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High throughput screening assay to identify small molecule inhibitors of Marburg virus VP40 protein

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Abstract

Marburg virus (MARV) causes sporadic outbreaks of severe disease with high case fatality rates in humans. To date, neither therapeutics nor prophylactic approaches have been approved for MARV disease. The MARV matrix protein VP40 (mVP40) plays central roles in virus assembly and budding. mVP40 also inhibits interferon signaling by inhibiting the function of Janus kinase 1. This suppression of host antiviral defenses likely contributes to MARV virulence and therefore is a potential therapeutic target. We developed and optimized a cell-based high throughput screening (HTS) assay in 384 well format to measure mVP40 IFN antagonist function such that inhibitors could be identified. We performed a pilot screen of 1280 bioactive compounds and identified 3 hits; azaguanine-8, tosufloxacin hydrochloride and linezolid; with Z-scores of >3 and no significant cytotoxicity. Of these, azaguanine-8 inhibited MARV growth at non-cytotoxic concentrations. These data demonstrate the suitability of the HTS mVP40 assay for drug discovery and suggest potential directions for anti-MARV therapeutic development.

Marburg virus (MARV) is a member of *Filoviridae* family of enveloped filamentous, non-segmented, negative-stranded RNA viruses ¹. Several filoviruses belonging to either the *Ebolavirus* genus or the *Marburgvirus* genus, have caused outbreaks of severe disease in humans ^{1–3}. The first MARV outbreak occurred in Germany and Serbia in 1967 resulting in 31 cases and 7 deaths ^{4, 5}. Since then MARV has resulted in several sporadic cases and outbreaks with the largest occurring in Democratic Republic of the Congo (DRC) (1998 - 2000) ^{6, 7} and Angola (2004-2005) ⁸. The Angola outbreak was notable for a reported case fatality rate of nearly 90%. The potential for filoviruses to cause large outbreaks has been highlighted by the West Africa *Zaire ebolavirus* epidemic from 2016-2019 and an outbreak

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in Democratic Republic of the Congo that began in 2014 and has continued well into 2020^{9–12}. The public health impacts of filoviruses highlight the need for medical countermeasures, such as small molecule therapeutics.

Suppression of type I interferon (IFN) responses by viral gene products contribute to the virulence of the filoviruses¹³. IFNs are cytokines that induce expression of antiviral genes that play a central role in innate antiviral defense. IFNs act by activating IFN alpha receptor-associated Janus kinase 1 (Jak1) and Tyk2 tyrosine kinases. These phosphorylate STAT1 and STAT2, leading to formation of STAT1–STAT2 heterodimers¹⁴. The phosphorylation and dimerization of STAT1 allows recognition of a non-conventional nuclear localization signal on STAT1 that mediates nuclear import by any of the three members of the NPI-1 subfamily of karyopherin- α (KPNA) proteins, KPNA1, KPNA5 and KPNA6^{15–19}. Nuclear translocation of STAT1 and STAT2 leads to the activation of genes that possess interferon stimulated response elements (ISRE).

Both MARV and EBOV infections inhibit production of and cellular responses to IFN¹³. The VP35 proteins of both EBOV and MARV inhibit the production of IFN in infected cells by blocking the RIG-I receptor signaling pathway^{20–25}. However, the mechanisms by which EBOV and MARV block signaling induced by IFN differ. EBOV VP24 binds to KPNAs 1, 5 and 6 and prevents STAT1 nuclear translocation^{19, 26, 27}. In contrast, MARV VP40 (mVP40) abrogates IFN signaling by blocking the type I IFN-induced activation of Jak1²⁸. This prevents activation of STAT1 and STAT2 and blocks IFN-induction of interferon stimulated gene (ISG) expression. Although Jak1 function is inhibited by mVP40, the underlying mechanism remains to be fully elucidated. There is evidence that IFN inhibition by mVP40 contributes to MARV host range, suggesting a role for this function in virulence^{29, 30}. Because of its likely role in pathogenesis, the IFN-inhibition function of mVP40 is a potential therapeutic target. Understanding the role of mVP40 in MARV infection could be facilitated by molecular probes specific for this protein. To discover potential small molecule inhibitors of mVP40 IFN inhibition, we developed and optimized a high-throughput screening (HTS) assay in 384-well format to identify compounds that overcome mVP40 inhibition of IFN induced ISG expression. We completed a pilot screen of 1280 bioactive compounds and identified three hits, azaguanine-8, tosufloxacin hydrochloride and linezolid, that specifically induced ISRE activation in mVP40 expressing cells but not in control cells that do not express mVP40. Of these, azaguanine-8 also inhibited growth of MARV *in vitro* without affecting mVP40 expression. Overall, these studies have established a robust cell-based screening assay to identify small molecules inhibitors of mVP40 IFN-inhibition.

Results

Development of reporter cell lines to measure mVP40 inhibition of IFN signaling.

To identify small molecules targeting mVP40 inhibition of IFN signaling, we developed an ISRE-firefly luciferase reporter assay to allow quantification of ISRE induction and inhibition by mVP40 in a cell-based context. Treatment of cells with IFN activates Jak-STAT signaling and hence ISRE reporter activity, whereas expression of mVP40 inhibits this response. We hypothesized that a small molecule inhibitor of mVP40 function would relieve this inhibition leading to ISRE-reporter activity (Fig. 1A). Because this constitutes a gain-of-

function assay, cytotoxic compounds would not be expected to score as hits. To develop this approach as a screening assay, we generated a 293T-based stable cell line with an ISRE-firefly luciferase reporter gene. These cells were then transduced with a lentivirus that expresses from a single mRNA both mVP40 and green fluorescent protein (GFP), with the two open reading frames separated by an internal ribosome entry site³¹. This procedure yielded the cell line mVP40-ISRE, referred to as “mVP40 cells” from here forward and in the figures. The ISRE reporter cell line was also transduced with an “empty-vector” lentivirus that expresses GFP alone, which yielded the cell line ISRE. Clonal mVP40 and ISRE cell lines were obtained by sorting for GFP expression, and Western blotting confirmed expression of mVP40 in the mVP40 cells (Fig. 1B). Treatment of cells with IFN β typically leads to activation of promoters with ISREs³². Upon addition of 100 U/ml of IFN β , a strong upregulation of luciferase expression was detected in the ISRE cells, whereas the mVP40 cells exhibited a diminished response, consistent with mVP40 inhibition of IFN signaling and ISRE promoter activity (Fig. 1C). Examination of the mRNA for a representative endogenous interferon stimulated gene (ISG), IFIT1, yielded parallel results (Fig. 1D), demonstrating that the reporter gene accurately reflects the status of the endogenous ISRE response.

