DISCOVERING NOVEL POXVIRUS-SPECIFIC DRUG TARGETS

by

VALERIE ELISABETH CADET

(Under the Direction of Robert J. Hogan)

ABSTRACT

The goal of this work was to identify viral factors essential to orthopoxvirus (OPV) replication via a genome-wide library screen of individual short-interfering RNAs (siRNAs) targeting conserved OPV genes and to identify indispensable genes for OPV replication in mammalian cells. For the viral siRNA screen, 177 open reading frames (ORFs) associated with vaccinia virus (VACV)-WR strain, variola virus-China 48, and/or one of two strains of monkeypox virus were bioinformatically compared and the most conserved regions were analyzed. Four distinct siRNAs were designed for each of 138 ORFs, while for the remaining 39 ORFs, 1-3 siRNAs were designed with 94% targeted gene coverage. We have identified 26 siRNAs targeting 17 ORFs which, when depleted, inhibit cowpox (CWPV) virus replication in vitro. Upon further analysis, five siRNAs, each targeting a separate viral gene, showed superior inhibiting activity when administered prior to infection and all proved to be essential for both CWPV and VACV replication in vitro. Finally, experiments assessed in vivo efficacy of several targets in a murine model of poxvirus disease. Studies also address the deficiency in FDA-approved poxvirus drugs via utilization of a small molecule array comprised of 486 compounds. Nearly all compounds in the library have completed phases I-III trials and were selected

based upon purity, solubility, commercial availability, and safety. Following in vitro screening in Vero E6 cells, we were able to identify four compounds currently in use as chemotherapeutics and/or immunosuppressives which directly protect cells from both VACV and CWPV infections: methotrexate, idarubicin, homoharringtonine and raltitrexed. These four compounds were further examined in a dose-response manner and methotrexate was chosen for in vivo drug efficacy analysis. These results identify a candidate from which derivatives can be studied for potential administration to protect against human OPV infections and highlight several other potential drugs to be further explored. We have also extended the work of previously published studies using methotrexate to protect against VACV by providing means to counter the drug toxicity observed. Taken together, these results show the utility of genome-wide siRNA screening for studying viral replication factors, along with identification of potential therapeutic targets for anti-orthopoxvirus therapies.

INDEX WORDS: Orthopoxvirus; Vaccinia; Cowpox; Variola; Monkeypox; RNA interference; RNAi; siRNA; Short interfering RNA; Genome screen; Small molecule; High-throughput screening; Methotrexate; Raltitrexed; Homoharringtonine; Idarubicin; Folic acid; Folinic acid; Dihydrofolate reductase; Murine; In vivo

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DEDICATION

Special thanks is extended to my family and friends for all of the support and sacrifices that they have made for me to attain this PhD. Antonyio and Elena, my beautiful children who were just 5 and 7 at the start of this journey, Mommy is so proud of you and what YOU have accomplished during these stressful times. Nicol, you know that without you being a surrogate mama for me those first couple of years in the program, this wouldn't have been possible. To my siblings, parents and Mon Oncle Aly, I hope a simple thank you can convey the gratitude and love I have for each of you. From the bottom of my heart, I know I am extremely lucky for having you all in my corner. To AM, KJ, KFT, LB and TS: your encouragement and belief in me was always so timely and supportive, words cannot describe how grateful I am for your friendship.

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

OPV	Orthopoxvirus
VARV	Variola virus
VACV	Vaccinia virus
CWPV	Cowpox virus
MKPV	Monkeypox virus
CMPV	Camelpox virus
BFPV	Buffalopox virus
RNAi	RNA interference
siRNA	Short interfering RNA
MTX	Methotrexate
RTX	Raltitrexed
HHT	Homoharringtonine
IDA	Idarubicin
NCC	NIH Clinical Collection
DHFR	Dihydrofolate Reductase
FNA	Folinic acid
FA	Folic acid

CHAPTER 1

INTRODUCTION

Although smallpox disease was eradicated in 1980, poxvirus zoonoses have been well documented in recent years. In the midst of this, there is fear that smallpox (i.e. variola virus, VARV) will be released in an act of bioterrorism [1-3] while vaccinia virus (VACV), the live vaccine agent against smallpox, has been repeatedly shown to be transmissible through direct physical and indirect fomite contact [4-7]. Given that much of the general population in the U.S. has not been vaccinated nor possess immunity to poxviruses, coupled with the severity of complications that typically occur following VACV infection of immunocompromised persons, the need for continued research and development of therapies for poxviruses has been underscored. In addition to all of this, there presently is a significant lack of antivirals licensed to treat any of the myriad of poxvirus infections.

This study was designed with the aim of identifying novel orthopoxvirus (OPV)-specific drug targets. While there are numerous published reports of both small molecules inhibiting orthopoxviruses (OPVs) along with RNA interference (RNAi) being used to identify viral genes which when blocked inhibit viral replication, none have taken the comprehensive approach discussed here. This study examines the following specific aims:

Specific Aim 1. The overarching goal of this work was to identify viral factors which are essential to OPV replication. Work was initiated via a genome-wide library screen of individual short-interfering RNAs (siRNAs) targeting conserved OPV genes to identify those which are indispensable for replication in mammalian cells. For the viral siRNA screen, 177 open reading frames (ORFs) associated with VACV Western Reserve strain (WR), VARV-China 48, and/or one of two strains of monkeypox virus (MKPV) were bioinformatically compared and the most conserved regions within those genes were analyzed. Four distinct siRNAs each were designed for 138 ORFs, while for the remaining 39 ORFs, 1-3 siRNAs were designed. This cross-functional tool exhibited 94% coverage of the genomic ORFs, thereby providing an extremely global view of several OPVs causing zoonoses today.

We have identified 26 siRNAs targeting 17 ORFs which, when depleted, inhibit cowpox virus (CWPV) replication in vitro. Upon further analysis, five siRNAs, each targeting a separate viral gene, showed superior inhibiting activity when administered prior to infection and all proved to be essential for both CWPV and VACV replication in vitro. Finally, experiments aimed at assessing in vivo efficacy of several of the targets in a murine model of poxvirus disease were carried out. Taken together, these results show the utility of the genome-wide siRNA screen for studying viral multiplication factors, along with identification of potential therapeutic targets for anti-orthopoxvirus therapies.

Specific Aim 2. In addition to a global viral RNAi screen, studies were undertaken to directly address the deficiency in FDA-approved poxvirus drugs. A small molecule array comprised of 486 compounds was acquired [8]. By using a library composed of compounds which have almost all completed phases I-III trials, the results obtained had great potential to be expanded upon to directly address the lack of specifically approved drugs to combat OPVs. The compounds in the library were selected for inclusion based upon favorable attributes such as purity, solubility, commercial availability, and safety. This array, obtained from the NIH, was unique in itself in that it was the first time any of these chemicals were found in such a collection. We were able to identify four compounds which directly protect the cells from both VACV and CWPV infections. We have also extended the work of previously published studies using methotrexate to protect against VACV by providing means to counter the induced toxicity observed upon compound administration.

The library was screened in vitro for individual compound ability to inhibit both CWPV and VACV replication. From this screen, four drugs currently in use as chemotherapeutics and/or immunosuppressives were identified: methotrexate (MTX), idarubicin (IDA), homoharringtonine (HHT), and raltitrexed (RTX). The four compounds were further examined in a dose-response manner and methotrexate was chosen for in vivo drug efficacy analysis. These results identify a candidate from which derivatives can be studied for potential administration to protect against human orthopoxvirus infections. They also highlight several other potential compounds which can be further explored for in vivo protective efficacy in future studies.

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CHAPTER 2

LITERATURE REVIEW

Origin and History of Smallpox

Poxviruses have plagued humankind for thousands of years. Variola virus, the causative agent of smallpox disease, has made an impact throughout history with case-fatality rates of 5-40% among previously unvaccinated individuals [1, 2]. There are many isolates of VARV which fall into one of two Primary Clades. Less virulent Alastrim Minor isolates along with West African intermediate isolates grouped into Primary Clade II caused up to 13% case fatality rate [1, 3]. Smallpox is highly infectious and has no known animal host reservoir. In 1980, after killing millions of people worldwide, VARV became the first (and only) human disease ever eradicated. Yet despite this, poxviruses continue to affect individuals throughout the world.

The origin of poxviruses are unknown, however, the following theories exist: 1) Man acquired the virus from an animal host in which it was endemic and 2) Humans harbored an ancestral VARV that eventually evolved to become the virulent one that we know of as having caused smallpox disease [4].

When examining the first theory, we see that the closest OPV related to VARV is camelpox virus (CMPV), with the two forming a subgroup [5, 6]. CMPV is believed to have evolved in the Americas approximately 3 million years ago, directly parallel with the evolution of camelids. Currently, despite forming a subgroup with VARV, it is highly host-specific and an entirely distinctive virus [2], though the sequence homology doesn't

preclude the possibility of VARV being derived from it [4]. Other evidence indicates the emergence of an ancestral highly pathogenic VARV taking place in the 18th and 17th centuries BC in the Indus river valley. A hole in this theory, however, is that the size of the human population hovered at approximately 5 million in the region. This number may be insufficient for VARV to become endemic although the epidemic of disease led to the collapse of the Indus Civilization along with the disappearance of the new virus. Yet, the possibility remains that this VARV ancestor persisted in a zoonotic rodent reservoir [7].

Monkeypox virus (MKPV) is clinically similar, though less pathogenic than VARV, in humans [3, 8-10]. However, its' genome does not group closely with VARV upon phylogenetic analysis [5], therefore, it is not likely that VARV emerged from prior MKPV exposure and endemnicity. CWPV, on the other hand, has a wide host range and is maintained in the wild rodent population [11]. While CWPV is not phylogenetically similar to VARV, it has the largest of all the OPV genomes [5, 12], and thus lends the possibility that VARV has diverged because of deletion mutations in CWPV that have occurred over time.

The second theory hints at humans harboring an ancestral VARV that eventually evolved to become the virulent one that we know of as having caused smallpox disease[4]. Examination of the carrying capacity of the environment of our early ancestors allows for this possibility. If there was a "proto-variola" virus that persisted in small human groupings of hunter gatherers, for example, the environment thousands of years ago may have facilitated the evolutionary and physiological adaptations of that virus that allowed for VARV emergence [2]. Though studies of early human ancestors thus far indicate they did not harbor an early VARV, the possibility remains.

