have been verified independently through multiple lines of research.\(^\text{3,13,14}\) This discovery identified OSBP, ORP4, or both proteins as executing important cellular functions capable of being altered through small molecule compound interactions.\(^\text{8,15-17}\) The OSW-1 compound is reported to induce apoptosis,\(^\text{16,17}\) mitochondrial dysfunction,\(^\text{16}\) and intracellular calcium release,\(^\text{18}\) which are all consistent with the OSW-1 compound altering the reported ORP4 function in cells.\(^\text{10,11}\) Based on the role of ORP4 in cell proliferation and viability,\(^\text{10,11}\) the OSW-1 compound cytotoxicity is likely due to its interaction with ORP4 rather than OSBP.

OSBP is a critical mediator in the replication of a broad spectrum of clinically important human pathogens belonging to the \textit{Enterovirus} genus.\(^\text{14,19,20}\) Multiple independent lines of research evidence have established the antiviral activity of OSBP-targeting small molecules, including the OSW-1 compound.\(^\text{14,19,21-23}\) Human viral pathogens belonging to the genus \textit{Enterovirus} are ubiquitous and established public health menaces, causing significant societal morbidity and mortality.\(^\text{24-26}\) \textit{Enterovirus} genus pathogens cause a spectrum of diseases including the common cold, acute respiratory infections and pneumonias, myocardial infections, hand, foot and mouth disease (HFMD), acute hemorrhagic conjunctivitis, and the paralytic condition acute flaccid myelitis.\(^\text{24-27}\) The high mutability and large spectrum of serotypes limit vaccine and antiviral therapeutic development for these viral pathogens.\(^\text{20,24-26,28}\) OSBP is also reported to be a critical mediator of hepatitis C virus (HCV),\(^\text{29,30}\) encephalomyocarditis (EMC) virus,\(^\text{31}\) dengue virus,\(^\text{32}\) and Zika virus.\(^\text{12}\) OSBP function is implicated as having a critical role in the formation of the viral replication organelles (ROs), which form at the ER–Golgi interface.\(^\text{15,33,34}\) ROs are critical structures for the reproduction of \textit{Enterovirus} genera viruses as well as other classes of viruses.\(^\text{20,33}\)

Herein we describe the discovery that the treatment of mammalian cells with a single, nontoxic dose of the OSW-1 compound induces a significant reduction (i.e., \textasciitexttt{~}90\% reduction) of OSBP protein levels in cells that lasts for several days after the brief exposure to the compound. The reduction of OSBP levels remains even after the intracellular OSW-1 compound concentrations have dropped to undetectable levels. Our results suggest that an unidentified system of OSBP repression in cells can be triggered by small molecule binding to OSBP, and this OSBP repression is inherited through multiple rounds of cell division before abating. Triggering the persistent reduction of OSBP levels with the OSW-1 compound reduces the replication of two \textit{Enterovirus} pathogens 24 h after the compound had been removed from the cells. The inhibition of viral replication by triggering the reduction of a required host protein through small molecule treatment could be a new modality of antiviral prophylaxis and potential therapeutic development.

\section*{RESULTS AND DISCUSSION}

\textbf{OSW-1 Compound Treatment Induces a Pronounced Reduction of OSBP Proteins Level Up to 72 h after the Compound Treatment Has Stopped.} OSW-1 compound (Figure 1A, 1) treatment of cells is reported to reduce OSBP protein levels.\(^\text{12}\) OSBP levels are reduced by \textasciitexttt{~}90\% after 12 h continual treatment of 1 nM of the OSW-1 compound in multiple cell lines (Figure 1B, Supp. Figure 1A). OSW-1 compound treatment for 24 h continual treatment also partially reduces the levels of ORP4 protein, the closest paralog of OSBP, but the ORP4 reduction is far less pronounced and not as rapid as the OSBP reduction (Figure 1B, Supp. Figure 1A). Experiments designed to determine the minimal concentration and time required for the OSW-1 compound to reduce OSBP levels in cells produced an unexpected result. A brief exposure of cells to a nontoxic concentration of the OSW-1 compound, followed by the removal of the OSW-1 compound from the cellular media, is sufficient to reduce OSBP protein levels for extended periods time (i.e., up to 72 h) after the OSW-1 compound treatment had stopped. (Figure 1C,D,E). These experiments are termed OSW-1 compound washout experiments (abbreviated as WO in Figure 1). In the OSW-1 compound washout experiments, (1) media containing OSW-1 compound is added to cells; (2) at the specified time (i.e., 0–6 h), the compound-containing media is removed; (3) the cells are gently washed three times in complete media to remove any residual OSW-1 compound; (4) the cells are re-incubated in compound-free media for the indicated recovery period times postwashout (pWO) (i.e., 0–72 h). The washout experiment probes the cellular responses triggered by, but not continually caused by, the presence of the OSW-1 compound in cells.

