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Cell Kinase Inhibitor Panel Reveals Multiple Targets to Prevent Replication of Varicella-Zoster Virus

A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

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May 2014

Honors Capstone Project in Biology

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Abstract

The alphaherpesvirus that causes chicken pox and shingles, varicella-zoster virus (VZV), infects skin fibroblasts and keratinocytes. These cells are typically quiescent and it is known that VZV manipulates their intracellular environment to activate MAPK signaling cascades, cell cycle regulators, and many transcription factors for its replication. We hypothesized that inhibition of cell kinases would prevent VZV replication and also elucidate which pathways are most important. We evaluated 80 kinase inhibitors for cytotoxicity and anti-proliferative effects on human foreskin fibroblasts, and then determined their antiviral efficacy against VZV-ORF9-Luc strain. Ten well-tolerated, potent kinase inhibitors whose targets are critical mediators of VZV infection were identified. Receptor tyrosine kinases (EGFRK, NGFRK and PDGFRK) were pivotal for VZV replication and 3 potent compounds inhibited these targets (Tyrphostin AG1478, AG-879, Tyrphostin 9). Their common downstream substrates were also found to be necessary for VZV replication: protein kinase A (PKA, H-89•2HCl) and protein kinase C (PKC, GF 109203X and PKC-412). Other potent compounds blocked the activity of calmodulin-dependent protein kinase (CaMKII, KN-62) and glycogen synthase kinase 3-beta (GSK-3β, Indirubin-3’-monooxime and Kenpaullone). We also identified one compound with previously known antiviral activity, the cyclin-dependent kinase inhibitor Roscovitine. Thus VZV infection causes changes in the intracellular environment that expose antiviral targets in multiple pathways.

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Executive Summary

Varicella-zoster virus (VZV) is a human-restricted herpesvirus that causes chickenpox (varicella) and shingles (zoster). Primary viral infection results in chickenpox, a generally mild disease that is typically contracted during childhood and presents with scattered, itchy skin pustules. Following initial infection, VZV, like other viruses in the herpes family, becomes dormant in nerve cells. The virus can reactivate spontaneously to cause shingles, a disease characterized by a localized, painful skin rash. The most common complication associated with shingles is postherpetic neuralgia (PHN)—the persistence of debilitating pain for months or years even after the infection and rash have resolved.

The Centers for Disease Control and Prevention (CDC) estimate that 3.5 million cases of varicella occur every year and that by age 40, the virus has infected 99.5% of people born in the U.S. Approximately one in three people will develop shingles in their lifetime, leading to one million cases each year in this country. The incidence of zoster and PHN increases with age, and with millions of 'baby boomers' set to join the group at the highest risk, the development of more effective antiviral drugs is crucial.

Problems associated with the vaccines and current antiviral medications necessitate new methods of treatment. The varicella vaccines, Varivax and Zostavax, consist of a live virus that cannot be given to people who are immunocompromised, although they have a very high risk for zoster. The shingles vaccine is only approved for individuals over 50 years old, and zoster

occurs even in about half of people who are immunized. Acyclovir, the primary medication used to treat zoster, is effective only within 48 hours after the onset of symptoms, and this drug is ten times less potent against VZV than other herpesviruses. Although the vaccines have prevented many cases of chicken pox and shingles, and acyclovir has helped some people recover from the infections, there is a compelling need for new treatments for this virus.

VZV, like all viruses, cannot replicate outside of a living host—it lacks the machinery necessary to carry out its own reproductive processes and must hijack the infrastructure of host cells. In so doing, it activates host kinases that send signals throughout the cell to facilitate viral replication mechanisms. Kinases are proteins that transfer high-energy phosphate groups to specific substrates, a reaction that typically 'turns on' its target. These kinases are typically linked into cascades that transduce signals from a cell's exterior to intracellular effectors, which in the case of VZV infection are factors necessary for replication of viral DNA and the production of viral proteins. VZV is known to activate specific kinases during its life cycle to gain access to these mediators, but there are inevitably more that have yet to be identified.

The goal of this project, therefore, was to elucidate additional kinases important in VZV replication by inhibiting their activity with compounds that block their ability to transfer phosphate groups. This way, we could assess the relative importance of specific targets in the viral life cycle. We hoped that by identifying the most essential kinases we could expand our knowledge of VZV-host interactions and identify potential targets of antiviral drugs.

To do this, we purchased eighty kinase-inhibiting compounds with well-defined targets. The first step in the process was to assess the safety of each compound in lab-grown human fibroblasts, a type of skin cell. This was crucial because compounds that killed cells they were meant to treat would likely cause significant side effects in patients. We compared drug-treated cells with non-treated cells to determine the effect of each inhibitor on cell viability. We then eliminated compounds that were toxic since they would be of little value in a medical setting.

Next, we studied the effect of non-toxic compounds on cell division. Cellular division, of course, is an essential process of living organisms in which kinases play a particularly important role. To make sure that inhibiting specific proteins did not prevent normal cellular proliferation, we measured the amount of cell division that took place in drug-treated fibroblasts after 48 hours. Drugs that prevented an increase in the number of cells relative to the beginning of the experiment were removed from the study because they, too, would likely be disadvantageous in a clinical setting.

Seventy of the original eighty inhibitors were nontoxic in these tests. We subsequently determined the antiviral efficacy of the remaining compounds in VZV-infected skin cells. We found a wide range of antiviral activity exhibited by the inhibitors and narrowed our focus to the sixteen most potent compounds. For each of the most effective compounds, we calculated a Selectivity Index (SI), which measures a compound's ability to prevent viral processes without deleteriously affecting the host. This step identified ten compounds whose

targets represent important mediators in VZV replication. Excitingly, a number of these proteins were implicated by more than one compound, which confirms the importance of their role in virus replication.

The cellular targets implicated by our findings are constituents of multiple signaling pathways, a number of which were previously unidentified as mediators of VZV replication. Moreover, the kinases we identified as important antiviral targets act at multiple levels of these pathways and are capable of eliciting a variety of responses that are crucial in the viral life cycle. Given the hierarchical nature of these cascade proteins, it is possible that combining inhibitors to block multiple kinases at the same time would result in synergistic antiviral effects. Drug therapy targeting host cell functions is particularly promising because viruses are much less likely to develop resistance to these drugs than current antiviral medications. It is our hope that the identification of these antiviral targets will ultimately lead to novel clinical treatments of chickenpox and shingles.

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Introduction

Varicella-zoster virus (VZV) is an alphaherpesvirus that causes both chicken pox (varicella) and shingles (herpes zoster). The virus is highly contagious and is spread primarily through aerosols and contact with infected skin lesions. Varicella is a common illness that is typically acquired during childhood. The Centers for Disease Control and Prevention (CDC) estimate that approximately 3.5 million new cases of chicken pox develop each year. Varicella has an incubation period of 10-21 days and presents with systemic, itchy skin lesions and fever and, although the disease is generally mild, can cause severe complications or even death, particularly in people who are older or immunocompromised (Balfour, 1988; De La Blanchardiere *et al.*, 2000). Varicella also poses a threat to pregnant women, developing fetuses, and newborns, since perinatal transmission may cause severe neurological defects and organ damage (Gnann, 2002).

The virus establishes latency in dorsal root ganglia following initial varicella infection and can reactivate spontaneously years or decades later as zoster. Anyone with prior exposure to live virus is at risk for developing shingles. This is of particular concern because the CDC estimates that 99.5% of American-born citizens over the age of 40 have had varicella. The mechanisms and causes of reactivation are incompletely understood but once VZV is acquired, the threat of a reemerging infection remains for life. Reactivation of dormant virus can be the result of physical or psychological stress, the loss of cell-mediated immunity caused by immunosuppression or senescence, as well as

a number of other genetic and demographic factors (Thomas & Hall, 2004). Zoster is characterized by a painful, vesicular rash in localized regions of the body, typically along a dermatome enervated by the ganglion where latency was established. Complications of zoster include facial paralysis, pneumonia, encephalitis, and vision loss (Gnann, 2002; Volpi, 2007), although such serious problems are rare. The most common complication associated with zoster, however, is postherpetic neuralgia (PHN), a debilitating condition characterized by pain in the affected region that can persist for months or years even after the resolution of infection and skin lesions (Tenser, 2001). The incidence of herpes zoster in the United States is rising, with total cases increasing across all age groups between 1993 and 2006 (Leung *et al.*, 2011; Yih *et al.*, 2005). The CDC approximates the incidence of zoster in the US at 4 cases per 1000 persons annually and 10 cases per 1000 persons over the age of 60, with an estimated 1 million total cases occurring each year (2014). The CDC now estimates that 1 in 3 people will develop shingles in their lifetime.