The first documented emergence of smallpox in human populations occurred thousands of years ago. History has long recorded plagues that are purported to be a result of the disease. For instance, in the 20th Dynasty of Egypt (~1160BC), Ramses V reigned for only five years. Studies of his mummified remains show clear evidence of scarring on the face, neck and chest likely caused by smallpox [2, 13]. A similar exam of a mummified body found in Naples, Italy, estimated to be that of a two -year-old boy, revealed a widespread vesiculopustular exanthema that upon microscopy, suggests a possible case of smallpox disease on the face and other parts of his exposed body. Radiocarbon dating estimates the child's death to have been in the middle of the 16th century [14].

In yet another example, the Antonine Plague, lasting from 165-180AD, was thought to have been caused by smallpox, essentially playing an integral role in the decline of the Roman Empire. The epidemic is estimated to have killed between 3.5 and 7 million people including Marcus Aurelius and was caused by Roman troops carrying infected prisoners from the Near East back to ancient Rome after war. In the 18th century, approximately 60 million Europeans were killed by VARV infection [15]. Notably, in Asia, Europe and Africa smallpox victims were typically children, who, if they survived the ordeal, acquired the lifelong immunity that allowed for a mostly protected adult population [16].

The situation in the Americas was dramatically different. Upon initial contact with Europeans, the Native Americans lacked prior exposure to VARV, thus, when it struck in any given village virtually the entire population was infected, with a rate of mortality reaching a frightening 30-40%. Beginning post-1492, Spanish colonization of

Central and South America was responsible for the decimation of the native inhabitants, with death quickly rising into the millions. Due to lack of prior contact with Europeans, the Native Americans had no preexisting immunity to the wide variety of diseases present on the European continent and their importation allowed disease to quickly spread into epidemics . By the eighteenth century, it is estimated that between 25- 50% of the indigenous population died after the European introduction of the VARV into North America [16]. Very few native groups, with the exception of those on the coast of northwest North America, were unscathed by smallpox disease. Interestingly, the virus was never able to establish the equilibrium necessary for it to become endemic. Instead, it took hold in periodic waves upon encountering people.

The first documented evidence of VARV's utilization as a weapon of biowarfare was during the French and Indian War (1754-1767) by British soldiers in North America. In a letter to Sir Jeffrey Amherst, commander-in-chief of British forces, Colonel Henry Bouquet suggested the grinding up of smallpox scabs into blankets that were to be distributed among the displaced Indian tribes [16]. An estimated 50% of Native Americans were killed following exposure to the blanketing due to intentional infection. Shortly afterwards, during the British occupation of Boston (1775-1776), smallpox flared up in the city in multiple waves. As the vast majority of British soldiers had survived smallpox exposure as children [16], they simply had to variolate[1] the susceptible minority. On the other hand, the majority of the Continental soldiers were immunologically naïve to the virus and efforts at variolation would place the lives of the soldiers at risk and leave the troops virtually defenseless during the treatment. As a result, inbound troops were quarantined in order to prevent possible spread of the virus to the

[1] See Poxvirus Therapeutic Strategies section below for description of variolation.

rest of the army. The British are believed to have exploited this fact to their benefit at times when the Continental Army's reserves were low due to infection. In an anecdotal summary of the mid-1800s, a southern doctor with the Confederate Army allegedly sold smallpox-contaminated clothing to the Union Army during the Civil War, in the hopes of spreading disease among the soldiers.

Orthopoxviruses Today

Thirty years have passed since smallpox was declared eradicated. However, poxviruses continue to plague both animals and man as a zoonotic pathogen. For example, there is a continual threat of MKPV in Central and Western Africa, along with the possibility in other parts of the world through importation. In addition, based on analyses of sera for poxvirus-specific antibodies and experimental testing, a wide variety of animals including rats, rabbits, prairie dogs, cattle, birds and squirrels may also be possible reservoirs of poxviruses in many parts of the world.

Monkeypox is an emerging disease in Africa caused by the MKPV and was introduced into the United States. Having emerged from under the smallpox umbrella, it was documented for the first time in the 1970s in Western and Central Africa with a case fatality rate ranging from as low as 1% (W. African strain) to as much as 17% (C. African strain). Cases have appeared in sporadic clusters in Western and Central Africa since then, but many have been found to be the result of varicella virus, which causes chickenpox disease. Nonetheless, by 2005 the disease had spread to southern Sudan, likely due to a carry-over of the small rodent animal reservoir from the west or perhaps brought by refugees returning to Sudan.

MKPV is perhaps the most important human orthopoxvirus today and evidence has shown the virus has evolved in Africa to be able to sustain multiple rounds of human transmission; not simply zoonotic transmission. In the summer of 2003, the US saw its first cases of the virus upon accidental transmission due to importation of MKPV-infected rodents from W. Africa. There were 71 human cases reported with approximately 1/4th of them having to be hospitalized. However, the imported strain of MKPV caused mild illness, and no one succumbed to disease. In addition, no human-human transmission was noted during this outbreak and there is no data to suggest MKPV has become endemic. [3, 17-20]

A promiscuous virus in terms of its broad host range, CWPV transmission has been repeatedly noted in recent years. Environmental factors have led to an increase in the natural reservoir hosts for the virus such as voles, which results in a higher risk of infection for cats, zoo and circus animals and subsequently for humans [21]. Human cases are mainly caused by direct contact with infected animals, most likely their domestic housecat or pet rat. One example of this is a case of generalized CWPV infection with necrotizing facial dermatitis in a cat that transmitted the virus to its human keeper [21]. Another example is a case of human CWPV acquired from a circus elephant in Germany that likely became infected from contact with a rat [22]. Human-human transmission is possible in this scenario, and up to three rounds have been noted.

Early 2003 saw a reemergence of buffalopox in India. The outbreak involved 4,000 domesticated buffalo out of herds totaling 10,000 animals. In addition to this, 125 human infections were recorded, primarily among animal herders and milkers. This incident led to an approximately 40% reduction in milk production as well as a wide

variety of clinical disease symptoms among both animals and humans [23]. Other cases of emergent buffalopox have also been noted [24, 25] leading to a significant decline in milk production and public health impact. In later years, an outbreak of BFPV in humans in a Pakistani hospital had nosocomial origins: buffalo fat contaminated with VACV was used in first aid supplies. [3]

VACV is used as the immunizing agent in the live smallpox vaccine preparation. Likely due to the manner of inoculation, accidental infections from recent vaccinee to close household contacts continue to occur [26-28]. Vaccination is through scarification and inoculation of the live attenuated virus into the upper arm. An active VACV infection then proceeds, taking approximately three weeks to completely heal. During this infectious period, accidental transmission from the site of infection to the eyes of the vaccinee has been noted. Furthermore in one instance, infectious material was introduced into a break in the skin of the two-year-old child of a vaccinee, leading to his hospitalization and treatment for eczema vaccinatum [27]. In yet another example, the girlfriend of a vaccinee presented with a genital infection due to spread of the active infection site to her body [29]. VACV infections of cattle have also been documented in recent years, having potential widespread agricultural and public health implications [28].

Smallpox has reemerged as a potential bioterrorist threat in this post-September 2001 world. The threat of using VARV as a biological weapon has prompted efforts of some governments to produce smallpox vaccines for emergency preparedness. An estimate based on U.S. government census data revealed that approximately 40% of the current U.S. population has no immunity to poxviruses, due to the termination of the smallpox vaccination program in 1972. This fact, coupled with smallpox having the

potential to be used as a biowarfare agent has prompted additional studies into the viral biology and anti-viral therapeutics available. Recombinant poxviruses that bypass pre-existing immunity [30-32] also highlight the need to be prepared to combat poxvirus infections in general.

An additional aspect for the interest in OPVs is its potential use as a platform technology for vector vaccines, requiring a high safety level for the virus being used as the vector. Several tools currently utilized to address the function and expression of poxvirus genes include the use of microarray analysis [33-37], deletion- mutant viruses [38-43], temperature-sensitive mutant viruses [44-49] and RNAi [17, 50, 51]. Poxviruses including fowlpox, canarypox and vaccinia have each been used extensively in live vaccine preparations for both human and veterinary consequence [47, 52-56].

There has been a variety of cells, diseases and pathogens targeted by these pox vectors over the years. Oncolytic therapies targeted against liver tumors and metastases [57-60], hypoxic tumor cells [61], prostate-specific antigen [62-64], antiangiogenesis in renal cell cancers [65], pancreatic adenocarcinomas [61], and several other cancer and tumor states [66]. There have been advances utilizing modified VACV Ankara vectors as vaccines against malaria [67], tuberculosis [68] and HIV [53, 69]. In terms of veterinary vaccines, most notably, a successful campaign to minimize rabies in wild animals was achieved with a recombinant VACV expressing the rabies glycoprotein [70] among others.

Clinical Poxvirus Disease

Smallpox, caused by VARV, is extremely contagious, very serious and sometimes fatal. On average, 30% of infected individuals succumbed to the disease with mortality being highest among infants and children [2, 11]. The viral disease is generally transmitted via direct and prolonged face-to-face contact although it can also be spread through direct contact with infected bodily fluids or contaminated objects such as bedding or clothing. Though rare, smallpox has been spread by virus carried in the air in enclosed settings such as hospitals, buses, and trains. Unlike the bulk of OPVs, VARV is not known to be transmitted through zoonotic means and humans are the only natural host. To date, there is not a specific treatment for smallpox disease and the only effective preventative is vaccination.

Upon infection, a person with smallpox is not contagious until the onset of fever, and the risk of transmission increases even more at the onset of rash. At this stage, the infected person is typically quite ill and usually bed bound. Contagiousness lasts until the last pox scab falls off; with the scab itself deemed able to transmit the virus.

The initial viral incubation period averages 12-14 days but can range from 7–17 days. Typically, there are no symptoms, the infected person may feel fine and he/she is not contagious. Following this period, the prodrome phase arises and may last for 2 - 4 days. Symptoms experienced at this point of smallpox disease include fever, general malaise and occasional vomiting. Fever usually ranges between 101-104° F, at which point, the person is typically unable to carry on with normal activities.

The ensuing rash first emerges as small red spots, usually in the mouth and on the tongue. They develop into sores that break open and spread large amounts of virus into

the mouth and throat. At this time, the person becomes contagious. Around the time in which mouth sores burst, the skin rash begins to appear, quickly spreading from the face to the arms, legs, hands and feet. Within 24 hours of first appearance, the rash spreads to the rest of the body. Concurrent with rash appearance, the fever drops and body aches and pains typically regress. The rash transforms into raised bumps by the third day post onset. By the fourth day, these bumps fill with a thick, opaque fluid usually with a depression in the center, lending the appearance of a bellybutton, a major distinguishing characteristic of smallpox disease. Unfortunately, at this time fever often will again spike and remain high until scabs form. These first 4 days of rash is the most contagious period during the disease course.