The OSW-1 compound washout experiments in HEK-293, HCT116, and K562 cells demonstrate that a brief 6 h exposure to 1 nM OSW-1 compound reduces the OSBP protein levels up to 90\% for multiple days (i.e., 48–72 h) after the OSW-1 compound is removed from the culture media (Figure 1C). Cell lines tested under these OSW-1 compound washout conditions include several different cancer cells (i.e., HCT116, HeLa, K562, and MCF-7; Figure 1C,D), the nonmutogenic HEK-293 cells (Figure 1C), and the human lung fibroblast MRC-5 cell lines (Figure 1D). The \textasciitexttt{~}90\% reduction in OSBP levels in the washout experiment persists for either 48 h (HEK-293 and HeLa) or 72 h (HCT116 and K562) after the compound exposure ceased (Figure 1C; HeLa result in Supp. Figure 7). K562, a suspension leukemia cell line, is pelleted and transferred to a new culture condition (Figure 7). K562, a suspension leukemia cell line, is pelleted and transferred to a new culture condition. The OSW-1 compound treatments as minimal as 1 nM for 6 h followed by a 24 h washout, caused a substantial reduction of OSBP levels in all cell lines indicated (Figure 1D). OSW-1 compound treatments as minimal as 1 nM for 1 h followed by a 24 h recovery after washout of the OSW-1 compound reduced OSBP levels \textasciitexttt{~}75\% in HEK-293 and K562 cells (Figure 1E, Supp. Figure 1B). Bottom-up proteomic mass spectrometry (MS) also shows a significant reduction in the detected OSBP peptides in the OSW-1 washout cells (Supp. Figure 1C), which confirms the reduction of OSBP levels detected by Western blot (Figure 1B,C).
A two-dimensional low/high pH HPLC separation of the trypsin-digested lysates allows for the MS detection of the relatively low abundance OSBP peptides without enrichment or purification. Unlike in the continual treatment with the OSW-1 compound (Figure 1B), ORP4 levels are not substantially reduced by the OSW-1 compound washout (Supp. Figure 1D). Importantly, the 6 h, 1 nM washout treatment of cells with the OSW-1 compound does not affect cell proliferation rates, cell viability, or overall cellular morphology at any time measured (Figure 1F, Supp. Figure 1E). In contrast, the continual treatment of cells with 1 nM OSW-1 compound causes significant evident cytotoxicity and effects on cellular morphology following 24 h of continual treatment (Supp. Figure 1E). Western blot analysis of the cell lysis pellets shows no detectable OSBP, verifying that the protein is not trapped in the pellet after cell lysis (Supp. Figure 2B). Additionally, OSBP is not excreted from cells during the washout experiment, as determined through Western blot analysis of the culture media (Supp. Figure 2C). The OSW-1-washout induced reduction of OSBP levels occurs in both high and low confluent cell culture populations, and the OSBP reduction is not significantly affected by splitting the cell culture immediately after OSW-1 compound treatment (Supp. Figure 2D). In the process of splitting the cell cultures, the washout cells are transferred to new plasticware completely free of any OSW-1 compound. (Supp. Figure 2D). These results support the existence of an active cellular process that persistently reduces the OSBP levels after the OSW-1 compound washout experiment.