To establish that the diminished response of the mVP40 cells to exogenous IFN β was due to the presence of mVP40, the effect of a previously described mVP40-targeting small interfering RNA (siRNA) on response to IFN β was assessed³³. ISRE responses were stimulated by IFN treatment in the scrambled siRNA-transfected ISRE cells. In the scrambled siRNA-treated mVP40 cells, response to IFN was impaired, consistent with the results of the prior experiments. However, upon siRNA knockdown of mVP40, the response to IFN in the mVP40 cells approached that of the IFN-treated, scrambled siRNA ISRE cells (Fig. 1E). Western blotting confirmed that this corresponded with reduced mVP40 expression levels in the mVP40 siRNA transfected cells compared to a scrambled siRNA transfected cells (Fig. 1E). This data indicates that the assay will allow identification of mVP40 inhibitors as indicated by enhanced expression of the reporter gene.

Optimization of an mVP40 assay for 384 well format.

Next, the reporter cell lines were used to develop an assay of mVP40 function in 384-well format to enable high-throughput screening. The approach optimized cell number to determine conditions that resulted in Z-factor (Z') > 0.5, signal to background (S/B) ratio >10 and % coefficient of variance < 20%. The ISRE and mVP40 cells were plated in the range of 10,000 - 50,000 cells per well in 384-well plates using a multi-well liquid dispenser, and the plates were then incubated for 2h at 37°C. Cells were subsequently mock-treated or exposed to 100 U/ml of human IFN β . 20h post treatment, the luciferase signal was measured (Fig. 2A–D). We determined Z' and S/B for each cell line to define the optimum number of cells to be used to achieve a robust assay (Table 1). Comparison of mock (media only) vs. IFN β -treated ISRE cells, mock vs. IFN β -treated mVP40 cells or IFN β -treated ISRE vs. mVP40 cells yielded a Z' >0.5 for most conditions. As might be expected, S/B was greatest when comparing mock vs. IFN β -treated ISRE cells. The mock vs. IFN β -treated mVP40-ISRE cells also consistently yielded a Z' > 0.5 but lower S/Bs. Comparison of IFN β -treated ISRE vs. mVP40 cells mostly yielded Z' >0.5 and S/B >10 (Table 1). For

further assays, the 20,000 cells per well condition was chosen because this yielded a good S/B window of >10 and a Z' of 0.6 for all the tested conditions for both ISRE and mVP40 cells (Fig. 2B and Table 1). The robust activity and Z-factors are consistent between experiments and from day to day (data not shown).

For an HTS screen, it was desirable to include a positive-control that would induce the ISRE promoter even in the presence of mVP40. Because no small-molecule inhibitor of VP40 has been described, we took advantage of the fact that at 100 U/ml IFN β led to a measurable increase in signal in the mVP40 cells. We therefore compared 10 or 100 U/ml of IFN β . In mVP40 cells, 10 U/ml IFN β did not yield activation of the reporter and signals were not distinguishable from DMSO-treated cells. However, addition of 100 U/ml IFN β increased reporter signal in the mVP40 cells such that S/B was 27 and Z' was 0.7 when compared to either the DMSO or the 10 U/ml wells (Fig. 3A).

In parallel, we assessed the robustness of responses of the ISRE cells under these conditions as a counterscreen. In ISRE cells, 10 U/ml IFN β treatment led to enhanced reporter activity with the S/B of 16 and a Z' of 0.7 (Fig. 3B). With 100 U/ml IFN β , the S/B was 310 and the Z' was 0.6 (Fig. 3B). Comparison of the ISRE and mVP40 responses after 10 U/ml IFN β yielded a 10-fold induction of the ISRE cells over the mVP40 cells and a Z' of 0.7 (Fig. 3C). Based on these data, we chose to screen for inhibitors of mVP40 function in the presence of 10 U/ml of IFN β . Two sets of positive controls were used; first, treatment of mVP40 cells with 100 U/ml of IFN β , which demonstrates that the reporter gene can be activated, and second, ISRE cells treated with 100 U/ml of IFN β to capture the ISRE activation. We also ran a counterscreen with ISRE cells to identify compounds that activate reporter gene expression independently of effects on mVP40 function.

Pilot HTS of the mVP40 assay.

Using the optimized conditions for the 384-well format, the mVP40-ISRE cells and the ISRE cells were screened using a library of 1280 bioactive compounds. We used as the positive control 100 U/ml of IFN β treatment in the presence of diluent (0.1% DMSO). Cycloheximide-treated wells were included to inhibit all protein biosynthesis including reporter expression and were used to calculate Z' for each plate as a negative control. Twenty hours post-addition, luciferase activity was measured (Fig 4A). Each of the four plates used to screen the library yielded a $Z' > 0.5$ (Fig. 4B). The layout for the screening plates and controls are indicated in Fig. 4B, and the signals from all the controls used with each cell line, are shown in Fig. 4C. The Z-scores, which indicate how many standard deviations a given value is from the mean, were calculated for each compound, and compounds with Z-scores > 3 were classified as hits (Fig. 4D–E; Table 2). We identified three hits specific for mVP40, seven hits unique to ISRE cells, and 12 hits common to both cell lines (Fig. 4F and Table 2). Interestingly, compounds used as cancer chemotherapeutic drugs that are known to activate a DNA damage response, such as doxorubicin and daunorubicin, scored as hits common to both the mVP40 and ISRE cells. Previously, we have identified these compounds as activating an IFN- α/β response that induces ISGs³¹. This induction occurs because these compounds activate signaling through the ATM and cGAS-STING pathways, which are not suppressed by filovirus IFN antagonist VP35

proteins and, by extension, should also not be blocked by mVP40³¹. These findings demonstrate that the mVP40 HTS assay is capable of identifying compounds that bypass mVP40 IFN antagonist function. The seven compounds unique to the ISRE cells likely induce type I IFN, which would induce the ISRE promoter in the absence of mVP40 but be suppressed in the presence of mVP40. The three hits specific to the mVP40 cell line represented potential inhibitors of mVP40 function.