The next stage in the infection is the pustular rash which arises approximately 5 days post initial rash onset. At this time, the bumps become pustules, sharply raised, usually round and firm to the touch, as if a small object is present under the skin. The pustules begin to form a crust and then scab. By the end of the second week after the rash appears, most of the sores have scabbed over. Resolving scabs begin to fall off leaving marks on the skin that eventually become pitted scars. Most scabs will have fallen off three weeks after the rash appears. The person is contagious to others until all of the scabs have fallen off [2, 11].

Contrary to the severity of smallpox disease, infection with VACV typically causes a localized rather than systemic disease which does not present for as long of a clinical duration in healthy individuals. It is a vesicular and exanthemous disease developed after direct contact with an infected lesion. The localized lesions typically start as itchy spots on the part of the body where transmission occurred, closely followed by

the appearance of local edema. After about 3 days, general malaise will advance and 3 or 4 days after the fever and headache have set in, the vesicles will become ulcerated lesions characterized as umbilicated pustules.

CWPV infections are characterized by localized pustular skin lesions, typically on the hands. Disease can spread to other parts of the body based upon the integrity of the skin, i.e. scratches and abrasions will allow entry of the virus. Patients have presented with eye, neck, face, arms, hands, feet and other localized spots. Similar to VACV infection, disease usually is not systemic in healthy individuals.

Poxvirus Therapeutic Strategies

Historically, once VARV infected a person, its host either died or gained lifelong immunity. For the virus to survive, it needed a constant pool of new victims, usually children. Unfortunately, in the urban centers of Europe, Africa, and Asia, smallpox was provided with a pool of naïve individuals enabling evolution thereby leading the masses to devise a method of protection.

The history of vaccination against smallpox is an interesting one. It is known that in ancient China and India until the end of the 18th Century (18th C.), the only way to prevent smallpox disease was with a form of inoculation called variolation. In practice, either the powdered scab from an infected person was blown through the nostrils of an uninfected person or the powder was inoculated via a scratch in the skin. This led to a milder infection and resulted in a much reduced death toll (1-2%), leading this practice to spread across several continents. Although inoculation had a mortality rate of 0.2 to 0.5 percent, it was far safer than the alternative risk of the full disease. Although in broad use

in Asia and Africa, the practice was rarely used in Europe until the 18th C. due to controversy. [16, 71]

In 1796, variolation was replaced with a much safer alternative through the work of Edward Jenner. The English doctor observed that milkmaids infected with CWPV had a much less severe infection, if any, and were left immune to smallpox. Through a series of experiments, he proved his theory that infection with CWPV induced cross-protective immunity and in so doing, created the first vaccine [16, 71-73]. He called his method "vaccination," from the Latin word vacca, or cow, and today we use *vaccination* to refer to immunization against any disease. The vaccine formulation was later found to have changed from CWPV to VACV, a genetically related but distinct virus with approximately 90% homology to variola.

By the mid-1900's, smallpox deaths worldwide had reached between 300-500 million people due to its high virulence. However, vaccination efforts were being revised as the World Health Organization (WHO) had targeted the disease for eradication. There were several reasons eradication was deemed possible to attempt. First, there is only one host for variola and no animal reservoir. Second, the social and economic impact was devastating Third, it is infectious only after a long incubation period, infection confers long-term immunity, and there was an affordable vaccine available. Finally, it is quite easy to spot an infected person and therefore proper quarantine procedures can be implemented in a timely manner. [4, 74]

Following successful vaccination campaigns throughout the 19th and 20th centuries, the last naturally occurring case of smallpox occurred in 1977 in an unvaccinated hospital cook in Somalia [74]. The disease was officially declared

eradicated by the World Health Organization in 1980, and it was mandated that any remaining stock of VARV should either be destroyed or transferred by all researchers worldwide to either the Centers for Disease Control and Prevention, Atlanta, GA, USA or the State Research Center of Virology and Biotechnology, Koltsovo, Russia. Since the last documented "naturally occurring" case in 1977, there have been two reported deaths from smallpox disease. They both occurred in 1978 in Birmingham, England resulting from a laboratory accident. [7, 75]

With the ending of global immunization approximately thirty years ago, there is now a large population that is immunologically naïve and although the vaccine is available, there is the small but significant risk of the serious aforementioned complications. These complications are exceptional and occur greater than ten times more often among primary vaccinees than among re-vaccinees and are more frequent among infants than among older children and adults. Coupled with the health concerns of vaccination due to complications in immunocompromised people, this has confined the administration of the vaccine to health care personnel, members of the military, and laboratory workers.

The vaccine in use today is a live virus preparation of infectious vaccinia virus, given via use of a bifurcated needle dipped into the vaccine solution to prick the skin of the upper arm. If there is a “take”, characterized by a red and itchy bump forming at the vaccination site, the vaccination is considered successful. The rash will progress into a vesicle and pustule which will eventually begin to drain and typically, within 21 days will clear up and the scab (which is infectious) will fall off (**Figure 2.1**).

In healthy individuals, the vaccine has historically been well-tolerated and elicited durable humoral and cellular immune responses with antiviral antibodies being detected up to 75 years and longer post-vaccination and humoral responses remaining stable for between 8 and 15 years post-vaccination. In addition, the vaccine has been effective as post-exposure prophylaxis to prevent or substantially lessen infection within a few days of exposure.

Currently, antiviral therapy against poxviruses is restricted to one drug, cidofovir. This drug is currently FDA approved for the treatment of AIDS-related retinal cytolomegalovirus infection, with successful off-label use as an anti-poxviral drug. [76-78]. Cidofovir, a nucleoside analog, is recognized by viral DNA polymerases and acts as a DNA chain terminator to inhibit OPV replication. Published reports of cidofovir-resistant strains have shown that new strains of orthopoxviruses are emerging which are attenuated but untreatable with cidofovir [31], again pointing to the need to develop alternative therapeutic interventions which would protect the immunologically naïve population against these viruses should an outbreak occur. Problems associated with cidofovir administration include kidney failure, decreased white blood cell count, an increase in ocular pressure, edema and pain of the eye, bile buildup that can eventually result in liver and pancreas complications that may lead to death. More mild complications are malaise, rash, hair loss, diarrhea, infection, pneumonia, dyspnea and vomiting.

Other drugs have been used with some efficacy against orthopoxviruses. These include Vaccinia Immune Globulin (VIG), which can be used to treat individuals who have developed generalized vaccinia, eczema vaccinatum, or progressive vaccinia post-

smallpox vaccination or accidental infection [79, 80]. A compound often used in concert with Cidofovir and/or VIG treatment, ST-246 prevents the virus from leaving the cell, thus preventing viral dissemination within the body. It acts by targeting VACV gene F13L and has potent anti-viral activity against multiple OPVs including MKPV, VACV, CWPV and VARV. ST-246 has also been shown to be active against a cidofovir-resistant strain of CWPV [81-84]. Given the potential impact on both animals of agricultural importance and human health, new treatments for poxvirus diseases are needed. Consequently, the basic biology of poxviruses needs to be explored to develop a thorough understanding of the genes, their functions, and interactions, with the goal of developing resources to combat the aforementioned issues.

Poxvirus Structure and Replication Scheme

Poxviruses, belonging to the family Poxviridae, can be divided into two subfamilies infecting either vertebrates or insects. They are composed of large DNA genomes, including all genes necessary for replication, transcription and assembly [85], allowing them to independently carry out all aspects of their life cycle in the host cell cytoplasm. Of the poxviruses infecting vertebrates, only two, variola (VARV), the causative agent of smallpox, and molluscum contagiosum viruses, utilize humans as their single host [86].

In order for poxviruses to replicate in the cytoplasm, they have to encode all proteins necessary for mRNA synthesis and DNA replication. This includes a DNA-dependent RNA polymerase, modifying enzymes to methylate, cap and add a poly-A tail to mRNA and additional accessory proteins needed for transcription elongation, DNA

resolution, and all other proteins needed for successful DNA replication that would normally be found in the nucleus.

Binding. Poxviruses initiate their infection of host cells by attachment and fusion. There is no clear consensus on the mechanism of fusion; however, in terms of binding, we know that cellular tropism is directed towards cells expressing heparin sulfate/glycosaminoglycans (GAG), i.e. most cells. In addition, components of the extracellular matrix may also be determinants of binding [85]. Mature Virion-associated (MV) proteins L1 and A26 have been implicated as receptor-binding proteins in a non-GAG manner, instead binding to extracellular matrix protein laminin as well as an unidentified protein [86, 87]. Interestingly, the soluble form of L1 seems to antagonize viral binding to the cell via competition for glycosaminoglycan-independent binding [87]. Other proteins involved are F9, which along with L1, associates with the entry fusion complex (EFC) of at least eight MV proteins. Additionally, MV membrane proteins A27, H3 and D8 mediate binding to GAGs [86].

Fusion and Entry. Poxviruses enter the cell in one of two ways: *Method 1* Following GAG-independent binding of host cell receptors, the virus enters the host by either directly fusing with the plasma membrane in a pH-independent manner [88] or uses clathrin-coated pits. *Method 2* The IMV (intracellular mature virion) binds to host GAGs and fuses with the cellular membrane followed by endocytosis into the cell [86]. The pox fusion complex includes several proteins including A16, A21, A28, G3, F9, G9, L5, H2, and J5 [89-91]. The late viral protein I2 has also been implicated in viral entry [92] although whether it associates with the EFC is to be determined.

Initial Uncoating and Early Transcription/Translation. Upon entry, the viral core which contains all the genes necessary for viral replication, goes through its initial uncoating. The core moves along microtubules to sites where virus factories can form with the aid of proteins A10 and L4 [93]. Within thirty minutes of entry, early gene transcription occurs. Transcription elongation factors G2, H5 and J3 [94], the RNA polymerase proteins A24, D7 and seven others along with associated proteins RAP94 and VETF are transcribed and translated. These are the proteins required for DNA replication[95].

Translocation and secondary uncoating. The viral core then translocates to just outside the cell nucleus where it undergoes a secondary uncoating and the nucleoprotein (NP) complex containing the viral genome is released. Transcription and translation of intermediate genes, which mainly code for late transcription factors, occurs at this time. The viral genes essential for genomic DNA replication (A20, B1, I3, H5, E3 and E8, and at least 10 other proteins) are then transcribed. Replication is triggered at approximately 100 minutes post-infection., and DNA is synthesized as large concatemeric molecules that are later resolved into the linear genome [96, 97].