Mass Spectrometry Measurements Show Intracellular OSW-1 Compound Levels Reduced below Detectable Limit 24 h after Compound Washout. Two complementary mass spectrometry analytical methods show that the intracellular concentration of OSW-1 is reduced to undetectable levels 24 h after compound washout (Figure 2, Supp. Figures 3 and 4). Quantitative LCMS measurements using a deuterated OSW-1 analog (i.e., d-OSW-1, Figure 1A, 2) as an internal standard measures OSW-1 in cell lysate from treated HCT116 cells (Figure 2A). The intracellular OSW-1 concentration of HCT116 cells treated for 1 h with 100 nM compound is $1.74 \pm 0.74 \mu M$ (Figure 2C). Postwashout, followed by a 24 h recovery, the intracellular OSW-1 concentration is below the measurable threshold of $\sim 100$ pM (Figure 2A, Supp. Figure 3). Single cell mass spectrometry (SCMS) is a complementary method to LCMS for determining intracellular compound concentration. Single cell mass spectrometry does not require any sample preparation, and therefore there is no possible loss of analyte during lysate preparation from treated cells.\textsuperscript{36,37} Semiquantitative SCMS of OSW-1 compound treated HCT116 using the single-probe sampling technology\textsuperscript{36,37} confirms the loss of detectable intracellular OSW-1 compound after the washout experimental conditions (Figure 2B, Supp. Figures 3 and 4). No potential OSW-1 compound metabolites are evident in either the LCMS
or SCMS analyses. These MS analyses show that the cellular changes observed during the OSW-1 compound washout experiments (Figures 1 and 3−6) are not likely due to the continued presence of the compound, but instead are the result of the brief exposure to the OSW-1 compound triggering a persistent cellular response.

Cell Imaging of OSW-1 Compound Washout Cells Shows the Remaining OSBP Returning to a Normal Cellular Localization. OSBP is predominately located at ER/Golgi membrane contact sites.3,7−9 Ligand binding, including to the OSW-1 compound, alters OSBP cellular localization to a condensed area colocated with the Golgi.8,12,21 Immunofluorescent experiments in OSW-1 compound treated HCT116 cells, under both continual OSW-1 compound treatment (Figure 3A) and OSW-1 washout treatment (Figure 3B), show a clear reduction of OSBP levels and change in OSBP localization. Continual treatment of OSW-1 compound induces a rapid change (<2 h) in OSBP localization to a complete punctate colocalization with the Golgi marker TGN46. After altering OSBP localization patterns, the continual treatment of OSW-1 compound induces a time-dependent decrease in OSBP fluorescence in cells, which

Figure 3. OSW-1 compound continual treatment versus washout condition treatment show differential staining patterns. (A) Continuous 1 nM OSW-1 compound treatment in HCT116 cells shows localization of OSBP to the Golgi with eventual loss of both OSBP signal and overall Golgi structure. (B) HCT116 washout conditions (1 nM OSW-1 for 6 h followed by compound removal shows a reduced OSBP and Golgi signal, but OSBP and Golgi localization patterns are restored to similar to DMSO by 24 h postwashout. All indicated times are after removal of the compound (i.e., postwashout).
reaches consistent low levels after 12 h (Figure 3A). This reduction of OSBP upon OSW-1 compound continual treatment is consistent with the Western blot OSBP reduction (Figure 1B, Supp. Figure 1A). The OSBP signal decreases with time and becomes increasingly punctate during the continual OSW-1 compound treatment. OSW-1 compound continual treatment alters the Golgi marker TGN46 from an evident perinuclear concentration to a dispersed punctate signal. This indicates that the OSW-1 compound continual treatment induces a pronounced dissolution of the Golgi consistent with previous reports (Figure 3A).12

Immunofluorescent microscopy of HCT116 cells during the recovery phase of the OSW-1 compound washout experiment (Figure 3B) shows an increasingly weak and diffuse OSBP localization pattern over time consistent with the OSBP reduction observed via Western blot (Figure 1C). During the initial phase of recovery after washout (i.e., 0−4 h), the OSBP signal is heavily colocalized with the Golgi maker in a nonperinuclear punctate pattern (Figure 3B), consistent with the continual OSW-1 compound treatment (Figure 3A). The TGN46 Golgi marker returns to a more normal perinuclear localization by 12 and 24 h in the washout cells (Figure 3B). Cells subjected to continual treatment of OSBP do not show an apparent reformation of the Golgi body (Figure 3A). The apparent reformation of the Golgi at the perinuclear localization indicates the washout cells returning to a more unaffected state despite the reduced OSBP levels (Figure 3B, 12 and 24 h). The low levels of OSBP signal in the 12 and 24 h washout cells have returned to a perinuclear colocalization pattern with the TGN46 Golgi marker similar to the DMSO vehicle-treated cells.