Validation of hits.

As small molecule inhibitors of mVP40 were our primary objective, we chose the three hits specific to the mVP40 cells for retesting. Doxorubicin was also further examined as a representative common hit between the ISRE and mVP40 conditions. Dose response studies were performed in both ISRE and mVP40-ISRE cells, starting at 50 μ M with three-fold serial dilutions in the presence of either 10 U/ml IFN β or DMSO. In parallel, 50% cell cytotoxic concentrations (CC₅₀) were determined in HEK293T cells. A secondary assay that evaluated the effect of compounds on a constitutively-expressed firefly reporter was also performed to triage compounds that non-specifically activate global gene expression or enhance firefly luciferase expression levels or activity.

We observed a dose-dependent activation of ISRE reporter activity in the mVP40-ISRE cells treated with 10 U/ml IFN β , with over 2-fold induction of activity apparent at concentrations >1 μ M of azaguanine-8, tosufloxacin hydrochloride and linezolid. Although, tosufloxacin hydrochloride and linezolid showed modest activation in DMSO treated mVP40 cells, no activation was seen in the ISRE cells suggesting these compounds are likely targeting mVP40 function (Fig 5 A–F). Doxorubicin showed robust activity in both ISRE and mVP40 cells with maximum activity around 1 μ M (Fig 5G–H). When assessed for cytotoxicity, doxorubicin resulted in cell toxicity with a CC₅₀ of 22 μ M, consistent with our prior studies³¹.

Azaguanine-8 exhibited some toxicity at the highest concentration tested with a CC₅₀ of 37 μ M, but no obvious toxicity was seen with the other two compounds (CC₅₀ >50 μ M) at the concentrations tested (Fig 6 A–D). None of these compounds resulted in enhancement in the constitutively expressing firefly luciferase assay, suggesting the activity is specific to ISRE promoter activation (Fig 6 A–D). We further examined whether the presence of these compounds affects the expression of mVP40. The stable mVP40 cells were treated with different doses of compounds (5 or 50 μ M) for 20h and mVP40 expression was evaluated by Western blotting (Fig 6E). No apparent effect on mVP40 expression or an internal control (β -tubulin) was observed in presence of either of the compounds that specifically affect mVP40. However doxorubicin treatment at 50 μ M showed a slight decrease in mVP40 expression, likely reflecting its toxicity. These data suggest these compounds are not functioning by inhibiting mVP40 expression.

Azaguanine-8 inhibits Marburg virus growth *in vitro*.

We further assessed the antiviral activity of the primary hit compounds against MARV. Titrations of the compounds were performed in HeLa cells against MARV infection at an MOI of 0.1 to 0.25 and assessed for MARV antigen by using immunostaining with anti-

MARV antibody at 24h post-infection as previously described³⁴. Treatment with azaguanine-8 showed a modest effect on virus growth, while other compounds failed to show any effect against the virus (Fig 7 A–C). To further examine the antiviral effects of azaguanine-8, we performed a dose response assay starting at 250 μ M that yielded an IC₅₀ of 17 μ M (Fig 7D). Importantly, these conditions did not result in toxicity against HeLa cells.

Activation of ISRE by Azaguanine-8 is inhibited by a Jak inhibitor.

As mVP40 functions by inhibiting Jak1 activation, it was of interest to determine if activation of ISRE in the presence of mVP40 by the hit compounds requires Jak kinase activity. Therefore, we assessed the effect of the hits in the absence or presence of the Jak1/Jak2 kinase inhibitor ruxolitinib. The ISRE or mVP40-ISRE cells were treated with 20 μ M of each compound in presence or absence of IFN β (10 U/ml) for 20h, followed by a luciferase assay to measure ISRE reporter activity (Fig. 8A). As seen before, mVP40 blocked activation of ISRE reporter activity in the presence of IFN β . However, treatment with compounds reversed this inhibition. Interestingly, addition of ruxolitinib resulted in suppression of ISRE activation by the compounds, suggesting Jak1 activity is needed for the compounds to overcome mVP40 inhibition of the IFN signaling pathway. These data are also consistent with previous studies and propose a model whereby ruxolitinib and mVP40 inhibit Jak1 by distinct mechanisms^{28, 29}.

mVP40 inhibition of Jak1 activation results in suppression of IFN- α/β -induced tyrosine phosphorylation of Jak1, Tyk2, STAT1, and STAT2. We examined the effect of the compounds on STAT1 phosphorylation as a means to determine whether azaguanine-8 could restore IFN- α/β -induced tyrosine phosphorylation events. The HEK293T cells were co-transfected with plasmids that express a human STAT1-GFP fusion protein and either an empty expression plasmid or plasmid that encodes mVP40. At 24 h post-transfection, cells were treated with DMSO or azaguanine-8 (20 μ M) and after 24 h exposed to IFN β for 30 min. IFN-induced phosphorylation of STAT1 was examined by Western blotting for total STAT1 and phospho-STAT1 levels (Fig 8B). IFN β but not azaguanine-8 alone led to STAT1 tyrosine phosphorylation. This IFN β induced STAT1 phosphorylation was inhibited in the presence of mVP40. Addition of azaguanine-8 led to a modest increase in STAT1 phosphorylation in the mVP40 cells upon IFN β treatment suggesting that azaguanine-8 is capable of reversing the IFN antagonist function of mVP40 (Fig. 8B).

Discussion

The VP40 proteins of filoviruses are matrix proteins that underlie the membrane of virus particles^{35, 36}. All VP40s direct budding and release of new virions from the plasma membrane of infected cells^{37, 38}. Unique to mVP40 and the VP40 from the filovirus M nglá virus, a closer relative to MARV than EBOV, is the capacity to potently inhibit IFN-signaling^{28, 39, 40}. In contrast, the VP40s from EBOV, other members of the *Ebolavirus* genus, or Lloviu virus, which is more closely related to EBOV than MARV, do not exhibit the same anti-IFN activity⁴¹. While MARV infection and mVP40 expression inhibit cellular responses to IFN by blocking Jak1 and downstream signaling events, the precise mechanism

by which Jak1 is inhibited remains unknown²⁸. For MARV, there is evidence that mVP40 IFN inhibition is relevant to the capacity of the virus to cause disease. Lethal infection of immune-competent mice required adaptation of MARV to this species by serial passage^{42, 43}. Once adapted, MARV can cause lethal disease in mice. Among MARV genes that consistently change in mouse-adaptation studies is mVP40^{43, 44}, suggesting that host species-specificity of mVP40 activity is critical for viral pathogenesis. When the IFN inhibitory function of mVP40 is compared between parental and mouse-adapted versions in human and mouse cells, only the mouse-adapted mVP40 is able to efficiently inhibit IFN signaling in mouse cell lines^{30, 44}.

