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# Inhibitors of the sodium potassium ATPase that impair herpes simplex virus replication identified via a chemical screening approach

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**Abstract**

Small molecules can provide valuable tools to investigate virus biology. We developed a chemical screening approach to identify small molecule inhibitors of poorly understood, pre-early gene expression steps in herpes simplex virus infection, using green fluorescent protein fused to an early protein. Our assay identified ouabain, a cardiac glycoside. Ouabain reversibly decreased viral yield by 100-fold without affecting cellular metabolic activity in an overnight assay. The antiviral potencies of other cardiac glycosides correlated with their potencies against the known target of these compounds, the cellular sodium potassium ATPase. Ouabain had a reduced effect if added 8 h post-infection. It did not inhibit viral attachment or entry, but did reduce the expression of viral immediate–early and early genes by at least 5-fold. Collectively, these results implicate a cellular target that was hitherto not considered important for a stage of HSV replication prior to viral gene expression.


*Keywords:* Herpes simplex virus; Chemical screening; Ouabain; Cardiac glycosides; Sodium–potassium ATPase; Attachment; Entry; Capsid trafficking; Uncoating; Immediate–early gene expression

## Introduction

Perturbation of biological systems with small molecule inhibitors has shed much light on the function of those systems in their uninhibited states. This is especially true for the replication of viruses, where pharmacological research has yielded greater understanding of the function of viral gene products and viral interactions with the host cell, in addition to clinically useful antiviral drugs.

Herpes simplex virus (HSV) is a clinically significant human pathogen (reviewed in Roizman et al., 2007). The process of HSV infection begins with attachment to a cellular receptor, followed by entry into the cytoplasm via one of a number of host cell-type-dependent pathways (reviewed in Spear, 2004), trafficking of the viral nucleocapsid to the nucleus on the cellular microtubule network (Sodeik et al., 1997), and uncoating at the nuclear pore to insert the viral DNA and trans-activators into the nucleus (Batterson et al., 1983). Immediate–early and early rounds of gene expression occur, permitting replication of the viral genome and maximal expression of late gene products (Honess and Roizman, 1974). Progeny virions are then assembled and egress from the host cell. The stage of HSV DNA replication is relatively well understood, thanks in part to a large number of known pharmacological interventions which target this event (reviewed in Coen and Schaffer, 2003). Comparatively less is known about other stages in HSV infection, especially events prior to DNA replication. More could be learned about these earlier events with the identification of small molecule inhibitors that target them.

Towards this end, we designed a cell-based screening approach using a virus that expresses a reporter with early kinetics. We previously reported the construction and characterization of a recombinant HSV-1 ICP8-GFP, which expresses the green fluorescent protein (GFP) fused to the viral single stranded DNA binding protein, ICP8 (Taylor et al.,

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2003). ICP8, a viral early gene product, is expressed prior to DNA replication, and its expression indicates that the prior steps of viral infection have already occurred successfully. We hypothesized that this virus could be used as a reporter in a screen to identify compounds that inhibit viral replication at a stage prior to expression of this early protein. Our screen and subsequent analysis identified ouabain, a well characterized inhibitor of the cellular sodium–potassium ATPase (reviewed in Xie and Cai, 2003), as an inhibitor of HSV replication after entry and before immediate–early gene expression.