**Long-Term Reduction of OSBP Levels in the OSW-1 Compound Washout Cells Is Not Due to Proteasomal or Calpain OSBP Degradation.** Proteasome inhibitors are reported to block the OSW-1 compound induced reduction of OSBP levels in cells.12 Additionally, the cellular protease calpain is implicated in OSW-1 compound cellular activity in HeLa cells.18 Co-incubation of the OSW-1 compound with any of the three structurally diverse proteasome inhibitors—MG-132, bortezomib, or carfilzomib—blocks the initial reduction of OSBP (Figure 3A). Inhibition of translation with 177 nM of cycloheximide shows that OSBP half-life in HCT116 cells is >24 h. p21, a protein with a known half-life of a few hours, is used as a control to verify cycloheximide inhibition of translation. (D) RT-PCR quantification of OSBP mRNA levels shows no reduction in transcript up to 72 h pWO during the OSW-1 washout experiment (6 h, 1 nM treatment) in HCT116 cells. Example Western blots for panels A−C are shown in Supp. Figure 6.

Figure 4. Long-term OSBP repression in washout cells is not due to proteolysis, transcriptional repression, or rapid turnover of the protein. (A) Co-treatment in HCT116 and HeLa cell lines with either proteasome inhibitors (25 nM bortezomib (BTZ), 25 nM carfilzomib (CFZ), and 170 nM MG132) or the calpain inhibitor ALLN (10 μM) with 1 nM OSW-1 results in significant protection of OSBP levels after 24 h continuous treatment. (B) Co-treatment with proteasome or calpain inhibitors do not reverse the reduction of OSBP levels in the OSW-1 compound washout experiment. Cells were treated with 1 nM OSW-1 for 6 h, compound was washed out, and then cells were allowed to recover for 24 h. After 24 h recovery, washout cells were treated with BTZ (25 nM), CFZ (25 nM), MG132 (170 nM), or ALLN (10 μM) for 24 h additional hours (48 h total recovery postwashout). BTZ treatment resulted in a partial recovery (~35%) of OSBP levels (C) Inhibition of translation with 177 nM of cycloheximide shows that OSBP half-life in HCT116 cells is >24 h. p21, a protein with a known half-life of a few hours, is used as a control to verify cycloheximide inhibition of translation. (D) RT-PCR quantification of OSBP mRNA levels shows no reduction in transcript up to 72 h pWO during the OSW-1 washout experiment (6 h, 1 nM treatment) in HCT116 cells. Example Western blots for panels A−C are shown in Supp. Figure 6.
rescue of OSBP levels (∼25%) in HeLa cells (Figure 4B). These results show that the initial reduction of OSBP levels in OSW-1 compound treated cells is caused by proteolysis, probably by the proteasome, but the long-term repression of OSBP levels in the washout cells is not likely the result of degradative proteolysis of OSBP.

**Figure 5.** OSW-1 compound treatment induces transient autophagy and minimal changes to the proteome in HEK-293 cells. (A) Inhibition of autophagy-induced proteolysis using 25 μM chloroquine (CQ) in HEK-293 cells does not rescue OSBP levels in OSW-1 washout experiment (i.e., 6 h, 1 nM OSW-1 treatment, followed by compound washout and 24 h recovery). CQ treatment increases OSBP levels compared to DMSO vehicle control in cells. (B) Treatment of HEK-293 cells for 6 h with 1 nM OSW-1 decreases p62 and increases LC3B (lower band on Western blot), which are markers of cellular autophagy. Rap = Rapamycin, a known autophagy inducing compound. (C) iTRAQ LC/MS/MS analysis (n = 3) of OSW-1 compound treated lysates relative to vehicle control. All significantly changed proteins were decreased (24 h pWO), with the exception of SSBP1 (single stranded binding protein 1), which showed a slight increase in protein levels 72 h pWO. (D) Table of proteins with significant changes (<0.05). See Supp. Figure 9 and 10 for OSBP Western blots for lysates used for iTRAQ.

**OSBP Is a Long-Lived Cellular Protein Not Rapidly Turned over in Cells.** The lifetime and rate of turnover of OSBP in cells have not been reported. The half-life of OSBP in HCT116 cells is over 24 h, as determined using cycloheximide to block protein translation (Figure 4C). p21, with a reported half-life of approximately 2 h, is used as a control to verify cycloheximide inhibition of translation (Figure 4C).42 The
extended half-life of OSBP indicates that the protein is not normally rapidly turned over in cells and that the rapid reduction of OSBP levels upon OSW-1 treatment (Figure 1B, Figure 4A) is a nonstandard cellular response. Additionally, the low turnover of OSBP under normal conditions (Figure 4C), in combination with the failure of the proteasome inhibitors to restore OSBP during washout condition (Figure 4B), suggests that reduction of OSBP under washout conditions is likely not caused by regulation of OSBP at the protein level.