Inhibitors of mVP40 anti-IFN function would likely be valuable molecular probes that could help define how suppression of Jak1 function contributes to virus replication. Because of the link between mVP40 function and virulence, such inhibitors might also serve as leads for therapeutic development. These considerations motivated the development of the HTS assay described in this study. Because inhibition of Jak1 leads to a block to IFN-induced gene expression, our assay was based on ISRE-luciferase reporter genes. We produced a stable cell line with an ISRE-luciferase reporter gene. We then introduced into this parent cell line either an “empty vector” lentivirus or a mVP40 expressing lentivirus. Using these cell lines, we were able to optimize assay conditions for 384-well format that could differentiate DMSO from IFN β treatment and where the activity of mVP40 could readily be differentiated from the empty vector ISRE cell line. Importantly, the inhibitory effect of mVP40 could be reversed by knocking down VP40 expression and could be overcome by adding larger amounts of IFN β (Figure 1E and 3A), thereby providing a positive control for gene activation. By screening against both the mVP40 cells and the ISRE cells, we identified compounds that only activated gene expression in the presence of mVP40. Such compounds are most likely to hamper mVP40 IFN inhibition. The compounds that activated ISRE signal in both cell lines are likely to turn on the ISRE luciferase by activating non-canonical pathways that stimulate the ISRE promoter bypassing inhibition by mVP40. Alternatively, they may induce IFN to sufficient levels such that they overcome the inhibitory activity of mVP40. Because small molecule inhibitors of mVP40 function result in a gain of signal, this assay avoids the common pitfall of loss of signal cell-based assays, where cytotoxic compounds score as hits.

After optimization, a screen of bioactive compounds was designed to further test the performance of the assay. Of the 1280 compounds tested, three compounds, namely azaguanine-8, tosufloxacin hydrochloride and linezolid, activated ISRE reporter activity in IFN treated mVP40 cells but not in ISRE cells, suggesting that these compounds likely affect mVP40 function. Additionally, none of these compounds impaired constitutive firefly luciferase expression or induced any profound cell toxicity at tested conditions.

Azaguanine-8, tosufloxacin hydrochloride and linezolid are known for their antibacterial properties. Azaguanine-8 has anti-neoplastic activity and has been used for treatment of acute leukemia⁴⁵. Interestingly, azaguanine-8 has been shown to inhibit virus infections in the context of several plant virus diseases and also has anti-bacterial activities^{46–51}. However, the activities can be reversed by addition of adenine and guanine^{47, 50}. Although the relevant antiviral target is not known, it has been suggested that azaguanine-8 acts by

blocking an enzyme involved in the utilization of purines by the virus⁵⁰. Azaguanine-8 has also shown to inhibit the synthesis of vaccinia virus deoxyribonucleic acid via disabling of the enzymic system involved with the incorporation of thymidine monophosphate into the DNA^{51–53}.

Tosufloxacin hydrochloride is a fluoroquinolone antimicrobial agent with broad spectrum antibacterial activity. This class of quinolones are effective in treating most pneumococci, which are associated with respiratory infection. Additionally, pleiotropic effects of fluoroquinolone drugs as immunomodulators^{54, 55}, anti-oxidant agents⁵⁶ and nitric oxide regulators⁵⁷ have also been reported. These functions may contribute to the antibacterial effects of these drugs against secondary infections in a murine influenza infection model^{58, 59}.

Linezolid belongs to the class of oxazolidinone antibiotics and blocks bacterial protein synthesis through binding to rRNA. It is FDA approved for use against wide variety bacterial infections including multidrug resistant and drug-resistant tuberculosis, community acquired pneumonia hospital-acquired pneumonia caused by *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* infections⁶⁰. Interestingly, in a murine model of post influenza bacterial pneumonia, linezolid exerted immunomodulatory effects marked by a decrease in chemokines and inflammatory cytokines⁶¹. However, a direct antiviral effect of tosofloxacin hydrochloride or linezolid has not been established, including in our study. It may be that, in cell culture experiments, the effects of disabling mVP40 inhibition are minimized by other MARV encoded factors that inhibit IFN responses such as the VP35 protein.

Azaguanine-8 did act as specific mVP40 inhibitor and it also suppressed MARV growth in cell culture. Consistent with this finding, azaguanine-8 not only restored IFN-induced ISRE gene expression but also partly reversed the inhibition of Jak1 function, as assessed by STAT1 phosphorylation (Figure 8B). To what extent virus inhibition by azaguanine-8 depends on the strength of host cell IFN responses remains to be determined. As noted above, there are several reports of azaguanine-8 having antiviral activity *in vitro*. Examples include activity against vaccinia virus, HIV-1, encephalomyocarditis virus, lymphocytic choriomeningitis virus and plant RNA viruses^{46–48, 51–53, 62–64}. That our assay can identify compounds with dual activities, targeting viral RNA synthesis and IFN evasion functions, is intriguing. The availability of the HTS assay described here provides a means to perform larger scale screens and identify additional mVP40 inhibitors.

Methods

Plasmids and Antibodies.

pGL4.45 (luc2P/ISRE/hygro) was purchased from Promega. This reporter plasmid has five repeats of an ISRE upstream of a minimal promoter that drives transcription of the optimized *luc2* firefly luciferase gene. Plasmids expressing Flag tagged mVP40 (MARV Musoke strain) and GFP-STAT1 have been previously described^{28, 29}.

The replication-defective lentiviruses were generated using the following plasmids: pHCMV-G encoding the vesicular stomatitis virus (VSV) glycoprotein; packaging plasmid pNL4.3-gag-pol; and mVP40 or empty vector encoding lentiviral vectors derived from pHR-SIN-CSIGW³¹. The Flag-tagged mVP40 lentivirus contains the mVP40 open reading frame (ORF) followed by an internal ribosomal entry site, which is followed by the green fluorescent protein (GFP) ORF. The control lentivirus is the same except that it only encodes GFP.