Late Transcription. Following this, the late genes are transcribed and translated, producing all structural proteins and enzymes. This occurs between 140 minutes to 48 hours post-infection. The genes required for concatamer resolution are synthesized in this round of transcription.

Virion assembly. Assembly of progeny virions begins with concatemeric genomic DNA intermediates that were previously formed being resolved into linear dsDNA with the help of the following proteins: DNA ligase A50, A22 along with four flanking

hairpin sequences which act as *cis*-acting motifs. This DNA is packaged with late viral proteins into immature virions that mature into the brick-shaped IMV and are transported to the cell periphery where they are released. Morphogenesis is mediated in part by A13 and A17. Wrapping is accomplished partly by E8, A40 and A32, and is indirectly assisted by I6's telomere binding capabilities [96].

Viral release. Release can occur in four different ways. 1) The cell can lyse and the virion be released where it will remain an IMV. 2) IMVs can be released in an inclusion body with the assistance of a 94kDa ATI protein late in the infection cycle. 3) A subset of IMVs will become wrapped by a double-membrane derived from the trans-golgi network or endosomes, becoming an intracellular enveloped virus (IEV). The wrapped virion then fuses its outermost membrane with the cellular plasma membrane becoming a cell-associated enveloped virus (CEV). Upon release from the cell surface, the virus is an extracellular enveloped virus (EEV). 4) A subset of the EEVs remain attached to the cell surface after release and and leave the cell along actin tails as cell-associated enveloped virions (CEV) to infect a neighboring cell. This mechanism is thought to facilitate cell-to-cell spread of VV. Genes involved in release include F12 and A36 (movement to the cell surface). Actin tail formation is mediated by A36, A34, A33 and B5 [97, 98].

Running Interference with RNAi

RNA interference (RNAi) is an evolutionarily conserved pathway which has proven to be a useful tool for silencing cellular or viral gene expression through the targeted degradation of mRNAs [99]. The process involves small interfering RNA (siRNA)

molecules that can modulate the activity of their gene targets. siRNA s can bind to specific messenger RNA (mRNA) molecules and decrease their production by degrading mRNA and/or inhibiting protein translation, for example. This process is important in cellular viral defense as well as gene expression regulation in general.

The RNAi pathway is initiated by the enzyme Dicer, which cleaves long double-stranded RNA (dsRNA) molecules into short fragments of ~20 nucleotides that are called siRNAs. Each siRNA is unwound into two single-stranded (ss) ssRNAs, namely the passenger strand and the guide strand. The passenger strand will be degraded, and the guide strand incorporated into the RNA-induced silencing complex (RISC). The most well-studied outcome is post-transcriptional gene silencing, which occurs when the guide strand base pairs with a complementary sequence of a messenger RNA molecule and induces cleavage by Argonaut, the catalytic component of the RISC complex. In some organisms this process is known to spread systemically despite the initially limited concentrations of siRNA [100].

The selective and robust effect of RNAi on gene expression makes it a valuable research tool, both in cell culture and in living organisms. Multiple studies have shown this naturally occurring process can be mimicked using synthetic siRNAs [99-101]. The exogenously introduced siRNAs are useful at inhibiting viral replication for several viruses both *in vitro* and *in vivo*, including influenza [102], hepatitis B [103], respiratory syncytial virus [104], herpes simplex 2 virus [105], and more recently, ebola virus [106]. Orthopoxvirus-specific siRNA-mediated replication inhibition has also been demonstrated recently targeting VACV genes B1R, G7L, and D5R [50, 107] both prophylactically and therapeutically, along with MKPV genes E8L and A6R [17].

However, these represent only a small fraction of the ORFs encoded or predicted by orthopoxviruses.

Other applications of RNAi include using it as a forward genetics approach to identify genes which affect a given phenotype. RNAi may be used for large-scale screens that systematically shut down each gene in the cell, which can help identify the components necessary for a particular cellular process or an event such as cell division or viral-specific determinants of replication while elucidating potential targets for novel antiviral strategies.

Genome-wide RNAi screening in mammalian cells is critical for uncovering factors affecting host cellular functions, proteins essential for viral propagation and various other examples. For instance, using a human-genome-wide RNAi screen facilitated the identification of over 300 host proteins that affect West Nile Virus infection [108], almost 300 cellular cofactors required for influenza A replication [79], 311 genes which are implicated in HIV-1 replication [109, 110], along with many Drosophila screens that identify and confirm a variety of viral- host protein interactions [51, 111-113]. RNA interference has also proven useful in discovery of viral target proteins using siRNAs in complementation studies [39, 114] as well as elucidating signaling pathways implicated in obesity [111] and intestinal bacterial pathogenesis [115].

Poxvirus siRNA Proof of Concept. siRNAs have been used as a strategy to silence genes in several different studies including those that silence candidate genes expressed prior to performing HIV p24 assays, HIV inhibition assays, influenza and SARS-CoV replication

inhibition assays. In recent years, several manuscripts have been published which utilize viral gene-specific siRNAs to silence poxvirus gene expression and inhibit poxvirus replication. These have prompted our lab to conduct a preliminary screen of multiple siRNAs targeting viral genes to determine if they are effective at the inhibition of CWPV or VACV replication. Studies utilized CWPV-Brighton Red strain along with a recombinant vaccinia virus expressing green fluorescent protein (rVV-eGFP) under control of the VACV p7.5 early/late promoter [116]. The results confirmed the following: 1) As previously published, RNAi can be used to inhibit the replication of poxviruses; 2) transfection of cells with virus-specific siRNAs can reduce the ability of both VACV and CWPX viruses to multiply (**Figure 2.2**) and 3) a reduction in viral mRNA due to siRNA transfection and pox gene silencing corresponds with plaque assay results (**Figure 2.3**). (Hogan, RJ and Tompkins, SM—unpublished findings).

The Small Molecule Approach to Disease Intervention

Small molecules are organic compounds characterized by low molecular weight, usually less than 800 Daltons. The term is often limited to describing molecules with very specific binding properties: high affinity binding to a biopolymer such as protein, nucleic acid, or polysaccharide while altering the native function of that biopolymer. The small size of the molecule facilitates rapid diffusion across cellular membranes, thus allowing for quick access to the intracellular site of action. Small molecules can be derived from natural or synthetic substances and can have a beneficial effect on disease, as seen with small molecule drugs, or be detrimental, as seen with carcinogen and teratogenic compounds.

Several published studies have exploited the use of small molecule libraries to discover inhibitors to various disease states, including orthopoxviruses specifically. Some identify nucleoside analogs to orthopoxviruses, with a cidofovir derivative (CMX001) [117] and ST-246 showing promise in pre-clinical settings or with limited clinical use [118, 119], although viral resistance has been noted. Recently, a non-nucleoside analogue (CMLDBU6128) has been identified as capable of inhibiting multiple orthopoxviruses in vitro [120].

Methotrexate, Raltitrexed, Homoharringtonine, and Idarubicin: A Unique Role for Molecular Inhibition of Orthopoxviruses

Methotrexate (MTX) (aminomethylpteroylglutamic acid) is an antimetabolite, antifolate, nucleoside analogue drug introduced for clinical use in 1950s [121]. Historically, it has been used to treat a broad range of neoplastic disorders including acute lymphoblastic leukemia, non-Hodgkin's lymphoma, bladder carcinoma, breast cancer and testicular tumor [122-125]. Recent literature reports indicate that it is also effective in treating the autoimmune diseases rheumatoid arthritis and severe psoriasis [126] (**Figure 4.2**).

Methotrexate is a structural analogue of folic acid (FA) and acts via competitive inhibition of FA metabolism. FA is a water-soluble vitamin found in a variety of foods including: green vegetables, legumes, whole grains and citrus fruits. It is also commercially available as a folate supplement for the required increase needed by women during child-bearing age, pregnancy and in patients with elevated levels of plasma homocysteine. [125]. Folic acid is an essential co-factor for the enzyme

dyhydrofolate reductase (DHFR) involved in the *de novo* synthesis of DNA nucleotides, purines and pyrimidines.

Methotrexate directly competes with FA for its binding site on DHFR and this inhibition of DHFR leads to imbalanced nucleotide pools, disrupting DNA synthesis [121, 125, 127]. This typically leads to cellular toxicity either due to its non-target specific mode of action or the resulting lack of late replicative cells. It is often associated with genotoxic damage like strand breaks, chromosomal abnormalities, extensive incorporation of uracil in place of thymine into the DNA, defective DNA repair, anomalous DNA methylation patterns and increased somatic mutation rates [125]. The immunosuppressive effect of the drug is dose-dependent [128] and intracellularly, MTX is polyglutamated, allowing for a 7-fold increase in tissue accumulation in the first 24 hours post-administration [123] This polyglutamated form is more potent than administered MTX in inhibiting folate-dependent enzymes [129]. The increased toxic effects of MTX after repeated administration can be attributed to its intracellular accumulation and subsequent DHFR inhibition.

Methotrexate antagonist leucovorin [folinic acid (FNA)] has been shown to bypass the MTX-induced inhibition of DHFR as it directly provides the tetrahydrofolates (THF) intracellularly. FNA is the 5-formyl derivative of tetrahydrofolates and supplies folates intracellularly since it remains unaffected by the inhibition of DHFR. The protective effects of exogenous FNA against MTX-induced chromosomal damage has been noted in various studies upon administration of FNA from 0.5-6 hours post-MTX treatment [121, 125, 127-129]. Further, post-treatment with FNA ameliorates the toxicity of MTX without compromising its therapeutic efficacy [128]. Vaccinia virus, a DNA

virus has been shown to be inhibited to varying degrees in cells treated with MTX [130-133]

Raltitrexed is an antimetabolite drug used in cancer chemotherapy and is key in the treatment of colorectal cancer since the late 1990s. Chemically, it is similar to folic acid (FA) and is in a class of drugs called folate antimetabolites, which inhibit one or more of three enzymes that use folate and deriavtives as substrates: DHFR, 5-phosphoribosylglycinamide formyltransferase (GARFT) and thymidine synthase(TS). Upon TS inhibition, formation of precursor pyrimidine nucleotides is hindered. Ultimately, raltitrexed prevents DNA and RNA formation [123, 134].