Vaccines against varicella and zoster have proven effective in reducing annual cases of VZV infection and associated complications but come with their own problems. The varicella vaccine, Varivax (Merck), has substantially reduced the incidence of varicella-associated mortality and morbidity in the US since its use began in 1995 (Davis *et al.,* 2004; Nguyen *et al.,* 2005). However, breakthrough cases occur in 15-20% of inoculated individuals (Schmid & Jumaan, 2010; Ampofo *et al.,* 2002). Furthermore, because Varivax is a live virus, it is not 100% effective at preventing establishment of latent virus and

protecting against reactivation to zoster (Hambleton *et al.,* 2008.) and live vaccines are not safe for immunocompromised patients. The varicella vaccine may also be contributing to the recently observed rise in cases of herpes zoster by lowering the extent of exogenous VZV exposure, which may act as a natural "booster shot" for previously infected individuals (Wagenpfeil *et al.,* 2004). Without this boost, cell-mediated immunity diminishes continuously with age and the risk of reactivation increases across the lifespan. Data implicating the role of the varicella vaccine in this theory, however, have thus far proven inconclusive (Reynolds *et al.,* 2008).

The zoster vaccine, Zostavax (Merck), is approximately 50% effective at preventing shingles and 90% effective against postherpetic neuralgia (Sanford & Keating, 2010), but the vaccine is only approved for people 50 and older. While that covers most individuals at the highest risk, a large portion of susceptible hosts are not eligible to receive the vaccine. In addition, Zostavax is expensive compared to other vaccines and is not covered by all insurance plans, further limiting access to it (Harvard Family Health Guide). The zoster vaccine, like the varicella vaccine, also uses a live, attenuated virus and is not appropriate for inoculation of immunocompromised individuals who are at highest risk for reactivation.

A number of antiviral medications are currently used for clinical treatment of varicella and zoster but like the vaccines, each presents their own challenges. The most commonly used drugs, acyclovir, famciclovir, and valaciclovir, are modified to their active forms by the viral enzyme thymidine

kinase. The active forms of these compounds are nucleoside analogues that incorporate into viral DNA and prevent replication. Acyclovir, while useful in treating VZV infection, is ten times less effective against VZV than the related herpes simplex viruses, and, because it is poorly absorbed, must be administered five times a day (Arvin, 1996). Famciclovir and valaciclovir are better absorbed than acyclovir but are not approved for use in treating zoster in immunocompromised patients (Arvin, 1996). Furthermore, treatment with these drugs must be started within 96 hours after appearance of skin lesions to decrease the severity of illness. These antivirals also do not prevent or remove latent virus and therefore offer no protection against viral reactivation (Arvin, 1996).

These drugs have the advantage of selectively targeting VZV-infected cells but also promote the emergence of drug resistance. The thymidine kinase gene is not essential for viral replication, so prolonged use of these nucleoside analogues can select for thymidine kinase-negative mutants that are unresponsive to acyclovir (Arvin, 1996). Such acyclovir-resistant strains have indeed been well documented in immunocompromised patients (Linnemann *et al.,* 1990; Léger *et al.,* 2001; Pahwa *et al.,* 1988). Acyclovir-resistant infections can be treated with Foscarnet, a drug that is highly toxic and can cause significant metabolic side effects (Coen & Schaffer, 2003), making it a less than desirable alternative.

The existence of these vaccines and antiviral medications, while important in minimizing the impact of varicella and zoster, has reduced the urgency for the development of safer and more effective drugs. The problems

associated with the virus-centered method of current treatment programs necessitate a new approach. To this end, a new paradigm has emerged in antiviral research-- the therapeutic targeting of host enzymes that are important mediators of viral replication. Viruses are, by nature, obligate intracellular parasites and depend on the action of many host signal transduction pathways to carry out processes like gene expression, replication, and packaging (Münter *et al.,* 2006). Blocking access to host proteins involved in cellular signaling pathways has the potential to disrupt normal virus-host interactions that are essential for the viral life cycle. This approach also has the advantage of presenting a more substantial barrier to the development of drug resistance than current treatments. Viruses rely on host signaling machinery because they lack genes for the proteins that carry out replication processes. It is unlikely that mutations in the VZV genome would give rise to resistance to these treatments because the virus is totally dependent on the activity of those specific proteins and has no way of subverting the signaling pathways when they are blocked.

Given the importance of these virus-host interactions, the primary goal of this project was to identify cellular signaling pathways involved in VZV infection. Previous studies have shown that VZV affects the activity of host protein kinases involved in phosphorylation cascades (Zapata *et al.,* 2007; Liu *et al.,* 2012; Liu & Cohen, 2013) that regulate transcription, cell survival, and cell cycle progression among other processes. With this information in mind, we hypothesized that we could elucidate specific pathways and mediators VZV uses for replication by evaluating a kinase inhibitor panel in cultured human skin

cells. Similar approaches have been used in other areas of infectious disease research and are now accepted methods for studying pathogen-host pathway interactions (Hussain *et al.,* 2010; Goswami *et al.,* 2012).

The goal of this project was to identify compounds that would be well tolerated by host cells but also effective at preventing viral replication. Such compounds would point to a cellular target whose activity was more important to VZV than the host. This difference in selectivity could be exploited in the clinical treatment of varicella and zoster.

To perform the study, we purchased a kinase inhibitor library containing 80 different compounds with well-defined molecular targets. The first objective was to demonstrate which compounds could be safely administered to the skin cells without causing cell death or inhibiting growth. The second objective was to test whether treatment with the kinase inhibitors resulted in a reduction of VZV infection. The final objective was to further assess the *in vitro* therapeutic efficacy of the most potent drugs identified in the second step by quantifying their selectivity for interrupting viral replication over host processes. Classifying the inhibitors in this way would allow us to discern the most important protein targets on which to focus future drug development efforts.

Materials and Methods

Cell and Virus Culture. Human foreskin fibroblasts (HFFs) (CCD-1137Sk; American Type Culture Collection, Manassas, VA) and MeWo cells, an immortalized human melanoma line, (a gift of Dr. Charles Grose, University of Iowa) were grown in Eagle minimum essential medium with Earle's balanced salts and L-glutamine (HyClone Laboratories, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Benchmark FBS; Gemini Bio Products, West Sacramento, CA), amphotericin-B (250µg/mL), penicillin-streptomycin (5000 IU/mL), and non-essential amino acid solution (all Mediatech, Manassas, VA) at 37°C under 5% CO_2. HFFs, a primary cell line, were grown for up to 20 passages. VZV-BAC-Luc (Zhang *et al.*, 2007) was derived from the Parental Oka strain, a wild type clinical isolate from Japan (Accession number: AB097933). Dr. Hua Zhu (University of Medicine and Dentistry of New Jersey) provided a master stock of VZV-BAC-Luc (passage 10). VZV-BAC-Luc was stored at -80°C and grown on HFFs for up to 10 passages.

Drug Handling and Preparation. A panel of 80 kinase inhibitors, the Screen-Well® Kinase Inhibitor Library (Product Number: BML-2832-0100), was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). Drugs were supplied as 100 µL of 10 mM stock dissolved in DMSO in deep, 96-well plates. We prepared 30 µL of 2 mM (MTT and Dose Response) and 10 mM (Neutral Red) replica plate aliquots for indicated assays. The library and aliquots were

stored at -80°C and protected from light until use. Drugs in all experiments were thawed at room temperature before dilution to final concentration in complete tissue culture medium (TCM) at 37°C.