**Long-Term Reduction of OSBP Levels in the OSW-1 Compound Washout Cells Is Not Due to Reduction of OSBP mRNA Levels.** RT-PCR analysis of OSBP mRNA transcript levels in HCT116 cells showed no change during the 72 h washout experiment (Figure 4D). This result indicates that the reduction of OSBP in the washout experiment is not due to repression of OSBP mRNA transcription.

**OSW-1 Compound Treatment Induces Autophagy in Cells, But Autophagy Does Not Cause the Long-Term Reduction of OSBP Levels.** In addition to the proteasome, autophagy is a major pathway for cellular protein degradation.33 OSW-1 compound treatment of HEK-293 cells induces autophagy under the washout experimental conditions, as determined through monitoring the cellular markers of autophagy, p62 and LC3A/B (Figure 5B). Autophagy results in an increase in LC3B and a decrease in p62 protein levels. Chloroquine (CQ) is a chemical inhibitor of the proteolysis of autophagic substrates, and therefore coadministration of CQ is required to measure LC3B levels during autophagy. Rapamycin (Rap) is a mTORC1 inhibitor that induces autophagy in cells. Similar to Rap/CQ treatment, OSW-1 compound/CQ treatment causes a large increase in LC3B levels relative to CQ treatment alone in HEK-293 cells over the time course observed (Figure 5B, lower band), indicating the induction of autophagy (Figure 5B). Additionally, 1 nM OSW-1 compound treatment for 6 h resulted in ~60% reduction in p62 levels (Figure 5B). The p62 levels returned to normal 24 h pWO, indicating that the cessation of the transient autophagy triggered during the OSW-1 compound washout experiment (Figure 5B). Importantly, cotreatment of HEK-293 cells with 25 μM CQ and 1 nM OSW-1 compound for 6 h, followed by compound washout and retreatment with CQ alone (i.e., without OSW-1 compound) for 24 h does not rescue OSBP protein levels (Figure 5A). This result shows the loss of OSBP is likely not due to autophagy-induced proteolysis. Co-incubation of the OSW-1 compound and CQ is cytotoxic at time points longer than 24 h.

**iTRAQ Proteomic Analysis Shows the OSW-1 Compound Washout Does Not Cause Reduction of Global Protein Levels.** Cellular iTRAQ proteomic analysis demonstrates that the OSW-1 compound washout treatment in HEK-293 cells does not induce broad degradation of cellular proteins (Figure 5C). Only a few of the (469) proteins confidently identified and quantified in the iTRAQ experiment showed significant changes in measured level (p < 0.05) at 24 h pWO, and the expression levels for these proteins return to levels similar to the vehicle control 72 h pWO. At the 24 h recovery time point pWO, many of the quantified proteins exhibit a small nonsignificant (p > 0.05) reduction in expression levels (Figure 5C). The iTRAQ results, in combination with the lack of cytotoxicity and growth arrest in the OSW-1 compound washout experiment (Figure 1F, Supp. Figure 1F), indicate that the reduction of OSBP levels is targeted for that specific protein and not the result of a widespread reduction of cellular proteins.

**Long-Term Reduction of OSBP Levels in the OSW-1 Compound Washout Cells Provides Prophylactic Antiviral Activity in Cells.** Consistent with the previously reported anti-Enterovirus activity of the compound,14 OSW-1 treatment inhibits the viral replication of two clinically isolated pathogenic Enterovirus viruses, Coxsackievirus A9 and Echo2, in HeLa cells in a concentration-dependent manner (Figure 6A). Continual OSW-1 compound treatment reduced the viral titer of 10-h viral infection in HeLa cells approximately ~1000-fold (Figure 6A). Importantly, HeLa cells subjected to a 6 h OSW-1 compound treatment followed by compound washout and a 24 h recovery period prior to viral infection show a reduction of viral titers of approximately 100-fold for the Echo2 virus and ~1000-fold for the Coxsackievirus 9A virus (Figure 6B). In HeLa cells, the 1 nM, 6 h OSW-1 compound washout experiment reduces OSBP levels by 24 h pWO ~90% (Figure 1D), and the protein levels are still somewhat reduced 72 h pWO (Supp. Figure 7). Based on the quantitative mass spectrometry of intracellular OSW-1 compound levels 24 h pWO (Figure 2), this observed

![Figure 6](image-url)