Homoharringtonine (HHT) is a small molecule plant alkaloid, derived from the *Cephalotuxus fortuneii* tree. It has been utilized in China for at least 30 years in the treatment of both acute and chronic myeloid leukemia. HHT and its analogs affect multiple pathways by inhibiting protein synthesis, upregulating genes associated with apoptosis, and the inhibition of angiogenesis, all in a dose and time dependent manner. The primary inhibitory mechanism of action by HHT is the inhibition of protein synthesis by binding to ribosomes and inhibiting polypeptide chain elongation. Its cytotoxicity is cell-cycle specific, primarily affecting cells in G1 and G2 phases in a manner directly proportional to the extent of inhibition of protein synthesis. Due to its relatively mild extramedullary toxicity and lack of anthracyclin-like myocardial toxicity, elderly patients are suitable candidates for treatment with HHT. [135, 136]

Idarubicin [(IDA) (4-demethoxydaunorubicin)] is an antineoplastic, anthracycline, antileukemic drug belonging to the family of drugs called antitumor antibiotics. It is a DNA-intercalating analog of daunorubicin. Mechanistically, it inserts itself into DNA,

thereby preventing it from unwinding which has an inhibitory effect on nucleic acid synthesis via its interaction with the cellular enzyme topoisomerase II. The absence of a methoxy group at position 4 of the anthracycline structure gives the compound a high lipophilicity and results in an increased rate of cellular uptake in comparison to other anthracyclines. As is the case with the majority of antileukemic drugs, when administered at therapeutic dosages, severe myelosuppression typically occurs. Other life-threatening side effects are myocardial toxicity leading to congestive heart failure. IDA is one of the primary treatments for acute myeloid leukemia. [121, 135, 137]

In summary, small molecules inhibitors have proven useful in treating a variety of diseases. They are most often discovered via the combination of initial cell-based high throughput screening and subsequent evaluation as part of *in vitro* and *in vivo* secondary assays. Small molecule inhibitors represent strong lead structures for human drug development and screening of compound libraries allows for previously identified drug-like molecules to be repurposed for application to other disease models.

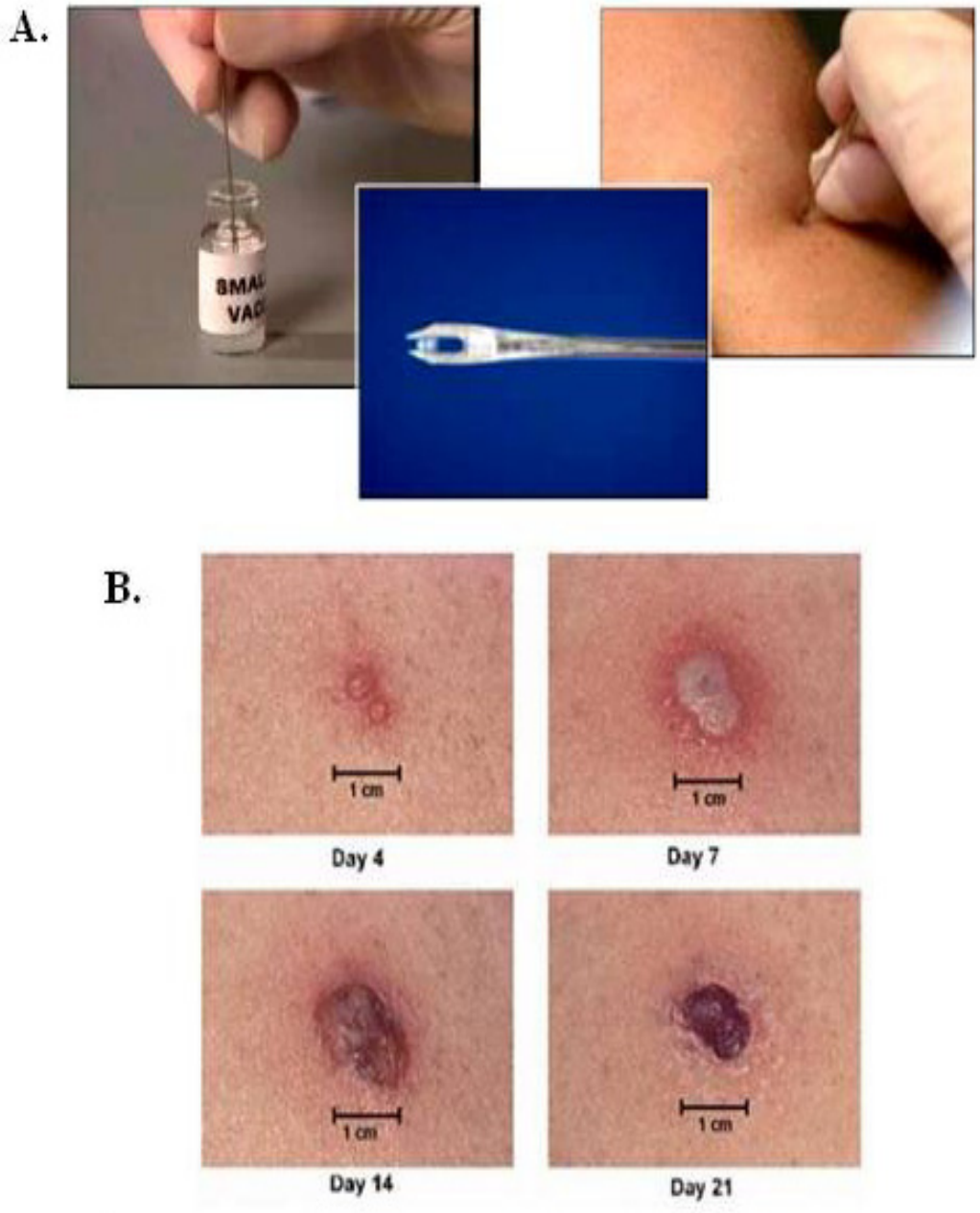
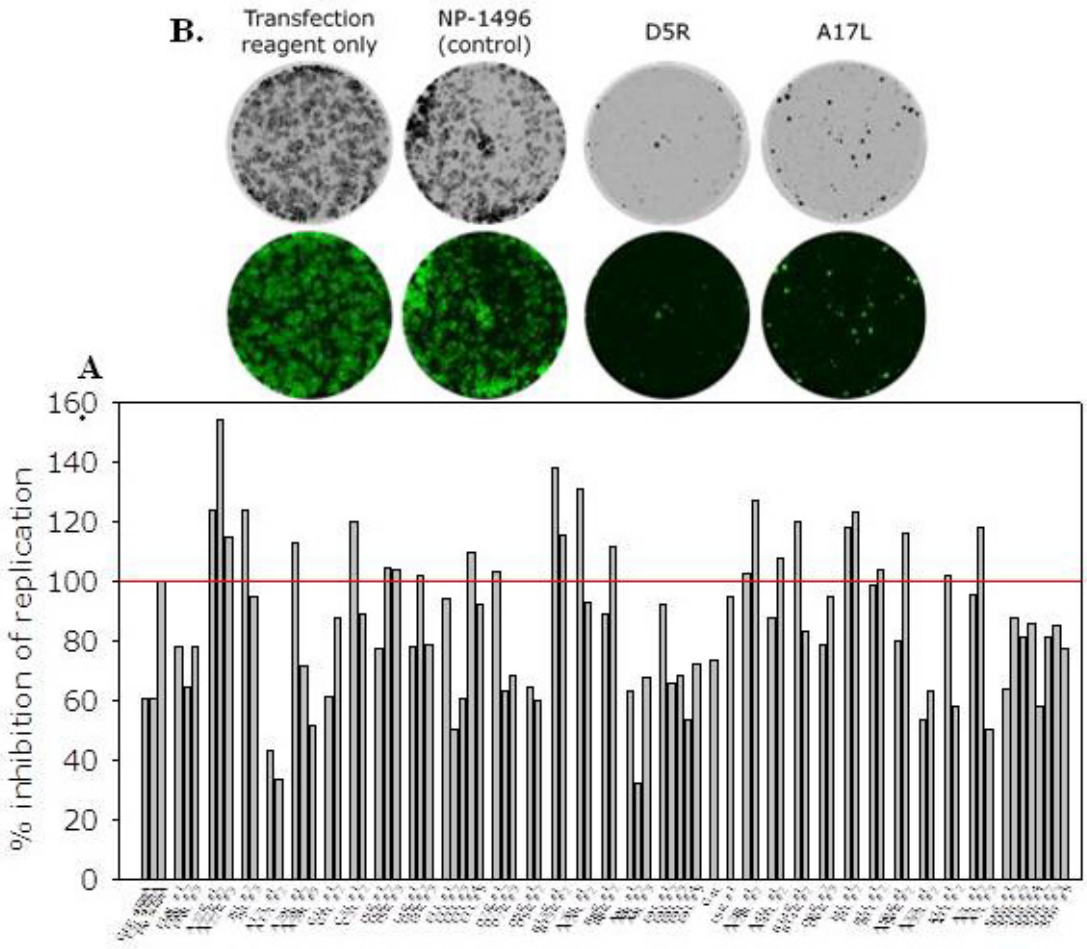
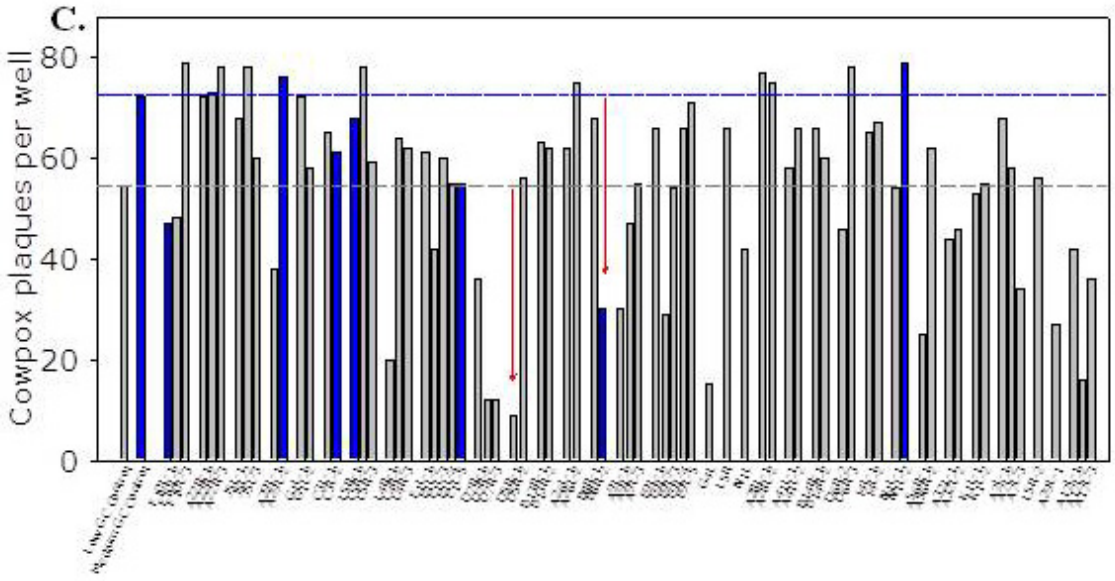


Figure 2.1 | Smallpox vaccination: Major (primary) reaction. **A**) Bifurcated needle and multiple pressure vaccination technique used. **B**) Expected vaccine site reaction and progression following primary smallpox vaccination or revaccination after a prolonged period between vaccinations. Days 4 through 21 after receiving the vaccine for the first time. *Source: CDC*.