Neutral Red (NR) Cytotoxicity Assay. The NR assay was performed in clear, 96-well plates with $5x10^{3}$ HFF cells/well. The cells were cultured for 24 h until reaching confluence, and then the medium was removed and replaced with 200 µL of medium containing the test drug. Drugs were evaluated at 12.5, 25, 50, and 100 µM. Four drugs were tested on each plate with inhibitors tested in triplicate and controls in replicates of six. The outside wells were filled with 200 µL of TCM during cell growth periods but received no cells or treatment (Figure 1). Following addition of drug, each plate was covered with an adhesive plastic sheet and incubated for 48 h. The drugs were removed before the NR stain solution was added [93% TCM, 1% non-essential amino acids, 5% FBS, 1% Neutral Red (Sigma-Aldrich, St. Louis, MO) dissolved in Dulbecco's Phosphate Buffered Saline with Ca^{2+} and Mg^{2+} (Mediatech Inc., Manassas, VA), filtered to clarify]. Neutral Red is absorbed by pinocytosis and accumulates in lysosomes; the amount of dye absorbed by the culture is proportional to the number of living cells. 250 µL of NR stain solution was added to each well and incubated for 2 h. Neutral Red solution was removed and cells were fixed with 250 µL of 0.5% formaldehyde (Fisher Scientific, Fair Lawn, NJ) in DPBS for 10 min at room temperature. The fixative solution was removed and replaced with 100 µL of desorb solution [50% ethanol (Ultra Pure LLC, Darien, CT), 1% glacial acetic

acid (Fisher Scientific, Fair Lawn, NJ) in distilled water]. Plates were then wrapped in aluminum foil and gently agitated on an orbital shaker for 25 min at room temperature. Following desorption of the stain, the amount of NR present in each well was measured by a spectrophotometer with absorbance at 540 nm. All absorbance measurements were compared to a diluent (DMSO) negative control that showed no toxic effects and to a positive control, staurosporine, which kills cells by apoptosis. Absorbance values substantially lower than the diluent were considered indicative of cytotoxicity.

	Stauro	Drug 1 100uM	Drug 1 50uM	Drug 1 25uM	Drug 1 12.5uM	Drug 2 100uM	Drug 2 50uM	Drug 2 25uM	Drug 2 12.5uM	Diluent	
	Stauro	Drug 1 100uM	Drug 1 50uM	Drug 1 25uM	Drug 1 12.5uM	Drug 2 100uM	Drug 2 50uM	Drug 2 25uM	Drug 2 12.5uM	Diluent	
	Stauro	Drug 1 100uM	Drug 1 50uM	Drug 1 25uM	Drug 1 12.5uM	Drug 2 100uM	Drug 2 50uM	Drug 2 25uM	Drug 2 12.5uM	Diluent	
	Stauro	Drug 3 100uM	Drug 3 50uM	Drug 3 25uM	Drug 3 12.5uM	Drug 4 100uM	Drug 4 50uM	Drug 4 25uM	Drug 4 12.5uM	Diluent	
	Stauro	Drug 3 100uM	Drug 3 50uM	Drug 3 25uM	Drug 3 12.5uM	Drug 4 100uM	Drug 4 50uM	Drug 4 25uM	Drug 4 12.5uM	Diluent	
	Stauro	Drug 3 100uM	Drug 3 50uM	Drug 3 25uM	Drug 3 12.5uM	Drug 4 100uM	Drug 4 50uM	Drug 4 25uM	Drug 4 12.5uM	Diluent	

Figure 1. Plate layout of Neutral Red Assay. Stauro wells indicate staurosporine-treated positive controls. Diluent wells indicate DMSO-treated negative controls.

MTT Cellular Proliferation Assay. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cellular Proliferation Assay is a rapid screen to assess the effect of all 80 inhibitors on cell growth and division. The assay was performed in clear, 96-well plates on sub-confluent HFFs (1.67×10^3 cells/well) that were cultured for 24 h. A Time 0 replicate plate for

each experiment was prepared for an initial measurement of cell number before drug was added. Each data point of the Time 0 plate was the average of 6 replicate wells. Following initial incubation, seeding media was removed and replaced with 200 μL of drug solution. Drugs were tested at 1.5, 3, 6, and 12 μM in triplicate wells. The outside wells were filled with 200 μL of TCM during cell growth periods but received no cells or treatment (Fig. 2). Following addition of drug, each plate was covered with an adhesive plastic sheet and incubated for 48 h. Twenty μL of 5 mg/mL MTT dissolved in PBS was added on top of existing drug solution and incubated for 3.5 h. MTT is a yellow tetrazolium salt that is absorbed and reduced to a purple formazan in metabolically active, dividing cells by dehydrogenase and reductase enzymes. Solutions were then removed and replaced with 150 μL of DMSO, a solvent of MTT. Plates were wrapped in aluminum foil and gently agitated on an orbital shaker for 15 min at room temperature. Once solubilized by the addition of DMSO, the amount of formazan was measured by a spectrophotometer. Absorbance was measured at 590 nm. The same quantification procedure was followed for 0 and 48 hour plates. Non-dividing cells do not reduce the solution and thus produce less formazan. Therefore, the absorbance is proportional to the amount of proliferative cells present. Absorbance measurements of treated wells were then compared to controls of 1 mM phosphonoformate (PFA), a compound that does not inhibit cell division, and blank wells receiving only TCM and MTT solution and ultimately assessed against absorbance measured in the Time 0 samples.

	Blank w/ MTT	Drug 1 12uM	Drug 1 6uM	Drug 1 3uM	Drug 1 1.5uM	Drug 2 12uM	Drug 2 6uM	Drug 2 3uM	Drug 2 1.5uM	PFA	
	Blank w/ MTT	Drug 1 12uM	Drug 1 6uM	Drug 1 3uM	Drug 1 1.5uM	Drug 2 12uM	Drug 2 6uM	Drug 2 3uM	Drug 2 1.5uM	PFA	
	Blank w/ MTT	Drug 1 12uM	Drug 1 6uM	Drug 1 3uM	Drug 1 1.5uM	Drug 2 12uM	Drug 2 6uM	Drug 2 3uM	Drug 2 1.5uM	PFA	
	Blank w/ MTT	Drug 3 12uM	Drug 3 6uM	Drug 3 3uM	Drug 3 1.5uM	Drug 4 12uM	Drug 4 6uM	Drug 4 3uM	Drug 4 1.5uM	PFA	
	Blank w/ MTT	Drug 3 12uM	Drug 3 6uM	Drug 3 3uM	Drug 3 1.5uM	Drug 4 12uM	Drug 4 6uM	Drug 4 3uM	Drug 4 1.5uM	PFA	
	Blank w/ MTT	Drug 3 12uM	Drug 3 6uM	Drug 3 3uM	Drug 3 1.5uM	Drug 4 12uM	Drug 4 6uM	Drug 4 3uM	Drug 4 1.5uM	PFA	

Figure 2. Plate layout of MTT Assay. Wells marked as Blank contained no cells and were treated only with MTT as a negative control. PFA wells received phosphonoformate as a positive control.

Dose-Response Assay. The Dose-Response Assay was performed in black, 96-well plates with $2.5x10^3$ HFF cells/well infected with VZV-ORF9-Luc as a rapid screening process to assess antiviral efficacy of non-toxic and non-inhibitory drugs. The strain of virus used in this assay encodes the gene for firefly luciferase, an enzyme that cleaves the D-luciferin substrate-- a reaction that releases photons that can be measured with bioluminescence imaging. Upon the addition of D-luciferin, cells that express the luciferase enzyme emit photons in proportion to the amount of VZV infection. Photon emission measurements were used to calculate VZV yield. Cells were plated with TCM and grown overnight for 24 h. TCM was then removed and cells were infected with 100 µL of TCM containing sonicated, cell-free VZV-ORF9-Luc showing more than 80% cytopathic effect (CPE) at a 1:25 ratio of infected to uninfected cells and adsorbed for two hours at 37°C. 100 µL of drug solutions were added on top of viral media for final concentrations of 1.5, 3, 6, and 12 µM in triplicate. Controls

of DMSO (diluent) at the same concentration as the highest drug dose and 1 mM PFA were used in replicates of six. Drug treatments were compared to the diluent control in order to calculate percent VZV yield, with the average of diluent-treated values taken as 100%. The outside wells were filled with 200 µL of TCM during cell growth periods but received no cells or treatment (Fig. 3). Following addition of drug, each plate was covered with an adhesive plastic sheet and incubated for 48 h. VZV yield was then determined by bioluminescence imaging using the IVIS-50® instrument (Caliper Life Sciences/Xenogen, Hopkinton, MA) and expressed as total flux (photons/sec/steradian/cm^2).