## Results

### *Design and execution of a chemical screening approach*

We designed a screen using a high content screening microscope at the Harvard Institute for Cell and Chemical Biology (ICCB). We used this assay to examine the ICCB's known bioactive compounds library, a selection of 480 compounds with previously described biological activities. Vero cells were plated in black, clear-bottomed 384-well plates and incubated overnight at 37 °C, yielding confluent monolayers of cells. Compounds were then robotically added to individual wells in duplicate at room temperature prior to infection. ICP8-GFP virus was added to the drug-containing media at a multiplicity of infection (MOI) of 20, and infection was allowed to proceed at 37 °C for 18 h. At the end of this incubation, cells were fixed with paraformaldehyde and treated with Hoechst 33258 to stain nuclear DNA. The screening microscope was used to record images of Hoechst and GFP fluorescence and amounts of fluorescence were quantified using image analysis software. Compounds that reduced the amount of GFP fluorescence by at least one standard deviation relative to the mean GFP signal for all compounds screened were scored as potential hits. From the 480 compound library, 17 compounds met this criterion. The images of the infected cells treated with these potential hits were then examined for signal from the Hoechst stain as a preliminary assessment of cytotoxicity. This eliminated twelve compounds that reduced the amount of Hoechst signal by at least one standard deviation relative to the mean DNA signal for all compounds screened. The remaining 5 compounds, approximately 1% of compounds screened, were ordered for secondary testing. Of these five, four compounds either caused visually apparent toxicity or did not cause an obvious reduction in GFP signal. The fifth compound, ouabain, caused an obvious reduction in the level of GFP expressed by infected cells that was visually apparent without software quantification (Fig. 1, compare panels B and D) and did not cause toxicity as qualitatively measured by examining the cells under light microscopy and reduction of signal in the Hoechst images (Fig. 1, compare panels A and C). We selected ouabain for further study.

### *Dose response of ouabain inhibition of GFP fluorescence*

We conducted infections in the presence of a range of concentrations of ouabain to quantify the potency and efficacy of ouabain's inhibition of GFP fluorescence resulting from infection with the ICP8-GFP virus (Fig. 2). The concentration of ouabain that reduced ICP8-GFP signal by 50% in this assay was between 14 and 140 nM. Treatment with $\geq$140 nM ouabain reduced nuclear GFP fluorescence produced by infected cells by five-fold, down to a level which corresponded to the amount of autofluorescent signal observed from untreated, uninfected cells.

### *Anti-HSV activity of ouabain*

Ouabain was assessed for effects on viral replication using wild-type HSV-1 strain KOS. Concentrations of ouabain $\geq$100 nM fully inhibited the formation of HSV-1 plaques on a monolayer of Vero cells after 72 h. A small number of rounded but still adherent cells, and dead floating cells, were visible in treated, but not untreated, cells after this length of exposure, and this effect was similar in the presence or absence of virus. As an alternative to the plaque assay, a single cycle growth experiment was performed to determine whether ouabain reduced viral yield. In this assay, the concentration of drug that reduced yield by 90% ($IC_{90}$) was 40 nM, and concentrations of 100 nM or higher consistently reduced viral yield by 100- to 10,000-fold (Fig. 3).

A cell viability experiment was conducted to assess whether the observed reductions in GFP expression and viral yield during overnight treatments were caused by cytotoxicity, such as we had observed during the longer duration treatment necessary for plaque formation. Confluent Vero cells were treated with a range of concentrations of ouabain for 24 h, and cellular metabolic activity was measured by cleavage of a tetrazolium salt, WST-1 (Fig. 4). No dose-dependent decrease in metabolic activity compared to untreated cells was observed. This indicated that the antiviral effects of ouabain in the yield assay were not associated with cytotoxicity.

### *Antiviral effect of other sodium–potassium ATPase inhibitors*

Ouabain is a well-characterized inhibitor of the cellular sodium potassium ATPase (reviewed in Xie and Cai, 2003), which is a clinically relevant drug target. As a result, detailed structure–activity relationship data on ouabain and a variety of other cardiac glycosides have been previously reported (Pullen et al., 2004). A selection of inhibitors of the sodium potassium pump was tested for effects on viral yield (Table 1). All compounds tested reduced viral yield by 100-fold at sufficient dosage. The antiviral potency of all of the plant-derived inhibitors correlated with their reported potencies against the pump (Pullen et al., 2004), though bufalin, an inhibitor derived from toad venom, was slightly more potent against viral yield than ouabain despite being slightly less potent against the sodium potassium pump.

### *Kinetics of the anti-viral effect of ouabain*

Next, we performed an experiment to determine if the antiviral effect of ouabain was reversible. Cells that were treated