Figure 2.2 | Vero E6 cells transfected with pox-specific or control siRNAs. A) Twenty-four hours later the cells were infected with recombinant VACV, which expresses EGFP under control from a VACV early/late promoter. Forty-eight hours post-infection (hpi), fluorescence was measured. In this system, virus load correlates with fluorescence (data not shown), so reduction in virus replication was measured by reduction on total fluorescence in each well, compared to control wells. **B**) Wells were photographed for reference. **C**) After 24h, the cells were infected with CWPV and 48 hpi wells were fixed and plaques visualized by crystal violet staining. The total number of plaques per well was determined by counting under a dissecting scope. In these experiments, % inhibition of replication was measured against the control siRNA (influenza NP-1496).


B.
Transfection reagent only
NP-1496 (control)
D5R
A17L
A
% inhibition of replication
160
140
120
100
80
60
40
20
0


C.
Cowpox plaques per well
0
20
40
60
80

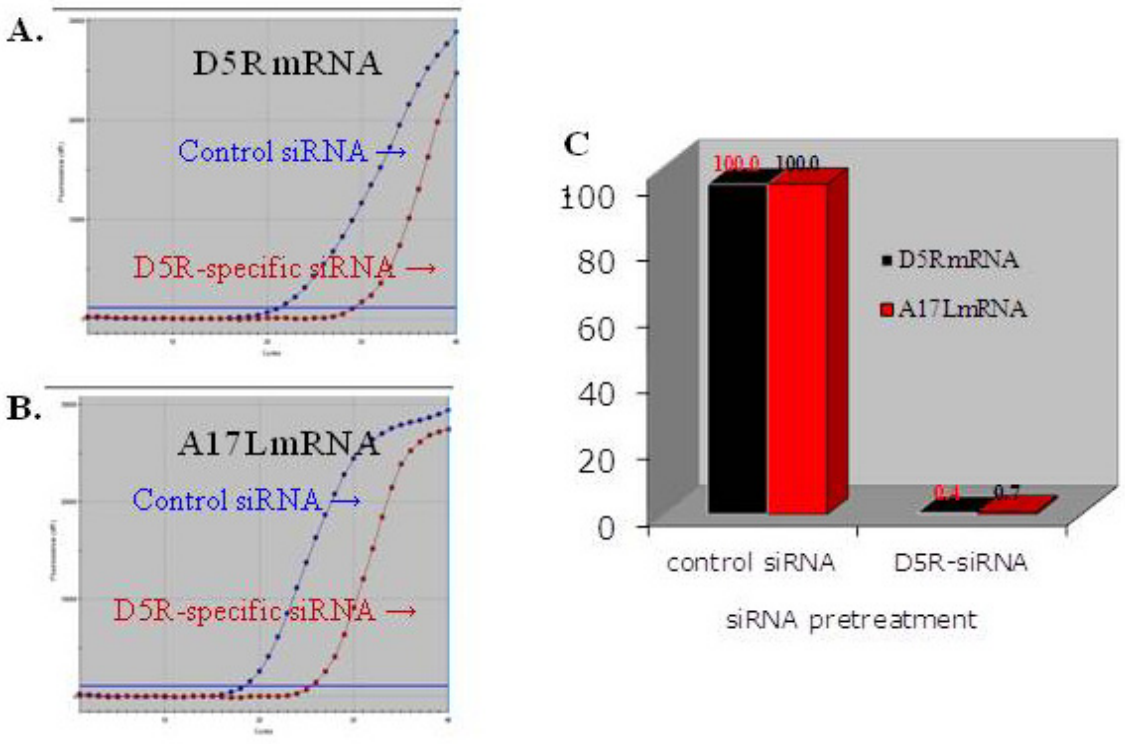


Figure 2.3 | Reduction in viral mRNA corresponds with plaque assay results. Vero E6 cells were transfected with control (Influenza NP-1496) or D5R (pox)-specific siRNA. After 24h, the cells were infected with CWPV and 24hpi RNA was purified from infected wells. **A**) D5R and **B**) A17L mRNA levels were measured by real-time PCR. **C**) mRNA levels shown as percentage of gene-specific expression when transfected with control siRNA.

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CHAPTER 3

EVALUATION OF A GENOME-WIDE POXVIRUS SIRNA LIBRARY TARGETING VIRAL GENES[1]

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Abstract

Orthopoxvirus (OPV) zoonoses have continuously emerged in Western and Central Africa, Brazil, Europe, the US and other locales around the world. Poxviruses are a complex family of viruses composed of very large, double-stranded DNA genomes that have not fully been characterized in terms of the function of many of the genes and their interactions within the host. To better understand the underlying biology associated with OPV replication, we have developed a genome-wide small interfering RNA (siRNA) library targeting conserved OPV genes. In a screen designed to identify those which are essential for viral multiplication, multiple siRNAs targeting 17 open reading frames (ORFs) were found to be effective at significantly decreasing viral multiplication *in vitro* when corresponding gene products are reduced or eliminated. In depth analysis of these data showed that siRNAs targeting viral genes D7R, I2L and L3L exhibit the most complete inhibition of multiplication when administered prior to infection with either cowpox (CWPV) or vaccinia (VACV) viruses. Using siRNA molecules, we also show two well-characterized viral genes, A17L and E3L, to be essential for viral multiplication, thus confirming previous data describing their role in this process. The efficacy of siRNAs targeting D7R and I2L were also examined in a Balb/c mouse model of CWPX infection. Results show that the mice experienced less weight loss and a longer median survival post-CWPV challenge. Taken together, these results highlight the utility of genome-wide siRNA screening for studying viral multiplication factors along with identifying potential therapeutic targets for anti-orthopoxvirus therapies.

Introduction

Members of the Poxviridae family consist of viruses with large DNA genomes including all genes necessary for replication, transcription and assembly [1], that infect either vertebrates or insects. Of the poxviruses infecting vertebrates, only variola (smallpox, VARV) and molluscum contagiosum solely infect humans [2]. However, cowpox (CWPV), vaccinia (VACV) and monkeypox (MKPV) viruses have shown zoonotic potential in Western Africa, Brazil, Europe, the United States and other locales around the world. In addition, smallpox is feared as a potential bioterrorist threat [3], particularly with the identification of recombinant poxviruses which bypass pre-existing immunity [4]. Due to this threat, military personnel, first responders and laboratory workers continue to be vaccinated. VACV is used as the immunizing agent in the live smallpox vaccine preparation. Likely due to scarification as the manner of inoculation, accidental infections from recent vaccinee to close household contacts continue to occur [5-7].

Buffalopox, a virus closely related to VACV, reemerged in India in early 2003. The outbreak involved 4,000 domesticated buffalo out of herds totaling 10,000 animals. One hundred twenty-five human infections were recorded, primarily among animal herders and milkers. This incident led to an approximately 40% reduction in milk production as well as a wide variety of clinical disease symptoms among both animals and humans [8]. Since then, other cases of emergent buffalopox have also been noted [9, 10] leading to a significant public health impact. Human-human transmission is possible in these scenarios, and up to three rounds have been noted. A promiscuous virus in terms of its broad host range, CWPV transmission has also been noted repeatedly in recent years. Environmental factors have led to an increase in the natural reservoir hosts for the

virus such as small rodents, which results in a higher risk of infection for cats, zoo and circus animals and subsequently for humans [11].

The disease caused by many OPVs is typically vesicular and exanthemous and characterized by localized pustular skin lesions.. Disease can spread to other parts of the body based upon the integrity of the skin, i.e. scratches and abrasions will allow entry of the virus. Patients have presented with infection of the eye, neck, face, arms, hands, feet and other localized areas of the body. Exceptions are noted, particularly as infections caused by MKPV can lead to systemic disease and widespread lesions Although systemic involvement does not typically result in a fatal outcome, the immunocompromised can suffer a variety of complications up to and including death. These complications are exceptional and include vaccinia eczematum, progressive vaccinia, generalized vaccinia, myocarditis, neurological disorders such as encephalitis and intraocular infections. [12-16]. Currently, there are no licensed antivirals for anti-poxvirus use. Cidofovir has shown antiviral effectiveness in off-label use although published reports indicate new strains of OPVs emerging which are attenuated but untreatable with cidofovir [17, 18]. Other drugs have been used with some efficacy against OPVs including vaccinia immune globulin (VIG) and investigational drug ST-246, both of which are effective but have drawbacks associated with their use [19-24]. With the success of smallpox eradication after widespread vaccination in the 1960s and 1970s, approximately 40% of the world's population has no immunity [25, 26]. Given the potential impact on both animals of agricultural importance and human health, new treatments for poxvirus diseases are needed.

Poxviruses have a distinct transcriptome pattern that is temporally regulated. Upon infection and entry into a cell, the viral core undergoes primary uncoating. Early transcription can take place within 30 minutes of entry, producing genes required for protein translation. Following viral core translocation and secondary uncoating, transcription and translation of intermediate genes occurs and DNA replication is triggered. Beginning approximately 2 1/2 hours post-infection, late genes transcribed and translated, producing the structural proteins and enzymes required for the virus to package its DNA and assemble. There are many genes produced by poxviruses during the various phases of transcription and translation though many of them are still not fully characterized in terms of whether the gene is essential for viral multiplication.