	PFA + VZV	Drug 1 12uM + VZV	Drug 1 6uM +VZV	Drug 1 3uM +VZV	Drug 1 1.5uM +VZV	Drug 2 12uM + VZV	Drug 2 6uM +VZV	Drug 2 3uM +VZV	Drug 2 1.5uM +VZV	Diluent + VZV	
	PFA + VZV	Drug 1 12uM + VZV	Drug 1 6uM +VZV	Drug 1 3uM +VZV	Drug 1 1.5uM +VZV	Drug 2 12uM + VZV	Drug 2 6uM +VZV	Drug 2 3uM +VZV	Drug 2 1.5uM +VZV	Diluent + VZV	
	PFA + VZV	Drug 1 12uM + VZV	Drug 1 6uM +VZV	Drug 1 3uM +VZV	Drug 1 1.5uM +VZV	Drug 2 12uM + VZV	Drug 2 6uM +VZV	Drug 2 3uM +VZV	Drug 2 1.5uM +VZV	Diluent + VZV	
	PFA + VZV	Drug 3 12uM + VZV	Drug 3 6uM +VZV	Drug 3 3uM +VZV	Drug 3 1.5uM +VZV	Drug 4 12uM + VZV	Drug 4 6uM +VZV	Drug 4 3uM +VZV	Drug 4 1.5uM +VZV	Diluent + VZV	
	PFA + VZV	Drug 3 12uM + VZV	Drug 3 6uM +VZV	Drug 3 3uM +VZV	Drug 3 1.5uM +VZV	Drug 4 12uM + VZV	Drug 4 6uM +VZV	Drug 4 3uM +VZV	Drug 4 1.5uM +VZV	Diluent + VZV	
	PFA + VZV	Drug 3 12uM + VZV	Drug 3 6uM +VZV	Drug 3 3uM +VZV	Drug 3 1.5uM +VZV	Drug 4 12uM + VZV	Drug 4 6uM +VZV	Drug 4 3uM +VZV	Drug 4 1.5uM +VZV	Diluent + VZV	

Figure 3. Plate layout of Dose Response Assay. All wells received media containing cell-free VZV. Wells with PFA received phosphonoformate as a positive control. Wells with Diluent indicate DMSO-treated negative controls.

EC_{50} Assay. The EC_{50} is the concentration of a drug that reduces viral yield by half. To determine the EC_{50}, it was necessary to lower the concentrations at which the compounds were surveyed in the Dose Response Assay, as many of them reduced VZV yield more than 50% at 1.5 µM. Drug concentrations used in

this assay were two-fold dilutions ranging from 0.094-24 μM (Fig. 4). The goal of this concentration expansion was to find a range of 0-100% viral yield that would allow the interpolation the EC_{50} from a resulting dose response curve. VZV yield from drug-treated wells was compared to the yield from untreated wells, with the average measurement from the untreated wells taken as 100% viral yield. The procedure of the assay was identical to that of the Dose Response assay with the exception that VZV-ORF9-Luc was used at a 1:20 ratio of infected to uninfected cells. Results were plotted as the percent virus yield versus the drug concentration on a Log_{10} scale, and then analyzed by non-linear regression to estimate the EC_{50} value. A plate containing DMSO with the same dilutions as the test plates accompanied each experiment as a control (there was no effect on virus yield).

	24uM + VZV	12uM + VZV	6uM + VZV	3uM + VZV	1.5uM + VZV	.75uM + VZV	.375uM + VZV	.1875uM + VZV	.09375uM + VZV	0 uM + VZV	
	24uM + VZV	12uM + VZV	6uM + VZV	3uM + VZV	1.5uM + VZV	.75uM + VZV	.375uM + VZV	.1875uM + VZV	.09375uM + VZV	0 uM + VZV	
	24uM + VZV	12uM + VZV	6uM + VZV	3uM + VZV	1.5uM + VZV	.75uM + VZV	.375uM + VZV	.1875uM + VZV	.09375uM + VZV	0 uM + VZV	
	24uM + VZV	12uM + VZV	6uM + VZV	3uM + VZV	1.5uM + VZV	.75uM + VZV	.375uM + VZV	.1875uM + VZV	.09375uM + VZV	0 uM + VZV	
	24uM + VZV	12uM + VZV	6uM + VZV	3uM + VZV	1.5uM + VZV	.75uM + VZV	.375uM + VZV	.1875uM + VZV	.09375uM + VZV	0 uM + VZV	
	24uM + VZV	12uM + VZV	6uM + VZV	3uM + VZV	1.5uM + VZV	.75uM + VZV	.375uM + VZV	.1875uM + VZV	.09375uM + VZV	0 uM + VZV	

Figure 4. Plate layout of EC_{50} Assay. All wells received media containing cell-free VZV. This layout was also used for the DMSO control plates.

Statistical Analysis. Statistical analyses were performed using Microsoft Excel 2011 and GraphPad Prism 5.02 for Windows and 5.0b for Macintosh (GraphPad

Software, San Diego, CA, www.graphpad.com). Data points represent mean values and all error bars represent the standard deviation. EC_{50} concentrations were determined by interpolation of non-linear regression curves and the Selectivity Index (SI) was calculated by dividing the cytotoxic concentration by the EC_{50} concentration. For drugs that were nontoxic at all levels of the NR Assay, 100 µM was applied as the cytotoxic concentration in calculating SI.

Results

As an obligate intracellular parasite, VZV relies on intimate interactions with its host cell. Limiting the viral life cycle by disrupting these relationships presents a unique set of challenges. Unlike the previously mentioned antiviral drugs that target VZV-specific processes, the compounds in this study target host proteins. Inhibition of host kinases has the potential to shut down crucial pathways and cause a number of deleterious alterations of the intracellular environment, even in uninfected cells. The primary objective in identifying cellular targets of VZV, therefore, was to determine which compounds could be safely administered within a range of concentrations that were both non-toxic and non-inhibitory to quiescent, uninfected cells. For this reason, the NR Cytotoxicity and MTT Cellular Proliferation assays were performed in human foreskin fibroblasts (HFFs), which are primary cultured cells that serve as a good host for VZV infection. The antiviral efficacy of nontoxic inhibitors was then assessed by the Dose Response Assay using VZV-ORF9-Luc infected HFFs. The most potent drugs were selected for further analysis by the EC_{50} Assay to determine their efficacy as inhibitors of viral replication and to calculate a Selectivity Index, an industry-standard quantification of the concentration range between which a compound is antiviral and adequately tolerated by the cell.

Neutral Red Cytotoxicty Assay. The NR Assay was performed in confluent HFFs to assess cytotoxicity of all 80 compounds. For values that were not judged to be cytotoxic but were less than the diluent, we suspect that some of the reduction in cell number was due to anti-proliferative effects of the drugs at high doses that prevented HFFs from growing and completely filling the wells. Furthermore, because the ultimate goal was to test as many compounds as possible for antiviral effects, we allowed some absorbance values considerably lower than the diluent value to pass the NR Assay. To qualify for further study, compounds had to be suitably nontoxic at 12.5 μM or higher. Compounds failing either this test or the MTT Assay were not used in the antiviral assays. Compounds that serve to illustrate the application of selection criteria of the NR Assay are shown (Fig. 5). Staurosporine was cytotoxic at all concentrations and thus was disqualified from antiviral assays. Wortmannin was toxic from 25-100 μM but was determined to be safe enough at 12.5 μM to warrant further study. Both AG-490 and Tyrphostin 9 passed at all concentrations. Most compounds in the library showed minimal cytotoxic effects and qualified for additional analysis (Table 1).