Mouse anti-tyrosine phosphorylated STAT1 (Tyr701, BD Transduction Laboratories), anti-Green Fluorescent Protein (anti-GFP) and anti-FLAG M2 epitope antibodies were purchased from Sigma. Mouse anti-actin antibody was purchased from Cell Signaling Technology.

Cell Lines.

HEK293T and HeLa cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone) and 1% penicillin-streptomycin (Gibco).

Generation of lentiviral expression vectors and stable cell lines.

The stable ISRE-reporter cell line was generated by transfecting the plasmid pGL4.45 (luc2P/ISRE/hygro) into HEK293T cells using Lipofectamine 2000 (Invitrogen). Two days post-transfection, hygromycin was used to select for cells that stably integrated the plasmid. Clonal cell populations were screened for luciferase activity in the presence of IFN β treatment (100 U/ml) and a single ISRE clone was selected for the optimal signal to background ratio.

Replication-deficient lentiviruses were produced as previously described³¹. Stable mVP40-expressing or control cells were generated by transducing the clonal ISRE cells with replication-defective lentiviruses that express mVP40 and GFP. As the lentiviruses express GFP under the control of an IRES, GFP was used to isolate stably-transfected cells and to establish clonal populations. ISRE control cell lines were screened for optimal ISRE luciferase activity following IFN β (10-100 U/ml) treatment; mVP40 cell lines were screened for mVP40 expression and optimal mVP40-suppression of IFN β induced ISRE luciferase activity. A single clonal population of each reporter cell line was selected for further use. The cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 2 μ g/mL of hygromycin.

Chemicals.

The chemical library used for the screens was the Prestwick Chemical Library (Prestwick Chemical). Dimethyl sulfoxide (DMSO), azaguanine-8, linezolid, tosufloxacin hydrochloride, doxorubicin and ruxolitinib were purchased from Sigma-Aldrich. All compounds were prepared as stock solutions at concentrations of 20 mM in DMSO before use and subsequent dilutions were made in culture media. IFN β was purchased from PBL Assay Science.

mVP40 HTS Assay.

mVP40 and ISRE (negative control) cells (2×10^4 cells/35 μ l/well) were distributed in 384-well plates using a MultiDrop Combi reagent dispenser (ThermoScientific). Plates were centrifuged for 5 min at 1000 rpm, after which cells were allowed to rest in the incubator for 2h at 37°C. The mVP40 cells were treated with 10 U/ml of IFN β (5 μ l/well) and ISRE cells were treated with media (5 μ l/well) before compounds were transferred using a high-density pin tool (V&P Scientific) attached to the pipetting head of a Nimbus liquid handler (20 nL/well; final concentration: 5 μ M). The appropriate positive and negative controls were added in columns 1, 2, 23 and 24. Twenty hours post compound addition, luciferase activity was read using the Neolite Reporter Gene Assay System (Perkin Elmer) and a H1 synergy plate reader (BioTek). Z-factor values were calculated using the equation $Z\text{-factor} = 1 - [3\sigma^+ + 3\sigma^-]/(\mu^+ - \mu^-)$; the positive control was either mVP40 cells treated with 100 U/ml of IFN β or ISRE cells treated with 10 U/ml or 100 U/ml of IFN β , and the negative cells treated with cycloheximide. The coefficient of variance (%CV) = $\sigma/\mu * 100$ and the hits were selected based on Z score = $(x-\mu)/\sigma$, where μ : population mean σ : population standard deviation. The compounds with Z score > 3 were scored as hits.

Secondary Screen.

The constitutively expressed firefly luciferase (ce-FF) assay was performed as follows. HEK293T cells (7.5×10^6) were transfected in a T75 flask with 3 μ g of a plasmid encoding constitutively expressed firefly luciferase (ce-FF) or pCAGGS empty vector. Twenty-four hours post-transfection, cells were trypsinized and resuspended in DMEM with 10% FBS and were distributed in 384-well plates (2×10^4 cells/40 μ l/well) using a MultiDrop Combi reagent dispenser (Thermo Scientific). After 1h, compounds were added in triplicate (0–50 μ M, 3-fold dilution series). Twenty-hours posttreatment, firefly luciferase activity was determined using the neolite assay.

Dose Response assays.

The stable cell lines, mVP40 or ISRE, were plated in a 384-well format (2×10^4 cells/40 μ l/well), and 1h post-plating, the cells were treated with media or 10 U/ml of IFN β and compounds were added in triplicate (0–50 μ M, 3-fold dilution series). Twenty hours post-treatment, luciferase activity was assessed as above.

Cell toxicity assay.

To determine the 50 percent cytotoxic concentration (CC₅₀), HEK293T cells (1×10^3 cells/40 μ l/well) were plated in 384-well plates and were centrifuged at 1000rpm for 5 min, after which they were allowed to rest for 1h. Compounds were then added as indicated (0–50 μ M, 3-fold dilution series) in triplicate. Twenty hours post-treatment, CellTiter-Glo (Promega) was added, and ATP content was determined by reading the luminescence using an EnVision plate reader. CC₅₀ values were calculated with Prism 8 using a four-parameter, nonlinear regression analysis.

siRNA ISRE Luciferase Assay.

ISRE and mVP40 cells (5×10^5) were transfected with 1 μ M of siRNA using Lipofectamine RNAiMAX (Thermo Fisher Scientific) against mVP40 or a scrambled siRNA control (D-001810-01 (Dharmacon)) in 96 well plates. The mVP40 siRNA were described previously (Bedoya et al., 2013); sense (5'-3')-GmUCUmGCCAUGCmUUCACmUUmUA and antisense-(5'-3'), AAmGUmGAAAGCmUmGGCmGACmU, where m denotes the presence of 2'-O-methyl modification at that ribonucleotide. Single stranded RNA oligonucleotides were annealed to form siRNA duplexes by denaturation at 95°C for 1 minute followed by 1 hour of incubation at 37°C. Forty-eight hours post-transfection, cells were treated with different amounts of IFN β (10-100 U/ml). Twenty hours post-treatment, luciferase activity was assessed with the ISRE Luciferase Assay.