**Figure 6.** OSW-1 compound treatment provides prophylactic activity against clinically isolated enterovirus pathogens 24 h after exposure to the compound. (A) Viral titers of infected HeLa cells incubated in the presence of the OSW-1 compound. Cells were treated with the indicated concentrations of OSW-1 compound for 6 h, followed by viral infection for 30 min (multiplicity of infection (MOI) of 1.0), followed by re-incubation for 10 h with OSW-1 compound. (B) Viral titers in OSW-1 compound washout HeLa cells. Cell were treated with the indicated concentrations of OSW-1 compound for 6 h, compound was removed, cells were allowed to recovery for 24 h in compound-free media. Then, cells were infected for 30 min with virus (MOI = 1.0) followed by 10 h incubation in compound-free media.
prophylactic antiviral activity in the OSW-1 compound washout experiment is not likely due to residual OSW-1 compound, but instead the antiviral activity is likely due to a sustained reduction of OSBP levels postwashout. The increased antiviral activity of the OSW-1 continual treatment (Figure 6A) compared to the washout cells could be due to the complete compound-induced inhibition of the OSBP protein, as opposed to the ~90% reduction of OSBP levels in the washout cells.

**CONCLUSIONS**

The results show that a nontoxic, short-duration treatment in several different cell lines with the OSW-1 compound triggers an unidentified regulatory mechanism that specifically represses OSBP levels for several days after the compound exposure is stopped. The persistence of the OSBP reduction for 48–72 h after the compound is washed out from cells indicates a stable effect passed on to multiple generations of cells. The OSW-1 compound washout cells do not exhibit growth arrest, cytotoxicity, or changes to cellular morphology (Figure 1, Supp. Figure 2, Figure 3B), which might be expected if global protein degradation or nonspecific protease activity is triggered. The initial degradation of OSBP triggered by OSW-1 compound treatment is mediated by the proteasome (Figure 4A), but the long-term repression of OSBP level after the OSW-1 compound is removed from cells is not due to proteasome degradation (Figure 4B). The reduction of OSBP levels is not a transcriptional response since OSBP mRNA levels are not changed due to OSW-1 compound treatment (Figure 4D). Global iTRAQ analysis also does not show a significant systematic reduction of protein levels or the reduction of any other protein to the magnitude of the OSBP reduction. Further, ORP4, the closest paralog to OSBP, is not reduced during the OSW-1 compound washout experiment (Supp. Figure 1D). The OSW-1 compound interacts with human OSBP and ORP4 with comparable binding OSBP and not ORP4 would be critical chemical probes in defining the regulation and antiviral therapeutic potential of compound-induced OSBP repression. Such OSBP-specific, noncytotoxic, small molecule effectors would be powerful chemical probes to study OSBP function, and these compounds could be potentially developed to inhibit a broad spectrum of severe human pathogenic viruses that currently cannot be prevented or directly treated.

**METHODS**

**Cell Lines/Viruses.** HCT116, HEK-293, HeLa, K-562, and RD (rhabdomyosarcoma, ATCC-CCL-136) cell lines were purchased from ATCC. MCF-7 cells were a gift from R. Cichewicz (University of Oklahoma, Norman). MRC-5 cells were a gift from E. Blewett (Oklahoma State University, Center for Health Sciences, Tulsa). Coxackievirus A9 (strain CoxA9-01) and echovirus 2 (strain Echo2-01) were obtained from the Oklahoma State Department of Health Laboratory.

**Antibodies.** Western Blotting Antibodies. Primary antibodies used for Western blot were OSBP A-5 (Santa Cruz sc-3657721), p21 C-19 (Santa Cruz sc-397), OSBP2 B-1 (ORP4; Santa Cruz sc-369522), SQSTM1 D-3 (P62; Santa Cruz sc-28359), and LC3A/B D3U4C XP (Cell Signaling 12741). Secondary antibodies used were goat anti-mouse IgG-HRP (Santa Cruz sc-2004), goat anti-rabbit IgG-HRP (Santa Cruz sc-2060), and goat anti-rabbit IgG-HRP (Cell Signaling 7074S).

**Immunofluorescent Imaging Antibodies.** Primary antibodies used for immunofluorescent (IF) imaging used were OSBP1 IF2 (Novus NBP2-00993) and TGN46 (Novus NBP1-49643). Secondary IF antibodies used were goat anti-mouse IgG H&L; Alexa Fluor 488 (Abcam ab150076) and donkey anti-rabbit IgG H&L; Alexa Fluor 594 (Abcam ab150076).