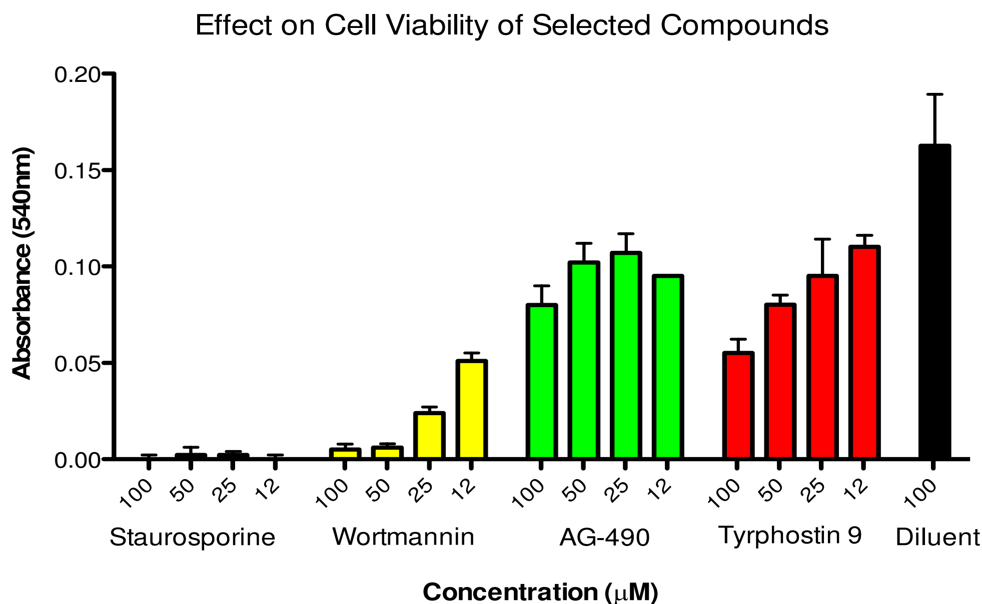


Figure 5. NR Assay for cytotoxicity. Absorbance of cells treated with indicated compounds at four concentrations (100, 50, 25, and 12.5 μM). Values are the average ± standard deviation of triplicate samples. Diluent represents numerical average of all control samples. Experimental measurements were compared to the diluent to determine degree of cell viability.

MTT Cellular Proliferation Assay. The MTT Assay was performed in sub-confluent HFFs in order to assess the level at which each drug inhibited cellular division. Cells were used at one-third the density of the NR Assay to give the cells space to divide. All 48-hour measurements were compared to an accompanying Time 0 plate to assess the degree of proliferation. Absorbance values below or equivalent to the Time 0 readings were interpreted as anti-proliferative. Compounds found to be anti-proliferative at 1.5 μM were disqualified. Examples illustrating the process used in assessing drugs by the MTT Assay are shown (Fig 6). Wortmannin was cytostatic at all concentrations tested. Although it passed the NR Assay at 12.5 μM, these results disqualified Wortmannin from antiviral assays. AG-490 and Tyrphostin 9 passed at all

concentrations. Most drugs that passed the NR Assay were also found to be non-inhibitory (Table 1).

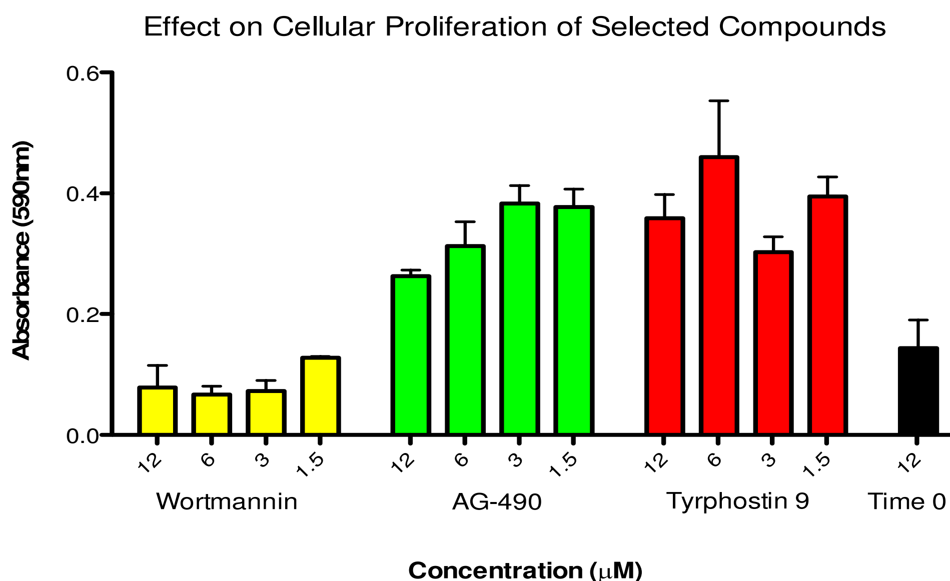


Figure 6. MTT Assay for cell proliferation. Absorbance of cells treated with indicated compounds at four concentrations (12, 3, 6, and 1.5 μM). Values are the average ± standard deviation of triplicate samples. Time 0 represents numerical average of all Time 0 absorbance values. Experimental measurements were compared to Time 0 to determine extent of cell proliferation.

Dose Response Assay. Ten inhibitors that were determined to be cytotoxic or cytostatic at low levels by the NR and MTT Assays, respectively, were removed from the panel and the remaining 70 drugs were evaluated for their antiviral potential. Drugs that reduced VZV yield to less than 20% at 12 μM were considered highly potent and were further analyzed by the EC_{50} Assay. Drugs that did not meet this cutoff were disqualified. Illustrative examples of antiviral analysis are shown (Fig 7). AG-490 reduced viral yield to only ~50% at 12 μM, well above the required 20% needed for further study. Tyrphostin 9 was a highly effective antiviral compound, nearly eliminating VZV infection at 12 μM. Indeed, the lowest concentration of Tryphostin 9 tested, 1.5 μM, reduced VZV

yield below 50%, indicating that the range of concentrations in this assay was too high for this potent compound. Most of the drugs displayed an antiviral effect, with seventeen compounds demonstrating considerable potency (Table 1).

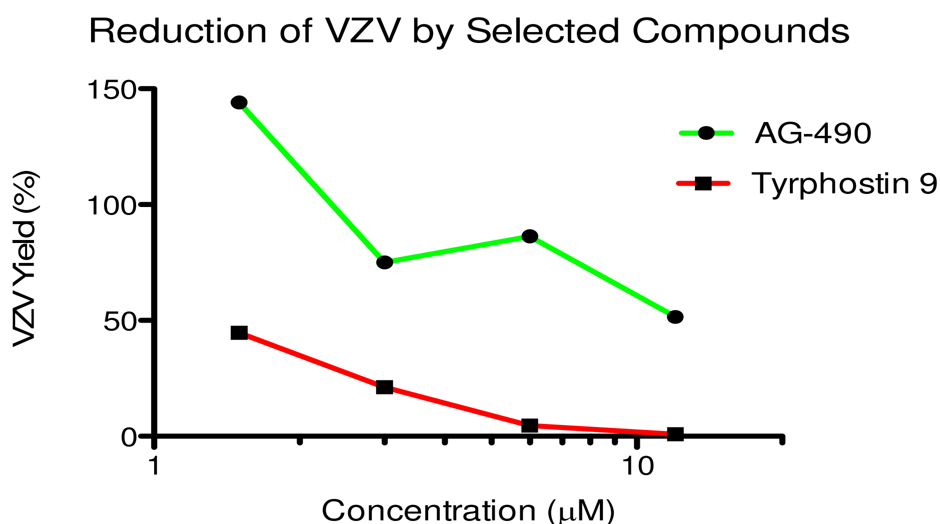


Figure 7. Percent VZV yield in cells treated with indicated compounds at four concentrations (1.5, 3, 6, and 12 μM). Photons/second of each compound at each concentration was divided by the average photons/second of diluent replicates on the plate on which it was tested to determine percent VZV yield

EC_{50} Assay. The EC_{50} Assay was performed on the 17 qualifying compounds to determine the concentration at which the inhibitors reduced viral yield by 50% for use in SI calculations. The results for Tyrphostin 25 were inconclusive and could not be fitted to a sigmoidal curve, so its EC_{50} and SI were not calculated. For both Tyrphostin AG1478 and PKC-412, the EC_{50} appeared to be outside the bottom range of the assay, but an approximate EC_{50} was still interpolated from the regression fit of each drug. An example of a dose response regression curve used to interpolate the EC_{50} is shown (Fig. 8). An EC_{50} was reliably interpolated

Table 1. Results of NR, MTT, and Dose Response Assays. All cytotoxicity and growth arrest numbers represent the lowest concentration, in µM, in which the respective effect was observed. Percent VZV yield represents data collected at 12 µM. * SP 600125 was not removed because it was previously shown to have antiviral effects in VZV (Zapata *et al*., 2007).