Several tools currently being used to address the function and expression of poxvirus genes include the use of microarray analysis [27-30], deletion mutant [31-35] and temperature-sensitive mutant viruses [36, 37] and RNAi [38, 39]. RNA interference (RNAi) is an evolutionarily conserved pathway which has proven to be a useful tool for silencing cellular or viral gene expression through the targeted degradation of mRNAs [40]. The natural process involves the synthesis of small interfering RNAs (siRNAs), typically 21-27 nucleotides in length from double-stranded RNA molecules. These then act as templates for their own replication and the degradation of target mRNA during RNAi [41]. Multiple studies have shown this naturally occurring process can be mimicked using synthetic siRNAs. The exogenously introduced siRNAs are useful at inhibiting viral replication for several viruses both in vitro and in vivo, including influenza [42], hepatitis B [43], respiratory syncytial virus [44], herpes simplex 2 virus [45], and more recently, ebola virus [46]. RNAi has also proven useful in discovery of

viral target proteins using siRNAs in complementation studies [47, 48]. Orthopoxvirus-specific siRNA-mediated replication inhibition has also been demonstrated in recent years targeting VACV genes B1R, G7L, and D5R [38, 39] both prophylactically and therapeutically, along with MKPV genes E8L and A6R [49]. However, these represent only a small fraction of the ORFs encoded or predicted by orthopoxviruses. Consequently, the basic biology of poxviruses needs to be explored to develop a thorough understanding of the genes, their functions, and interactions with host genes, with the goal of developing novel antiviral therapeutics.

In this study, we performed a genome-wide in vitro screen using individual siRNAs and CWPV as an OPV model in order to identify ORFs which are implicated in viral replication. We identified 17 viral genes that prove essential to OPV multiplication. Validation of a subset of ORFs was performed using both CWPV and VACV to confirm specificity of gene function. Knockdown of several ORFs are highlighted; three which have not been extensively explored, D7R, I2L and L3L and two fairly well-characterized ones, A17L and E3L. *In vivo* studies using siRNAs targeting I2L and D7R are also described. This strategy may be useful for other viruses with complicated genomes and/or replication schemes.

Materials and Methods

Cell lines and virus propagation. Vero E6 (Vero) cells were obtained from ATCC, Inc. (CRL-1586) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum at 37^{o} C and 5% CO_2. Cells used in all experiments were mycoplasma-free as determined by PCR and propagated without use of antimicrobial compounds. CWPV-Brighton Red (BR) and VACV-Western Reserve (WR) were the generous gift of Dr. Bernie Moss (NIH). Both viruses were propagated in Vero cells as previously described [50].

Poxvirus RNAi Library Synthesis. A list comprising 177 ORFs containing 701 unique mRNA transcripts associated with VACV-WR, VARV-China 48, and/or one of two strains of MKPV-Congo or MKPV-USA was compiled and categorized into similarity groups. The most conserved regions within those genes found in the above listed strains were analyzed. The groups and transcripts were imported into a custom relational database, and a proprietary C# algorithm implementing best-practice siRNA design steps [51] was used to select siRNAs targeting all transcripts within each similarity group. Specificity to mRNAs within a similarity group was insured by BLAST analyses of [52] candidate siRNAs against human mRNA transcripts from release 22 of the reference sequence (RefSeq) database [53] as well as the 701 viral transcripts. Via this process, 4 distinct siRNAs each were designed for 138 ORFs. For the remaining 39 ORFs, 1-3 siRNAs were designed (Thermo Fisher Scientific, Dharmacon, Inc.). This cross-functional tool exhibited 94% coverage of the targeted genes.

Screening Protocol. For each of the two independent screens carried out, Vero cells were first plated in standard 12-well plates ($3.6x10^5$ cells/well) in 0.8mL DMEM supplemented with 5% FCS. Upon reaching ~80% confluency, siRNAs were transfected into cells using Dharma*FECT* 1 Transfection Reagent (Thermo Fisher Scientific #T-2001-03) at a final concentration of 50nM according to the manufacturer's protocol. A non-targeting siRNA, siNTC, (siControl 1, Thermo Fisher Scientific #D-001700-01-20) was included to control for non-sequence-specific effects. At 24 hours post-transfection, cells were infected with CWPV (0.0006-0.0008 MOI), and incubated for 48 hours prior to being fixed with ice-cold methanol and stained with 0.13% crystal violet containing a final concentration of 5% methanol and 10% formaldehyde v/v. Plaques were then imaged and quantified using the AID ViruSpot plate reader (AID Diagnostika) (**Figure 3.1**).

Validation and candidate gene selection. siRNAs which inhibited viral replication by 50% or more in the plaque reduction assays were individually rescreened in Vero cells at 50, 25, and 12.5nM concentrations. After 24 hours, the transfection supernatant was removed, wells washed with PBS, and the cells subsequently infected with CWPV or VACV as described above. Surviving cells were fixed, stained, and plaques quantified as described above and/or visually using a dissecting microscope.

mRNA quantification. siRNAs were transfected into Vero cells as described above (50nM). After 24 hour incubation, cells were infected with CWPV or VACV at an MOI of 1. Total RNA was isolated in TRIzol (Invitrogen) 24 hours post-infection, cleared of contaminating DNA using TURBO DNase (Ambion) and converted to cDNA with the Transcriptor First Strand cDNA Synthesis kit (Roche) following manufacturers' instructions [54]. Quantitative real-time PCR (qPCR) was carried out using QuantiTect SYBR Green technology using an Mx3000p Multiplex Quantitative PCR System (Quiagen: 204056, Stratagene: Mx3000P) according to the manufacturer's instructions. Generally, reactions consisted of 1μL of cDNA in a final volume of 25μL. All assays were performed in duplicate in microtest 96-well plates and individual experiments repeated three to five times. Both non-template and no Sybr Green controls were included on each run. siRNA and primer sequences can be found in **Table 3.1**. Cyclophillin B was used as an internal reference gene to normalize transcript levels. Relative mRNA levels were determined through comparison of (*i*) the PCR cycle thresholds (Ct) for the gene of interest and Cyclophillin B (ΔCt) and (*ii*) ΔCt values for the experimental siRNAs versus siNTC (ΔΔCt).

Mouse treatment and infection protocol. Female BALB/c mice (National Cancer Institute) were maintained in microisolator housing with food and water provided ad libitum. All mice were specific-pathogen free and aged 8-10 weeks at time of experiment. Mice were administered 30ug of siI2L, siD7R and siNTC in 1mL PBS on days -1 and 3 post-infection via hydrodynamic tail vein delivery [42]. Control mice were administered PBS on the same schedule. Under light anesthesia (2,2,2-tribromoethanol by

intraperitoneal injection) mice were inoculated intranasally with 3 times the previously determined 50% lethal dose (LD_{50}) of CWPV diluted in 50µl PBS (final administered dose: $2.16x10^5$ PFU) or control diluent on day 0. Mice were weighed daily, observed for signs of illness and euthanized upon reaching 20% weight loss and/or an apparent clinical score of 3 (**Supplementary Table 3.1**). All studies were carried out in accordance of the University of Georgia Institutional Animal Care and Use Committee.

Lung analyses. Upon euthanasia, lungs were individually collected and homogenized with a glass dounce homogenizer in PBS supplemented with 1% antibiotic/anti-mycotic [(Cellgro: 30-004-CI) a combination of 10,000 units/ml penicillin G, 10 mg/ml streptomycin sulfate and 25 µg/ml amphotericin B with 8.5 g/L NaCI]. Supernatants were clarified via centrifugation at 13,000 rpm for 20 minutes and titered on Vero cells. Plaques were counted and imaged as previously described. Lung cytokine profiles were assayed from 50µl of clarified supernatant using the Mouse Th1/Th2 6-plex kit per manufacturer's instructions (Invitrogen: LMC0002) and analyzed on a Luminex 200 system.

Data Analysis. For identification of efficacious siRNAs, two parameters were studied: plaque counts in cells treated with experimental siRNAs compared to control siRNAs, along with siRNA toxicity based on visual inspection of the cellular monolayer upon crystal violet staining and microscopy. Data were analyzed using GraphPad Prism 5.01. Two-way Anova with a Bonferroni post-test was used to test for significant differences in weight along with lung cytokine profiles. Survival curve comparison was used to test for

significant differences in length of survival (days). Differences with $p < 0.05$ were considered significant.

Results

A pox-specific siRNA library screen identifies orthopoxvirus ORFs which are essential for virus multiplication. We used an siRNA library targeting the bulk of OPV genes conserved among the four variants analyzed to investigate which viral genes are essential for poxvirus multiplication in Vero E6 cells (**Figure 3.1**). The library was designed with between one and four siRNAs targeting each viral gene. After screening each siRNA individually in two separate experiments, along with further concentration- dependent validation studies (described below), 17 ORFs were identified as being important for CWPV replication in vitro (**Figure 3.2A**). The 26 siRNAs targeting those genes effectively reduced viral multiplication by at least 50% in comparison to cells transfected with a non-target control siRNA (NTC) (**Figure 3.2A and Table 3.2**). Representative data from the primary screen can be found in **Supplementary Figure 3.1**.

Validation and candidate gene selection. To identify gene targets in which to perform validation studies, the siRNAs which effectively inhibited CWPV replication by at least 50% in the library screens were re-synthesized and individually rescreened in Vero cells. Each siRNA was transfected at 50, 25, and 12.5 nM final concentrations and 48 hours post-infection with CWPV, plaques quantified as described above (**Supp. Figure 3.2**). The five most efficacious siRNAs identified in this experiment are shown in **Figure 3.2B and C**. siRNAs targeting CWPV genes D7R, E3L, A17L, I2L, and L3L (**Table 3.1**) inhibited viral plaque formation significantly as evidenced by very low numbers of

plaques (*n*=1-4), in comparison to 57 plaques seen when the cells were transfected with the NTC siRNA. For each of the genes validated in this study, the single siRNA which proved most efficacious at reducing viral multiplication was selected for further experiments: 1G4 (D7R), 7G8 (E3L), 6E8 (A17L), 8H5 (I2L) and 6A9 (L3L). As shown in **Figure 3.2B**, CWPV replication is inhibited by siE3L and siI2L 97% and 99%, respectively, while siRNAs targeting D7R and L3L decrease multiplication by approximately 95% each. siA17L, though the least potent of our selected group, inhibits CWPV multiplication by 83%. Plaque images of cells transfected with select siRNAs, including non-target control (NTC), 48 hours after infection are shown for reference (**Figure 3.2C)**. Effective inhibition of VACV multiplication was also noted, thus validating the utility of the siRNAs with an alternate OPV. Two doses of siRNA were required to achieve a reduction in viral replication similar to levels seen with CPWV infection (**Supplementary Figure 3.3**). These data support the role of the five genes as essential for viral multiplication.