**Cell Viability Assays.** Trypan Blue viability was performed utilizing a TC20 Automated Cell Counter using 10 μL of cell solution mixed thoroughly with 10 μL of Trypan Blue stain (Thermo 15250061). Alternatively, calcein AM and Hoechst stain were also used to determine cell viability. Five micromolar calcein AM (Thermo C1430) and 5.5 mg/mL Hoechst 33342 (Thermo H1399) were added to the cells and incubated at 37 °C for 1 h. Plates were imaged using an Operetta High-Content Imaging System (PerkinElmer) using brightfield, 488 nm, and Hoechst settings.

**Washout Experiments.** Cells were treated with complete media containing either DMSO (vehicle), 1 nM OSW-1 compound, or 1 nM Taxol for the indicated times (i.e., 0–6 h). Compound was removed, and cells were gently washed thoroughly with compound-free complete culture media three times, followed by re-incubation of cells with complete compound-free culture media. The cells are then allowed to recover in the compound-free media for the indicated times (0–72 h) followed by cell lysis and Western blot or proteomic mass spectrometry analysis.

**Intracellular OSW-1 Quantification.** HCT116 cells were treated with 100 nM OSW-1 compound for 1 h, with or without pWO recovery time. The cells were trypsinized and lysed using 1 mL of 50 nM d-OSW-1 dissolved in cold acetonitrile and methanol (1:1) with brief vortexing on ice for 10 min. The cells were then pelleted, and the supernatant was transferred to a new tube and dried using a speed vacuum (Savant SPD11 V, Thermo Scientific). Following treatment, the microchip was washed without pWO recovery time. The cells were trypsinized and lysed using 1 mL of 50 nM d-OSW-1 was added into the solvent.

**Single Cell Mass Spectrometry.** HCT116 cells were seeded out on a glass microchip (18 mm diameter) with chemically etched microwells (55 μm diameter; 25 μm deep) placed into each well of a 6-well plate. Cells were incubated in the same manner as described for nano-UPLC/MS. Following treatment, the microchip was washed using 1 mL of 50 nM d-OSW-1 was added into the solvent.

**Immunofluorescence.** HCT116 cells were seeded onto sterile 18 mm coverslips in 12 well plates and incubated for 24 h. Cells were
then treated with DMSO or 1 nM OSW-1 compound for the indicated times. Cells were fixed using 4% paraformaldehyde and permeabilized using 0.5% Triton X-100. Image-IT FX signal enhancer (Thermo 1369331) was added to the coverslips, followed by incubation with 1% BSA for blocking. Primary antibody was then added, and the coverslips were incubated overnight at 4 °C. Secondary antibody was incubated in darkness at RT for 1 h. After washing, the coverslip was soaked in 300 nM DAPI (Thermo D1306) solution for 10 min, and coverslips were mounted onto glass slides using VECTASHEILD HardSet Antifade mounting media (VECTOR laboratories H-1400). Imaging was performed with a Leica SP8 using a 63× glycerol objective with 2× digital zoom. Images were analyzed with ImageJ software.

Cycloheximide Translation Blocking Experiments. HCT116 and HEK-293 cells were treated with 177 μM cycloheximide (Sigma C7698-1G) for the indicated times. α-tubulin protein levels were used as a control to verify translational inhibition with cycloheximide.

Proteasome/Calpain Inhibitor Assays. Proteasome inhibitor co-incubation and washout experimental conditions were performed in HCT116 cells treated with DMSO (Sigma 472301), 1 nM OSW-1 compound, 25 nM bortezomib (Sigma 5043140001), 25 nM carfilzomib (AdooQ Bioscience A11278), 170 nM MG-132 (Sigma 474787), or a combination of treatments for the indicated period of time. For calpain inhibition, in both co-incubation and washout experimental conditions (6 h treatment, 24 h recovery), HeLa cells were treated with DMSO, OSW-1 compound (1 nM), ALLN (10 μM), or a combination of treatments.

RT-PCR Analysis. HCT116 and HEK-293 cells were treated in the same manner as the washout experimental protocol (6 h, 1 nM OSW-1 compound with 0–72 h recovery). RNA was extracted (see Supporting Information for protocol), and RNA concentration and purity were analyzed using a Nano-Drop spectrophotometer. cDNA was created from the isolated RNA using the Maxima First Strand cDNA Synthesis Kit (Thermo K1671). cDNA synthesis was confirmed by PCR with intron spanning β-actin primers. Once verified, RT-PCR was set up using Fast SYBR Green (Thermo 4385612) as outlined by the iTRAQ Reagents-8plex Multiplex Kit (Sciex 4381664) as outlined by the iTRAQ Reagents-8plex Protocol provided by Sciex. The HEK-293 cells were subjected to standard OSW-1 washout (1 nM, 6 h treatment). Please see Supporting Information for experimental details.