Compound Name	Cytotoxicity	Growth Arrest	% VZV Yield
Tyrphostin 9	>100	>12	0.9
RG-1462	25	>12	2.0
H-89 • 2HCl	50	12	2.6
GF 109203X	50	>12	2.8
Roscovitine	50	>12	5.3
PKC-412	100	6	5.3
Tyrphostin AG1478	100	12	6.0
Tyrphostin 25	25	>12	7.0
Terreic Acid	25	12	7.6
Indirubin-3'-monooxime	50	12	8.5
KN-93	25	>12	8.9
Kenpaullone	100	3	11.6
N9-isopropyl-olomoucine	50	>12	12.7
KN-62	25	>12	12.8
AG-879	100	>12	14.0
Triciribine	>100	12	16.9
Tyrphostin 23	>100	>12	18.0
U-0126	>100	>12	21.0
AG-494	>100	12	21.0
Lavendustin A	100	>12	21.0
BML-259	50	12	21.0
SP 600125*	12.5	6	21.7
Tyrphostin 47	>100	>12	23.0
PP1	100	>12	23.1
H-7 • 2HCl	50	>12	24.0
ML-9 • HCl	50	3	25.3
HA-1077 • 2HCl	>100	12	26.0
ML-7 • HCl	50	>12	26.4
GW 5074	>100	>12	27.1
SB-202190	100	12	29.2
Tyrphostin 51	100	>12	31.0
LY 294002	>100	12	31.0
Tyrphostin AG1288	>100	6	31.0
5,6-dichloro-1-β-D-ribofuranosylbenzimidazole	100	6	31.0
SB-203580	100	>12	32.0
Olomoucine	>100	>12	32.0
H-9 • HCl	50	>12	33.0
ZM 336372	100	6	34.6
SU 4312	100	12	37.9
Tyrphostin 1	>100	>12	39.0

Compound Name	Cytotoxicity	Growth Arrest	% VZV Yield
HA-1004 • 2HCl	>100	12	41.3
PD-98059	>100	>12	42.0
Y-27632 • 2HCl	50	12	42.6
LFM-A13	>100	12	42.7
Piceatannol	50	6	42.9
2,2',3,3',4,4'-Hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether	25	3	43.0
Indirubin	>100	3	43.3
ZM 449829	100	>12	43.5
2-Aminopurine	>100	>12	44.0
AG-126	>100	>12	48.0
AG-1296	50	>12	49.0
Apigenin	100	12	50.0
SC-514	>100	12	50.4
Daidzein	>100	3	51.0
2-Hydroxy-5-(2,5-dihydroxybenzylamino) benzoic acid	100	12	51.0
SU 1498	25	>12	51.9
AG-490	>100	>12	52.0
Genistein	>100	3	53.0
H-8	100	>12	53.7
Tyrphostin 46	>100	>12	54.0
Palmitoyl-DL-carnitine	100	>12	54.5
Rapamycin	25	12	54.5
Quercetin • $2H_2O$	>100	12	59.3
Iso-olomoucine	>100	12	59.8
Tyrphostin AG1295	>100	12	62.0
BML-257	>100	12	64.0
AG-825	100	>12	65.0
Hypericin	50	12	65.0
Hydroxy-2-naphthalenylmethylphosphonic acid	>100	6	72.9
AG-370	>100	>12	112.0
Wortmannin	25	<1.5	--
D-erythro-Sphingosine	12.5	6	--
PP2	12.5	6	--
Rottlerin	12.5	6	--
BAY 11-7082	12.5	6	--
BML-265	12.5	6	--
5-Iodotubericidin	12.5	3	--
Erbstatin analog	12.5	3	--
Staurosporine	12.5	<1.5	--
Ro 31-8220 mesylate	12.5	1.5	--

from most regression curves (Table 2).

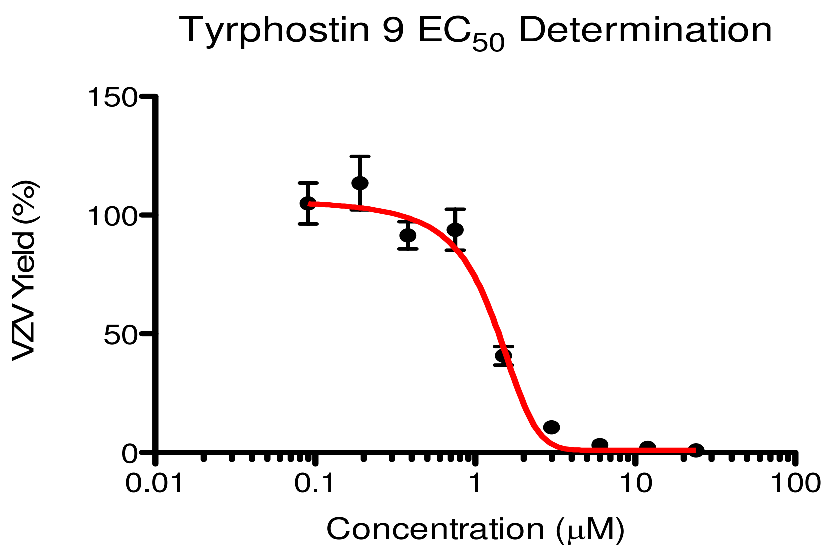


Figure 8. Non-linear regression of Tyrphostin 9 (R^2 = .9144). Concentrations are in two-fold dilutions from 0.09375-24 μM. EC_{50} = 1.3 μM, as determined by interpolation by GraphPad software.

Selectivity Indices. The Selectivity Index (SI) is a comprehensive ranking of the safety and efficacy of a drug. An SI value of 10 or greater was considered the cutoff for determining the best drugs. Of the sixteen compounds for which an EC_{50} concentration was determined, ten had an SI greater than 10 and are candidates for future study (Table 2). These ten highly effective compounds implicate a number of important pathways and enzymes necessary for VZV replication. Amongst them are three growth factor receptor kinase inhibitors (Tyrphostin AG1478, Tyrphostin 9, and AG-879) and a number of their downstream effectors, including two protein kinase C inhibitors (PKC-412 and GF 109203X), two glycogen synthase kinase 3 beta (GSK-3β) inhibitors (Kenpaullone and Indirubin-3'-monooxime), a calmodulin-dependent protein

kinase (CaMKII) inhibitor (KN-62), a cyclin-dependent kinase (CDK) inhibitor (Roscovitine), and a protein kinase A inhibitor (H89 • 2HCl).

Table 2. Results of EC_{50} Assays and SI values. Applied cytotoxicity represents the value used in calculating SI. Regression curves could not be reliably fit for drugs marked * so their SI values may actually be larger than indicated. Drugs marked ** were nontoxic at 100 µM so their SI values may actually be larger than shown.

Drug	Applied Cytotoxicity (µM)	EC_{50} (µM)	SI
Tyrphostin AG1478*	100	0.22	454.4
PKC-412*	100	0.45	222.2
Tyrphostin 9**	100	1.3	76.9
Kenpaullone	100	2.9	34.5
KN-62	25	0.86	29.1
GF 109203X	50	1.9	26.3
Indirubin-3'-monooxime	50	2.9	17.2
Roscovitine	50	2.9	17.2
H-89 • 2HCl	50	3.1	16.1
AG-879	100	9.7	10.3
N9-isopropyl-olomoucine	50	5.6	8.9
Terreic Acid	25	3.1	8.1
Triciribine**	100	13.0	7.7
Tyrphostin 23**	100	13.2	7.6
KN-93	25	4.3	5.8
RG-1462	25	4.6	5.4

Discussion

In this study we identified several important cellular kinases utilized by VZV during viral replication by inhibiting their activity with specific compounds. Understanding the roles of the identified host proteins in the viral life cycle will expand our knowledge of VZV infection and has the potential to lead to novel approaches to antiviral therapy.