Immunoblotting.

ISRE or mVP40 cells (5×10^5 /well) were treated with the indicated compounds at 5 and 50 μ M for 24 h. Cells were lysed in NP-40 lysis buffer, and samples were analyzed by Western blot with antibodies described in the text. To confirm expression of mVP40 in stable cell lines, cell lysates were subjected to Western blot analysis for Flag-tagged mVP40, GFP and endogenous β -tubulin expression.

For the STAT1 phosphorylation assay HEK293T cells (5×10^5 /well) were transfected with empty vector, STAT1-GFP or Flag tagged-mVP40 expression plasmid as indicated in the figure. The next day the cells were treated with compounds at 20 μ M. 20h later cells were mock treated or treated with 100 U/ml IFN β for 30 minutes. The cells were lysed in NP-40 buffer and samples were analyzed by Western blotting using the respective antibodies as described in the text.

Lysates were run on 4-12% Bis-Tris Plus polyacrylamide gels (Thermo Fisher) and transferred to a PVDF membrane (Bio-Rad). Membranes were probed with the indicated antibodies, developed by Western Lightning Plus ECL (PerkinElmer), and imaged on a ChemiDoc MP Imaging System (Bio-Rad).

RNA Isolation and Quantitative RT-PCR.

Total RNA was extracted from cells using Trizol (Sigma) with the Direct-zol RNA kit from Zymo as per the manufacturer's protocol. For cDNA synthesis, ~1 μ g RNA was reverse transcribed using a Superscript® IV First-Strand Synthesis kit (ThermoFisher Scientific) using an oligo dT primer. Real-time PCR was performed using the PerfeCTa SYBR Green FastMix (QuantaBio) on a CFX96 Real-Time PCR detection system (Bio-Rad), with a PCR temperature profile as follows: 95°C for 30 seconds and then 40 cycles of 95°C for 5 seconds, 55°C for 15 seconds and 68°C for 10 seconds. The expression levels of IFIT1 and β -actin mRNAs were analyzed using gene specific primers (IFIT1-Forward: 5'-AGTGTGGGAATACACAACCTACT-3' Reverse: 5'-GGTCACCAGACTCCTCACATTT-3', and β -actin-Forward-5'-ACTGGAACGGTGAAGGTGAC-3', Reverse-5'-GTGGACTTGGGAGAGGACTG-3'.

Expression levels were normalized to β -actin mRNA and copy number calculated relative to mock-transfected cells.

Virus infections.

MARV (*Musoke*) was grown and concentrated as described previously³⁴. Briefly, virus was amplified in Vero cells in DMEM containing 2% FBS for 7 days. Culture supernatants from infected Vero cells were centrifuged at 1800×g for 15 minutes to pellet any cell debris. The virus was then pelleted by overlaying the clarified culture supernatant over a 20% sucrose cushion in PBS and then centrifugation at 141,118 ×g for 2 h at 4°C. The pelleted virus was resuspended in PBS and stored at –80°C until used. To determine the titer of the stock, HeLa cells were incubated with 10-fold serial dilutions of the stock for 24 h.

For infection assays, HeLa cells were plated in a 384-well plate at a density of 6500 cells/well. 16-18 hours after plating, cells were pretreated with compounds for 1 hour at indicated doses. After pretreatment, cells were infected with MARV to achieve a MOI of 0.1-0.25. 24-30 hours after infection, plates were immersed in 10% neutral buffered formaldehyde to inactivate the virus and cells. Inactivated plates were washed to remove formalin followed by permeabilization, blocking and staining with and rabbit anti-MARV antibody (IBT Bioservices) from animals immunized with MARV Musoke strain virus-like particles that contain the viral glycoprotein, nucleoprotein and VP40. Primary antibody signal was visualized using an anti-rabbit alexa 488 secondary antibody (ThermoFisher scientific). Cell nuclei were stained with Hoechst 33342 (ThermoFisher scientific). Images were captured using a Biotek Cytation microscope followed by analysis using Cell Profiler software^{65–67}. Total nuclei count was used as a proxy for cell numbers and antigen positive cell numbers indicated infection. Rate of infection was determined (infected cell number/total cell number) for each treatment and normalized using untreated controls as 100%. Data was plotted and analyzed using GraphPad prism software. All work with MARV was done at Boston University's National Emerging Infectious Diseases Laboratory (NEIDL) at biosafety level 4 and was approved by institutional and local committees.

Statistical Methods.

For the screening assay, Z' was used as statistical factor to define the variation in the assay and screening window. To select hits, the Z score was used, which defines how many standard deviations from the mean is a given value. The Oneway ANOVA followed by tukey's test was used to assess significance between multiple groups in reporter gene assays. Student's two-tailed t test was used to identify significance between two unpaired groups. A p-value of <0.05 indicates a significant difference between experimental groups.

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Figure 1. Generation of mVP40 and ISRE stable cell lines.

A. Schematic diagram showing the screening strategy. (I) IFN activates Jak1 and Tyk2, which phosphorylates STATs. These dimerize and enter the nucleus through interactions with karyopherin alpha (KPNA) proteins. In the nucleus, a STAT1-STAT2-IRF9 complex activates ISRE promoters, leading to luciferase expression. (II) The expression of mVP40 blocks Jak1 and leads to decreased reporter activity. (III) A small molecule binds to mVP40 and blocks its function, restoring the reporter activity. B. Schematic diagram showing how the stable ISRE and mVP40 cell lines were generated. Expression mVP40 and GFP was

examined using anti-Flag and anti-GFP antibodies via Western blotting. C. An ISRE luciferase reporter gene assay was performed on ISRE or mVP40 cells. The cells were plated in 96-well format and the next day cells were mock treated or treated with 100 U/ml IFN β . Twenty hours later, reporter gene activity was measured. The data represent means $\pm$ standard deviations and are representative of three independent experiments. D. Quantitative RT-PCR was performed to measure endogenous IFIT1 mRNA levels in ISRE or mVP40 cells treated with 100 U/ml of IFN β . The RNA was isolated 20 h after treatment, and levels were normalized to those of β -actin mRNA. The data represent means $\pm$ standard deviations and are representative of two independent experiments. E. Knockdown of mVP40 relieves the inhibition of ISRE induction by mVP40. ISRE or mVP40 cells were transfected with scrambled (scr) siRNA or siRNA against mVP40. Forty-eight hours later, the cells were treated with the indicated amounts of IFN β and luciferase activities were measured 20h post treatment. The y-axis represents “fold increase” of firefly luciferase activity where the activity from DMSO treated mVP40 cells transfected with scrambled siRNA was set to 1. The knockdown of mVP40 was confirmed by Western blotting using anti-Flag and anti-GFP antibodies. *p value < 0.05, **p value < 0.01.