Autophagy Experiments. HEK-293 cells were treated with DMSO, 1 nM OSW-1 compound, 25 μM chloroquine, 100 nM rapamycin, or a combination of treatments for 6 h. After 6 h, the media was washed with three separate 5 mL compound-free media washes. Cells were then incubated with either drug free media, media was washed out with three separate 5 mL compound-free media, or 25 nM bortezomib (Sigma 5043140001), 25 nM carfilzomib (AdooQ Bioscience A11278), 170 nM MG-132 (Sigma 474787), or a combination of treatments for the indicated period of time. For calpain inhibition, in both co-incubation and washout experimental conditions (6 h treatment, 24 h recovery), HeLa cells were treated with DMSO, OSW-1 compound (1 nM), ALLN (10 μM), or a combination of treatments.

iTRAQ Experiments. iTRAQ experiments were performed using the iTRAQ Reagent-8plex Multiplex Kit (Sciex 4390812) and Multiplex Buffer Kit (Sciex 4381664) as outlined by the iTRAQ Reagents-8plex Protocol provided by Sciex. The HEK-293 cells were subjected to standard OSW-1 washout (1 nM, 6 h treatment). Please see Supporting Information for experimental details.

Viral Proliferation Inhibition Experiments. The CoxA9-01 or Echo2-01 clinically isolated viruses were passaged twice in RD cells and then stored at –80 °C until use.

Continual OSW-1 Treatment Antiviral Experiment. (Figure 6A): 2.0 × 10^5 HeLa cells were plated in 1 mL of media in 24-well plates and incubated (37 °C, 5% CO2) for 20 h. Cells were dosed with OSW-1 in 1 mL of media for 6 h in quadraplicate wells. Then, media was removed, cells were gently washed three times with 1 mL of FBS-free DMEM media and then infected with either CoxA9-01 or Echo2-01 viruses (estimated MOI = 1.0) in serum-free DMEM. The virus and cells were incubated for 30 min, at which point the virus inoculum was removed and the culture was washed once with serum-free media. Then, the infected cells were dosed again with OSW-1 compound at the same previous concentrations in 1 mL of complete media, incubated with the compound for 10 additional hours, and then snap-frozen and stored at –80 °C until processing. Viral titration was done through thawing the plates, scraping the cells into microcentrifuge tubes, and then centrifuging the samples at 10 000g at 4 °C to produce the virus containing supernatant, which is assayed for TCID-50 titration on subconfluent RD cells. Three independent experiments were averaged to produce the indicated results (Figure 6A).

Washout OSW-1 Treatment Experiment (Figure 6B). This experiment was performed identically to the antiviral assay described above, with the following changes. Cells were treated with the indicated compound-containing media for 6 h. Then, the cells were washed three times in compound-free complete media and incubated for 24 h in compound-free media. After 24 h washout recovery, the cells were then infected with CoxA9-01 or Echo2-01 viruses at an estimated MOI of 1.0 for 30 min, cultured for 10 h in complete media, and then subjected to analysis as described above.

Statistical Analysis. All results are expressed as mean ± SD and are n ≥ 3 unless otherwise stated. All statistical tests were performed using GraphPad Prism 7.0. Comparison between groups was made by using a one-way ANOVA with a follow up Dunnett’s test. The p values are reported using GraphPad Prism: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001.

Other Methods. Any remaining experimental procedures and additional details of the methods outlined above are described in the Supporting Information.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information, which includes Supplementary Figures, detailed methods, and experiments, is available free of charge on the ACS Publications Web site. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.8b00984.

Effects of OSW-1 compound treatment on OSBP levels and cells, data showing that reduction of OSBP levels due to OSW-1 compound washout is not an experimental artifact, full chromatogram of LCMS and SCMS 100 nM OSW-1 treatment of HCT116 cells, spectra of SCMS at 0 and 24 h recovery, LCMS limit of OSW-1 quantification, representative inhibition binding curves of OSBP and ORP4 for OSW-1 compound, Western blots for data in Figures 4 and 6, data showing that chemical inhibition of autophagy does not rescue OSBP levels in OSW-1 compound washout cells, OSBP levels in iTRAQ lysate samples, detailed methods, full Western blots, and characterization of deuterated OSW-1 (PDF).

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**Notes**

The authors declare no competing financial interest.

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