The results of the Neutral Red Assay identified a number of inhibitors that were too toxic in non-infected cells to warrant further study. The drugs used in this study were capable of causing deleterious consequences in host tissue — they directly targeted mediators of cell survival, growth, differentiation, division, and gene expression pathways that are crucial elements of living systems. Some of the drugs may prevent virus replication but at the cost of severely inhibiting host cellular processes or cell death. This would, of course, limit the potential value of such a drug in treating a non-malignant illness like varicella or zoster. The concentrations used in these experiments (0-100 μM) were much higher than would be administered in a clinical setting, where it is difficult to achieve a concentration greater than 10 μM *in vivo*. Compounds were required to be non-toxic at 12.5 μM because this concentration was closest to the real-world value. We were confident that host cells would tolerate compounds that were non-toxic at 12.5 μM or higher. This proved to be a reasonable cutoff, as it left us with 71 of the original 80 compounds that were safe for use in cultured cells. The eliminated compounds were simply too harsh and complications associated with their use could likely outweigh any potential antiviral activity.

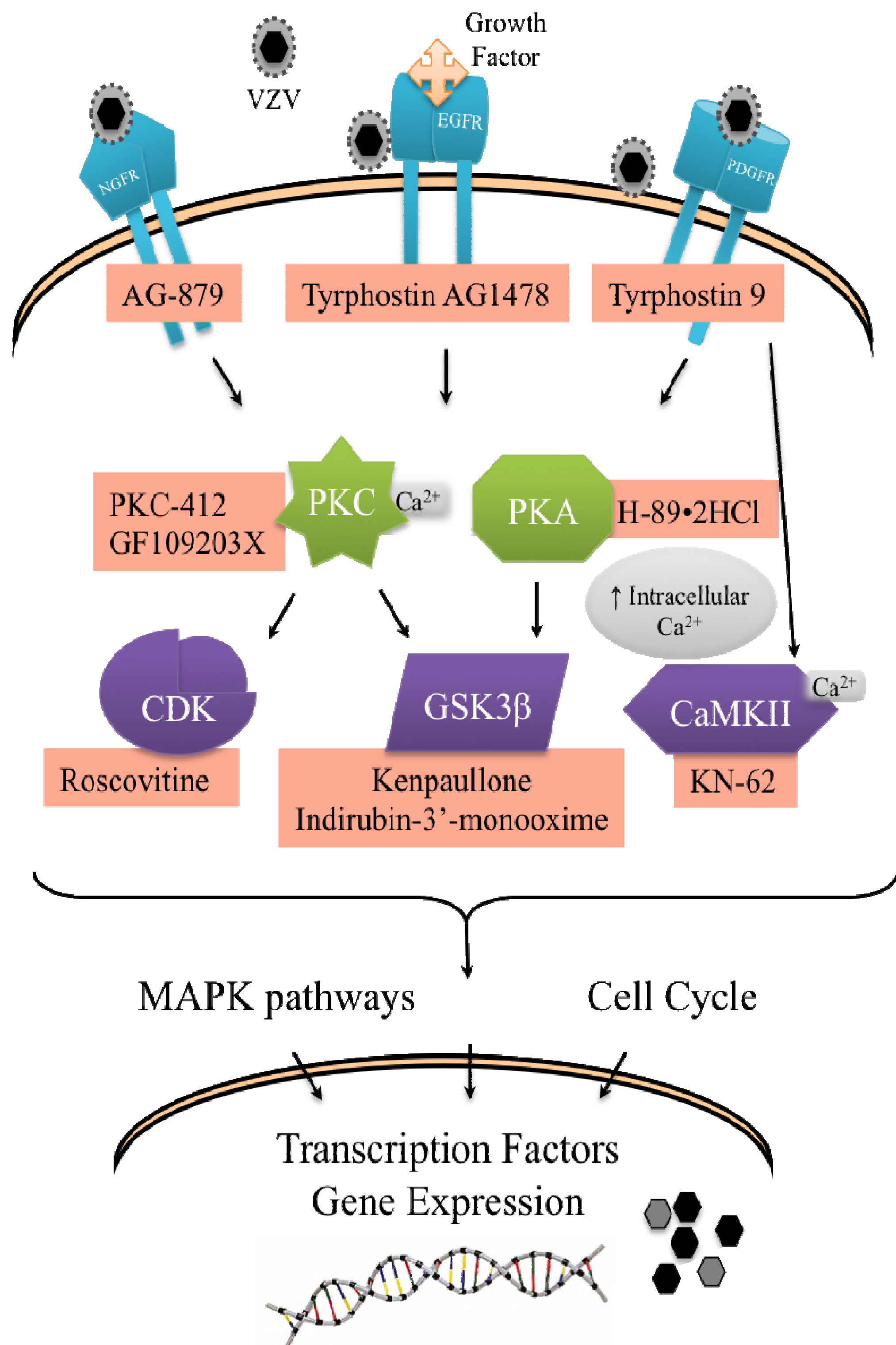
Results of the cellular proliferation assay indicated that the majority of compounds tested did not severely inhibit cell division. A non-toxic compound could still limit the rate of important metabolic processes crucial for normal cell activity without causing cell death. Like cytotoxic compounds, inhibitors that cause such problems would not be useful in the clinical treatment of VZV. The MTT Assay was performed to measure the effect of the inhibitors on cell growth and division. The cutoff used for these experiments was 1.5 μM but, for a number of reasons, it was not as important or strictly applied as the toxicity cutoff. For one, these compounds are designed to be anti-proliferative and, indeed, some of them are currently being studied as anti-cancer therapies (Giamas *et al.,* 2010; Hidalgo & Rowinsky, 2000; Y. Zhang *et al.,* 2008). Furthermore, most cells *in vivo* are not dividing, and, important for our purposes, VZV grows well in quiescent cells. The cutoff for the MTT Assay may have been a bit too conservative, as it eliminated only one additional compound (Wortmannin) from the study. The majority of the drugs used, however, including those that would prove most effective at blocking VZV replication, did not inhibit cell proliferation at the highest concentrations tested.

Assessment of the antiviral capacity of the remaining compounds distinguished the ten most effective inhibitors of VZV replication. Potency of compounds that were not eliminated by the Neutral Red or MTT Assays was first assessed by the Dose Response Assay, where a cutoff of twenty-percent viral yield at 12 μM was used to keep only the most efficacious inhibitors in the study for further analysis. The 12 μM cutoff was selected for its proximity to the

attainable *in vivo* concentration of 10 μM and the Neutral Red cutoff used earlier. Application of these criteria narrowed the list of compounds to the sixteen most effective drugs and ten compounds remained after EC_{50} concentrations and SI values were calculated. An SI value of 10 is the industry standard used in evaluating the potential of a compound for use *in vivo* and thus served as the cutoff in our study. The limits used in selecting this final group of compounds proved to be appropriate: it is unlikely that any highly effective drugs were eliminated too early. The rejected compounds simply lacked potency.

The compounds highlighted by this study affect targets that are critical mediators of the viral lifecycle. Identification of these targets as well as knowledge of their function has led to the development of a working model detailing their relationships and roles in VZV replication (Fig. 9). Of the ten most promising drugs identified in this study, three (Tyrphostin AG1478, Tyrphostin 9, and AG-879) are selective inhibitors of growth factor receptor tyrosine kinases (GFRTKs). This is a particularly exciting finding as the independent action of three inhibitors confirms the importance of these proteins as antiviral targets. GFRTKs are subdivided into epidermal growth factor receptor kinases (EGFRK), platelet-derived growth factor receptor kinases (PDGFRK), and neuronal growth factor receptor kinases (NGFRK) that are expressed in the fibroblasts used in these experiments and many other cell types. GFRTKs are important membrane-bound signal transducing proteins that initiate cell-signaling cascades that activate, among others, the Ras, MAPK, PI3K, Akt,

Figure 9. Antiviral Targets Required for VZV Replication



and JAK pathways. These pathways elicit a variety of responses that promote cell growth, proliferation, differentiation, survival, transcription factor production, and gene expression (Alberts *et al.,* p. 555-567). Given the multitude of activities initiated by these enzymes, GFRTKs have become a common target in the treatment of malignant and non-malignant disorders (Grimminger *et al.,* 2010). Activation of both EGFRK and PDGFRK signaling are required for human cytomegalovirus (HCMV) infection, a member of the herpes virus family (Evers *et al.,* 2004; Soroceanu *et al.,* 2008). Similarly, inhibition of GFRKs prevented viral pathogenesis during herpes simplex virus 1 (HSV-1) infection in mice (Sharma *et al.,* 2011). Moreover, AG-879 and Tyrphostin 9 exhibit antiviral activity against a wide variety of viruses including HSV-1 (Kumar *et al.,* 2011). It is no surprise then that the GFRTKs were implicated in this study as logical targets for the replication of VZV. It is possible that VZV interacts with these receptor kinases before or upon entry into the host cell to activate mechanisms necessary for expression of viral proteins.