Figure 2. Optimization of an HTS assay in 384 well-format.

A-D. Different numbers of ISRE or mVP40 cells (ranging from 10K - 50K, as indicated) were plated in 384-well format and allowed to rest for 2h, after which the cells were treated with 100 U/ml of IFNβ. Each data point represents the luciferase signal from a single well.

Figure 3. Effects of varying IFNβ amounts on the HTS assay.

ISRE or mVP40 cells (20K cells/40μl) were plated in 384-well format and allowed to rest for 2 h, after which the cells were treated with 10 U/ml or 100 U/ml of IFNβ. 20h later, luciferase activities were assessed, and Z-factor (Z') values and S/B values were calculated. A. Signals from mVP40 cells treated with 100 U/ml IFNβ were compared to the DMSO or 10 U/ml IFNβ conditions, and Z' and S/B were calculated. B. Signals from the ISRE cells were compared following addition of DMSO, 10U/ml IFNβ or 100 U/ml IFNβ. Z' and S/B

were calculated by comparing DMSO vs 10 U/ml IFN β or DMSO vs 100 U/ml IFN β . C. Signals from ISRE vs. mVP40 cells were compared following addition of 10 U/ml IFN β .

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Figure 4. Pilot assay to screen for inhibitors of mVP40.

(A) Schematic for high-throughput screening assay of VP40 function. Stable VP40 or ISRE cells were plated in 384-well plates. Two hours later, cells were treated with IFNβ (mVP40 cells) or media (ISRE cells). The appropriate controls were added, DMSO or cycloheximide (negative control) or 10 or 100 U/ml IFNβ (positive control). Compound addition was done via pin tool transfer. Twenty hours posttreatment, a luciferase assay was performed. The screening concentration of compounds was 5 µM. B. Results of HTS. A total of 1280 bioactive compounds were screened (4 screening plates). The Z' of each plate is indicated.

The layout of ISRE and mVP40 screening plate, including the relevant controls, is also indicated. C. The luciferase values of all the controls used in the screening of both the ISRE and mVP40 cells are indicated. D-E. Z-scores were calculated for each compound in both the primary mVP40 and secondary ISRE assays, and those with a Z-score greater than 3 were scored as hits. Each circle represents a unique compound. F. A Venn diagram showing the distribution of hits between the ISRE and mVP40 assays is shown.

Figure 5. Confirmation of hits in the reporter cell lines.

Dose responses in mVP40 cells for activation of the ISRE luciferase reporter gene following DMSO or IFN β treatment in the presence of Azaguanine-8 (A), Tosufloxacin hydrochloride (C), Linezolid (E) and Doxorubicin (G). Dose responses, performed as in panel A but in ISRE cells; Azaguanine-8 (B), Tosufloxacin hydrochloride (D) Linezolid and (F) and Doxorubicin (H). The axes represent percent ISRE luciferase activity in presence of the respective compound normalized to the DMSO control.

Figure 6. The effect of hits on a constitutively expressing firefly luciferase gene and their cytotoxicity.

The effect of compounds A, Azaguanine-8, B, Tosufloxacin hydrochloride, C, Linezolid, D, Doxorubicin, on expression of a constitutively expressed firefly (ceFF) luciferase gene was evaluated (orange line, left y-axis) as was cytotoxicity (red line, right y-axis). Percent normalized luciferase activity was calculated relative to a DMSO control. The data represent the means $\pm$ standard deviations and are representative of three independent experiments. E. ISRE or mVP40 cells were treated with 5 or 50 μM of the compounds-Doxorubicin (Doxo), Linezolid (Linz), Tosufloxacin hydrochloride (Tsufx) and Azaguanine-8 (Aza-8) or IFN β

(10 U/ml). 20h later cell lysates were prepared and expression levels of Flag-mVP40, GFP and β -tubulin were determined using anti-FLAG, anti-GFP and anti-tubulin antibodies respectively via Western blotting.

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Figure 7. Anti-Marburg virus effects of azaguanine-8.

HeLa cells were pretreated with two-fold serial dilutions of compounds starting from either 100 μM (A-C) or 250 μM (D). The percent infectivity was determined by quantitative immunostaining for Marburg virus protein. The DMSO treated, infected sample was set at 100%. The cell viability in HeLa cells was determined at 24h post compound treatment using the CellTiter Glo assay. A, Azaguanine-8, B, Tosufloxacin hydrochloride C, Linezolid, D, Azaguanine-8.

Figure 8. Azaguanine-8 blocks mVP40 antagonism of the IFN signaling pathway.

A. The control or mVP40 ISRE cells were plated into 96 well plates, pretreated with ruxolitinib (5 μ M) for 1h and then treated with DMSO or 20 μ M of Azaguanine-8 (Aza-8), Tosufloxacin hydrochloride (Tsufx) or Linezolid (Linz) in the presence or absence of IFN β (10 U/ml). The samples on the right side of the graph were also pre-treated with ruxolitinib, as indicated. Luciferase activity was measured at 20h post treatment. Error bars represent means $\pm$ standard deviations and are representative of two independent experiments.

**** $p < 0.0001$, ns-not significant. B. 293T cells were transfected with hSTAT1-GFP expression plasmid or either empty vector (pCAGGS) or the Flag-tagged mVP40 expression plasmid, as indicated. The next day, cells were treated with Azaguanine-8 (20 μ M). 20h later, the cells were treated with IFN β (100 U/ml) for 30 min and analyzed by Western blotting for STAT1 tyrosine phosphorylation. The total levels of STAT1-GFP were evaluated with an anti-GFP antibody, an anti- β -tubulin blot served as a loading control and anti-FLAG antibody was used to assess mVP40 expression.

Table 1.