Two of the final ten drugs are Protein Kinase C (PKC) inhibitors (PKC-412, GF 109203X). Like the GFRTKs, the implication of two separate drugs as high quality treatments validates the essential role of PKC in VZV replication. The importance of PKC in modulating the VZV life cycle was confirmed by Bontems *et al.* (2002) when they demonstrated that PKC phosphorylates the VZV protein IE63, which is a key regulator of viral gene expression previously shown to be essential for virus replication by Sommer *et al.* (2001). PKC is a central protein that is activated by a number of receptor signaling pathways,

including those initiated by the GFRTKs, which modulates a vast array of cellular processes. PKC functions vary by cell type, but common responses include cell growth, transcription regulation, and alteration of membrane structure (Newton, 1995). The activity of a multifunctional effector like PKC, in addition to its known interaction with viral proteins, makes it an important cellular target of VZV.

Two glycogen synthase kinase 3β (GSK-3β) inhibitors (Kenpaullone and Indirubin-3'-monooxime) were also potent antiviral compounds in the study. Like PKC, GSK-3β is another diverse signaling protein that regulates a variety of cellular functions. Most notably, GSK-3β is involved in metabolism, gene expression, neuronal development, and regulation of cell death by facilitating apoptotic pathways (Grimes & Jope, 2001). GSK-3β activity is regulated by epidermal growth factor (EGF) and the EGFRK pathway (Saito *et al.,* 1994). The nature of this regulation appears to be inhibitory, however, so its role in preventing VZV replication is unclear. Furthermore, GSK-3β is involved in both stimulatory and inhibitory crosstalk with a number of other signaling proteins like Protein Kinases A, B (Akt), and C (Grimes & Jope, 2001), making its role in VZV infection difficult to discern.

A Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) inhibitor, KN-62, was also found to have antiviral activity by this analysis. CaMKII requires activation by calcium ions. The relationship between VZV and intracellular calcium is poorly understood, but other viruses in the herpesviridae family are known to transiently increase the concentration of Ca^{2+} ions to induce calcium-

signaling pathways (Cheshenko *et al.,* 2003). CaMKII is another signaling hub that phosphorylates many downstream effector proteins and is thusly an important mediator in a number of pathways. CaMKII is expressed in all eukaryotic cells (Alberts *et al.,* 2010. p. 553) and, given its role in many signal transduction pathways, would be a logical target for VZV to hijack.

Roscovitine, a cyclin-dependent kinase (CDK) inhibitor, was also a drug of significant importance in our analysis. Roscovitine has previously been shown to disrupt VZV replication (S. Taylor *et al.,* 2004), further validating the findings of this study. Moreover, Leisenfelder *et al.* (2008) showed that a CDK protein co-localized with and phosphorylated a VZV tegument protein in HFFs, providing evidence of a direct interaction between VZV and host CDKs. CDKs regulate cell cycle progression and are targeted by herpes viruses to promote transcription and passage through the G_1 phase of the cell cycle to the S phase where viral DNA is replicated (Hardwick, 2000). This process is so important to viral replication, in fact, that many herpes viruses encode their own cyclin proteins to promote it (Mittnacht & Boshoff, 2000). It is not surprising then that inhibition of these effectors resulted in prevention of VZV replication.

One final drug implicated by the panel analysis was H89 • 2HCl, an inhibitor of Protein Kinase A (PKA), which is homologous to PKC and is an important mediator in the activation of cellular transcription factors, which are required for VZV mRNA synthesis (Rahaus & Wolff, 2003). A role of PKA in VZV infection was shown by Desloges *et al.* (2008) when they demonstrated that substrates of PKA are phosphorylated during VZV infection and that treatment

with an inhibitor of PKA dramatically reduced virus replication in fibroblasts. The effects of PKA activity vary depending on cell type, but it is certainly a crucial target for VZV.

Together, the results of this project have implicated specific host kinases as essential for VZV replication. Although the identification of these proteins is an important first step in the development of antiviral medication, a number of additional assays would provide critical evidence of the roles and effectiveness of kinase inhibitors as therapy for varicella and zoster. Five potential laboratory studies will be discussed here.

The first logical future study we would perform is the plaque assay, a more standard virology technique used to detect virus concentration in a sample. Plaque assays measure infectious particles such as virions, infected cells, or syncytia in a sample. These particles form countable plaques (cleared areas) on a monolayer of confluent cells. Each plaque represents an infectious particle present in the original sample. Such an assay could be performed with the top ten drugs identified in this study to validate our results.

Second, we would analyze the activation or inhibition of the cellular proteins we identified as antiviral targets. Cell kinases are typically regulated by phosphorylation on specific sites that can be detected by immunoblotting (Western blot) techniques (Rahaus *et al.,* 2005). To determine the effect of VZV on the identified proteins, the concentration of their phosphorylated forms would be compared in infected and uninfected cells as well as in the presence of their inhibitors.

Third, time of addition studies would be performed to help us further understand the antiviral mechanisms of potent compounds. Witvrouw *et al.* (1998) used this type of experiment to elucidate the mechanism of a drug that inhibits the replication of VZV and other viruses. In this assay, drugs are added to virus-infected cells at various times before and after infection. Virus replication is later quantified and the results may point to the phase of the virus lifecycle that is blocked by the compound. Some of the drugs identified in our study may have much lower EC_{50} values before, during, or after infection depending on the phase of infection they inhibit. Such a change in the EC_{50} concentration could make a compound even more effective than suggested by this study if administered under the right conditions. The results of treatment with GFRTK inhibitors would be of considerable interest in such an experiment because it is possible that prophylactic administration of these compounds could prevent virally mediated host-signaling and virus replication altogether. This would be important in the treatment of immunocompromised individuals who are at high risk for zoster. Results of time of addition studies would have a direct implication on how therapeutic compounds targeting these proteins would be used in a clinical setting.

Fourth, we would perform combination assays that could yield valuable information regarding synergistic or antagonistic interactions between the kinase inhibitors we identified and current antiviral medications. Such approaches have been used to demonstrate the additive antiviral effects of antiherpes drugs and interferon in treating VZV and HSV-1 (Baba *et al.,* 1984; J. Taylor *et al.,* 1998).

Combination therapy is common for many malignant and non-malignant diseases and is used most notably in the treatment of HIV and hepatitis C. In this case, the use of multiple kinase inhibitors may synergistically shut down a number of crucial pathways and have greater antiviral activity than the independent effect of each drug alone. Alternatively, because some of the targets identified in this study regulate the activity of others, it is possible that co-inhibition may cancel the effects of the individual inhibitors. These drugs can also be combined with current medications to block viral replication by two separate mechanisms, which could further suppress the development of drug resistance.

Finally, compounds that showed promise in cultured cells could be tested *in vivo* in a severe combined immunodeficient (SCID) mouse model. The SCID model is commonly used in the assessment of VZV pathogenesis (Arvin, 2006; Ku *et al.,* 2005; Zerboni *et al.,* 2010). In these assays, SCID mice are implanted with human skin and then VZV is inoculated directly into the tissue. The mice are treated with antiviral drugs and virus yield is measured by in vivo bioluminescence (Rowe, et al, 2010). Because the immune systems of SCID mice have been completely removed, any effect on viral replication is the result of treatment. This process is important in identifying how these drugs fit into a clinical setting and determining the proper route of administration (i.e., topically or systemically). Information from these studies would shed light on the processing of drugs in an *in vivo* tissue environment. This is a crucial step in assessing the potency and safety of drugs in a living system and serves as the bridge from bench to bedside.

Together, the results of this study have highlighted several important cellular targets of VZV and expanded our understanding of host-virus interactions that have the potential to lead to new treatments of VZV infection. Although the findings of this project provide further evidence of the merits of the host-centered paradigm, the medical community as a whole has thus far been resistant to accept this chemotherapeutic approach to treating varicella and zoster. We hope that the results of this project will strengthen the perception of these methods in combating these diseases and lead to continued efforts in the development of host-targeted antivirals.

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