Optimization of cell number

	ISRE cells			mVP40 cells			ISRE vs mVP40 cells	
	Mock vs IFN β (100 U/ml)			Mock vs IFN β (100 U/ml)			IFN β (100 U/ml)	
	Z'	S/B	%CV	Z'	S/B	%CV	Z'	S/B
10K	0.8	165	5	0.7	6	8	0.8	25
20K	0.6	58	12	0.8	13	6	0.6	16
30K	0.5	60	16	0.5	11	12	0.4	13
50K	0.7	41	8	0.6	7	10	0.7	10

Table 2.

List of hits identified in ISRE and mVP40 screening assays with Z-score>3

	Compounds	Structure	Smiles	Therapeutic Role
mVP40 cells				
1	Azaguanine-8		<chem>c12N=C(\NC(c1n[nH]2)=O)/N</chem>	antineoplastic
2	Tosufloxacin hydrochloride		<chem>c12N(/C=C(\C(c1cc(c(n2)N1CC(C1)N)F)=O)/C(=O)O)c1c(cc(cc1)F)F</chem>	antibacterial
3	Linezolid		<chem>C1(N(c2cc(c(N3C COCC3)cc2)F)C[C@@H](O1)CNC(=O)C)=O</chem>	antibacterial
ISRE cells				
1	Thiamphenicol		<chem>S(c1ccc([C@H]([C@H](NC(C(Cl)C)I)=O)CO)O)cc1)(=O)(=O)C</chem>	antibacterial
2	Ciprofloxacin hydrochloride monohydrate		<chem>C=1(/C(c2c(N(\C1)C1CC1)cc(c(c2)F)N1CCNCC1)=O)\C(=O)O</chem>	antibacterial
3	Dihydrostreptomycin sulfate		<chem>N(C1C(OC(C(C1O)O)CO)OC2C(OC(C2O)CO)C)OC3C(C(C(C3O)O)NC(=N)N)O)NC(=N)N)C</chem>	antibacterial
4	Chloramphenicol		<chem>[N+](c1ccc([C@H]([C@H](NC(C(Cl)Cl)=O)CO)O)cc1)([O-])=O</chem>	antibacterial

5	Josamycin		<chem>[C@@H]1([C@@H]([C@H]([C@@H]([C@H](O1)C)O[C@H]1C[C@@]([C@H]([C@@H]([C@H](O1)C)O)C(=O)CC(C)C)N(C)C)O)[C@@H]1[C@H]([C@@H]([C@@H](CC(O[C@@H](C/C=C/C=C/[C@@H]([C@@H](C[C@@H]1CC(=O)C)OC(=O)C)OC</chem>	antibacterial
6	Spectinomycin dihydrochloride		<chem>[C@@]12([C@](O[C@]3([C@](O1)([C@H]([C@H]([C@H]([C@H]3O)NC)O)NC([H])(H))(O[C@@H](C2=O)C)[H])O</chem>	antibacterial
7	Benzylpenicillin sodium		<chem>N12[C@@]([C@@](C1=O)(NC(=O)Cc1ccccc1)[H])(SC([C@@H]2C([O-])=O)(C)C)[H]</chem>	antibacterial
ISRE+mVP40 cells				
1	Riluzole hydrochloride		<chem>c1(nc2c(s1)cc(OC(F)(F)F)cc2)N</chem>	antispastic
2	Mupirocin		<chem>CC(=CC(=O)OC CCCCCC(=O)O)CC1OCC(C(C1O)O)CC1C(C(C(C)O)C)O1</chem>	antibacterial
3	Chlorhexidine		<chem>C(NC(=N)NCCC CCCNC(NC(Nc1ccc(Cl)cc1)=N)=N)(Nc1ccc(Cl)cc1)=N</chem>	antibacterial

4	Lincomycin hydrochloride	HCl	<chem>[C@@H]1(O[C@@H]([C@@H]([C@@H]([C@H]1O)O)O)SC)[C@H](NC([C@H]1N(C[C@@H](C1)CCC)C=O)[C@H](O)C</chem>	antibacterial
5	Minocycline hydrochloride	·HCl	<chem>[C@]12(/C(=C\3/C(c4c(c(ccc4O)N(C)C)CC3CC1[C@@H]/C(=C\C2=O)/C(=O)N)/O)N(C)C=O)/O)O</chem>	antibacterial
6	Daunorubicin hydrochloride	H-Cl	<chem>c12c(C(c3c(C1=O)cccc3OC)=O)c(c1c(c2O)C[C@@](C[C@@]1(O[C@@H]1O[C@H]([C@@H]([C@H](C1)N)O)C([H])(C(=O)C)O)O</chem>	Antibacterial/ antineoplastic
7	Doxorubicin hydrochloride	H-Cl	<chem>c12c(C(c3c(C1=O)cccc3OC)=O)c(c1c(c2O)C[C@@](C[C@@]1(O[C@@H]1O[C@H]([C@@H]([C@H](C1)N)O)C([H])(C(=O)C)O)O</chem>	Antibacterial/ antineoplastic

8	Chloropyramine hydrochloride	<p>• HCl</p>	<chem>N(c1ncccc1)(Cc1ccc(Cl)cc1)CCN(C)C</chem>	antihistaminic
9	Ribostamycin sulfate salt		<chem>[C@]1(C(O[C@@]2([C@@H]([C@H]([C@@H]([C@@H](O2)CN)O)O)N)[H])[C@H](C[C@H]([C@@H]1O)N)(O[C@H]1[C@@H]([C@@H]([C@H](O1)CO)O)[H])</chem>	antibacterial
10	Reserpine		<chem>c12[nH]c3c(c1CCN1[C@@]2(C[C@@]2([C@@H]([C@H]([C@H](OC(c4cc(c(c4)OC)OC)OC)=O)C[C@@]2(C1)[H])OC)C(=O)OC)[H])[H])ccc(c3)OC</chem>	antipsychotic
11	Epirubicin hydrochloride	<p>• HCl</p>	<chem>c12c(C(c3c(C1=O)cccc3OC)=O)c(c1c(c2O)C[C@](C[C@@]1(O[C@H]1O[C@H]([C@@H]([C@H](C1N)O)C)[H])(C(=O)CO)O)O</chem>	antineoplastic
12	Valproic acid		<chem>C(=O)(C(CCC)CC)O</chem>	anticonvulsant