Gene-specific knockdown of mRNA levels by siRNA transfection. In order to determine if siRNA transfection resulted in a decrease of the corresponding viral mRNA, RT-qPCR was performed. Mutli-step growth curve experiments confirmed that optimal viral gene silencing occurs at 24 hours post infection in vitro for all viral genes assayed with the exception of E3L. In the case of E3L, experiments showed that at 24hpi, mRNA levels could be measured adequately, therefore that time point was used (data not shown). (**Supplementary Figure 3.4**). The pattern of expression after infection once data are normalized to cyclophillin gene expression suggests that the cyclophillin gene is being

upregulated early after infection. Based on these results, Vero cells were transfected for 22-24 hours with viral gene-specific siRNA molecules followed by infection with CWPV at an MOI of 1 for 24 hours. The mRNA levels of each gene were measured using RT-qPCR with gene-specific primers (**Table 3.1**). Gene-specific downregulation was standardized against respective gene levels in samples transfected with the negative control, siNTC, using Cyclophillin B transcript levels for normalization as described above. When administered 24 hours prior to infection, D7R, E3L, I2L and L3L mRNA transcript levels were reduced to 3% of normal gene expression levels with cognate siRNAs (**Figure 3.3A**). A17L shows a similar pattern as in the plaque assays by showing an average mRNA level of 16% in comparison to levels seen in cells transfected with the NTC siRNA **(Figure 3.3A).** Taken together, these data show effective gene-specific mRNA reduction of each of the five genes being examined.

Inhibition of poxvirus multiplication by siRNA alters expression of an unrelated early and late gene. With gene-specific downregulation confirmed, we decided to investigate the effects of individual gene silencing on other viral gene transcript levels. Specifically, mRNA expression of E3L, an early gene, was measured in cells transfected with D7R, L3L, or I2L-specific siRNA molecules in comparison to cells transfected with siNTC or siE3L. As shown in **Figure 3.3B**, a reduction of D7R mRNA in the cell correspondingly reduces E3L transcript levels by 89%. In addition, when late genes I2L or L3L are silenced via transfection of their respective siRNAs, E3L gene expression levels are also reduced by 95%, levels relative to gene-specific silencing with siE3L.

In order to determine if reduction of early viral gene products would have an effect on the levels of late gene products, we examined A17L transcript levels. The experiment was carried out as described above with siA17L being transfected in Vero cells as a control. We observed that siD7R, siL3L and siE3L, in silencing their respective cognate gene, each led to reduced A17L mRNA levels at an average of 2% of gene-specific silencing levels (**Figure 3.3C**). Ultimately, we show that knock-down of an early transcript, an RNA polymerase subunit, alters late gene expression, while the converse also appears to be evident.

siRNA administration pre-CWPV infection prolongs mice survival. To assess whether the siRNAs effective at reducing viral multiplication in vitro had a protective effect *in vivo*, we used an established murine Balb/c model of CWPV infection. Mice were administered poxvirus-specific siD7R, siI2L or siNTC as a control via hydrodynamic tail vein injection (*n*=5 mice per group). The mock-treated group received PBS. 24 hours later, mice were infected intranasally with $3LD_{50}$ of CWPV-BR in 50uL total volume as described above. Two days post-infection, mice were given a second dose of siRNA. Animals were monitored for morbidity and mortality until 20% of initial body weight was lost, at which point they would be euthanized, or upon regaining initial weight lost (**Figure 3.4**). Morbidity as assessed by weight loss was significantly reduced in mice receiving siD7R in comparison to mice receiving PBS by d.9. Mice treated with siI2L had slight protection from weight loss by d.9 as well although significance was limited to comparison with the group of mice who had been treated with the control siRNA .(**Figure 3.5A**). In addition, mice treated with siRNAs targeting viral gene I2L or D7R

and challenged with CWPV survived 2 or 3 days longer than the group treated with a non-targeting siRNA (siNTC), respectively (**Figure 3.5B**). Interestingly, there was a single mouse which survived CWPV infection and was recovering by experiment termination ($p<0.03$) (**Figure 3.5B**).

Lung cytokine profiles are skewed to a Th1 response. Poxvirus infections normally induce a robust Th1 response, characterized primarily by IFNg production [55, 56]. In order to ensure the administered siRNAs are not altering the expected antiviral cytokine expression profile, lungs were individually collected upon euthanasia and homogenized in PBS supplemented with 1% antibiotic/anti-mycotic. The clarified supernatants were assayed for the classic Th1/Th2 cytokines as described above (**Figure 3.6**). Both IL-2 and IL-4 results were below the limit of detection for all groups (data not shown). As expected, IFNg was robustly induced by CWPV infection as was IL-10 to a lesser extent. Differences in expression levels were noted between the mice that received siI2L when compared to controls (*$p<0.05$) and siD7R (***$p<0.001$). Mice that received siNTC showed a slight depression in IFNg levels in comparison to all groups (***$p<0.001$). Despite the appropriate antiviral response, lung viral burdens were not statistically different among the treatment groups (**Figure 3.7**).

Discussion

With the threat of VARV being intentionally released via an act of bioterrorism, poxvirus research has focused on antiviral prophylactic and therapeutic drugs. In addition, the zoonotic monkeypox virus (MKPV) infections that have cropped up in western Africa and the Midwestern US [57] and VACV in Brazil and India [7, 14] along with the

agricultural implications of animal poxvirus infections worldwide all bolster the need for additional effort in that arena. There is a significant shortage of basic knowledge of many ORFs in the orthopoxviruses. The potential for OPV gene-specific silencing leading to multiplication inhibition using siRNA has been explored previously in small-scale experiments [38, 39]. To extend upon these findings, we developed a genome-wide siRNA library targeting conserved OPV genes in a manner designed to identify those which are essential for viral replication. Here we report additional viral genes which had not previously been linked to this function. In addition we explore the specificities of several of the siRNAs at silencing their respective gene targets in CWPV- or VACV-infected cells.

Table 3.2 summarizes the functions of 17 viral genes that were targeted by the 26 siRNAs shown in **Figure 3.1A**. Five genes encode proteins which are a component of RNA polymerases (A24R, A29R, D7R, H4L and J6R). Nine genes were found to encode the following functions: interferon resistance (E3L), a subunit of the ribonucleotide reductase (F4L), IMV membrane protein (A17L), structural protein (A12L), core protein (I7L), capping enzyme and transcription factor (D12L), disulfide bond formation (F9L), a Uracil DNA glycosylase (D4R) and a virion protein and transcription factor (L3L). Viral genes which have no published function or a putatively described function that proved to be essential to viral multiplication are C6L, I2L, L3L and H7R. siRNAs targeting each of the genes listed show an antiviral potency in reducing CWPV or VACV replication in Vero cells by 52% - 99% in standard plaque reduction assays. The functional groups of ORFs identified as inhibiting multiplication are depicted in **Supplementary Figure 3.5**.

Several genes were chosen out of this group of 17 to analyze further in terms of gene specific knockdown of viral transcripts and potency against VACV-WR. A17L has been extensively investigated and much information is available concerning its' gene function, role in morphogenesis and importance to the viral life cycle [58-60]. In the present study, there is a significant antiviral response when A17L mRNA is inhibited by siA17L along with the effects of inhibition of earlier proteins as well. siE3L inhibited viral multiplication by as much as 95% in this study. Its role in antagonizing the host's antiviral response to VACV infection has been documented several times since the identification of the protein in 1993 [61-64] which prompted us to look further into siE3L's gene-specificity. D7R is another viral gene that shows higher multiplication inhibition in these studies. Past research indicates this gene's role as a DNA-dependent RNA polymerase subunit would likely be indispensible to the virus [65, 66]. Our results indicate that I2L and L3L, both genes that haven't been extensively explored, contribute to an antiviral state that the virus cannot overcome in terms of multiplication when silenced by their respective siRNAs. I2L has been shown to be essential in virion entry [33], while L3L is a conserved protein involved in the transcription of early viral genes [67, 68].

In order to investigate the specificity of the select siRNAs in reducing mRNA transcripts of their individual genes, we performed RT-qPCR. Our results indicate that the multiplication inhibition seen in plaque assays is due to the decrease of the gene targeted by the siRNA tested (**Figure 3.3A**). We examined the possibility that the expression of an early gene (E3L) could be restricted when silencing genes normally expressed late in the infection cycle using siRNA. Our results show a corresponding

decrease in E3L gene expression comparable to gene-specific silencing when D7R-, I2L- or L3L-specific siRNAs were transfected into cells prior to CWPV infection. A similar phenomenon was observed when studying whether silencing genes expressed earlier in the infection cycle using siRNA would have an effect on A17L transcript levels (**Figure 3.3B,C**).

L3L, putatively described as a late promoter of early gene transcription [68] was identified in our screen. We hypothesize that silencing of L3L results in a single round of virus replication leading to the production of replication-deficient virions. I2L, encodes a membrane-associated protein expressed late in the infection cycle and plays an essential role in virion entry [33, 68]. In contrast to L3L, we hypothesize that when blocked by siRNA, I2L-deficient virions produce progeny which show a striking reduction in infectivity due to lack of the virions' ability to enter target cells after the initial round of replication. A third ORF identified, D7R, is a subunit of the viral RNA polymerase [65, 66]. Virions deficient in this protein exhibit near-normal levels of early gene transcription and DNA replication, however, they produce an abnormally low level of late proteins [37], thus blocking the virus' ability to complete the multiplication cycle. In addition, A17L and E3L, genes which have fairly well-characterized roles in the life cycle of orthopoxviruses, were also found to be specifically inhibited by siRNA, therefore diminishing the virus' capacity to efficiently replicate in mammalian cells. Confirmation of gene-specific silencing as well as validation with VACV was carried out by probing for mRNA reduction in infected Vero E6 cells. We also noted that inhibiting poxvirus replication by viral gene-specific siRNA molecules reduced the expression of unrelated early and late viral genes.

Despite the robust multiplication inhibition seen *in vitro*, *in vivo* results indicate that upon prophylactic administration of siRNAs targeting poxvirus D7R and I2L genes, death in Balb/c mice is delayed, but not completely prevented. Several potential reasons for this difference exist. 1) Replication kinetics of CWPV in vitro suggest that peak expression of these viral transcripts occurs at approximately 24hpi. In our experiment, the animals received the siRNA at days -1 and 3 pi. Perhaps treating the mice at day 0 or 1 pi would have caused a more significant decrease in the gene transcript levels, and therefore viral levels, allowing the mice to recover. 2) siRNA molecules were intravenously administered via the mouse tail vein with the expectation that delivery would be systemic. It is possible that siRNA delivery efficiency was low at the site of infection. Future studies will examine the timing of siRNA administration along with a dose response curve to determine optimal concentration. Finally, an obstacle to overcome with siRNA administration is delivery. Results seen could also be due to the inefficient delivery of the siRNAs. Small molecule inhibitors developed using these data, may prove to be more effective.

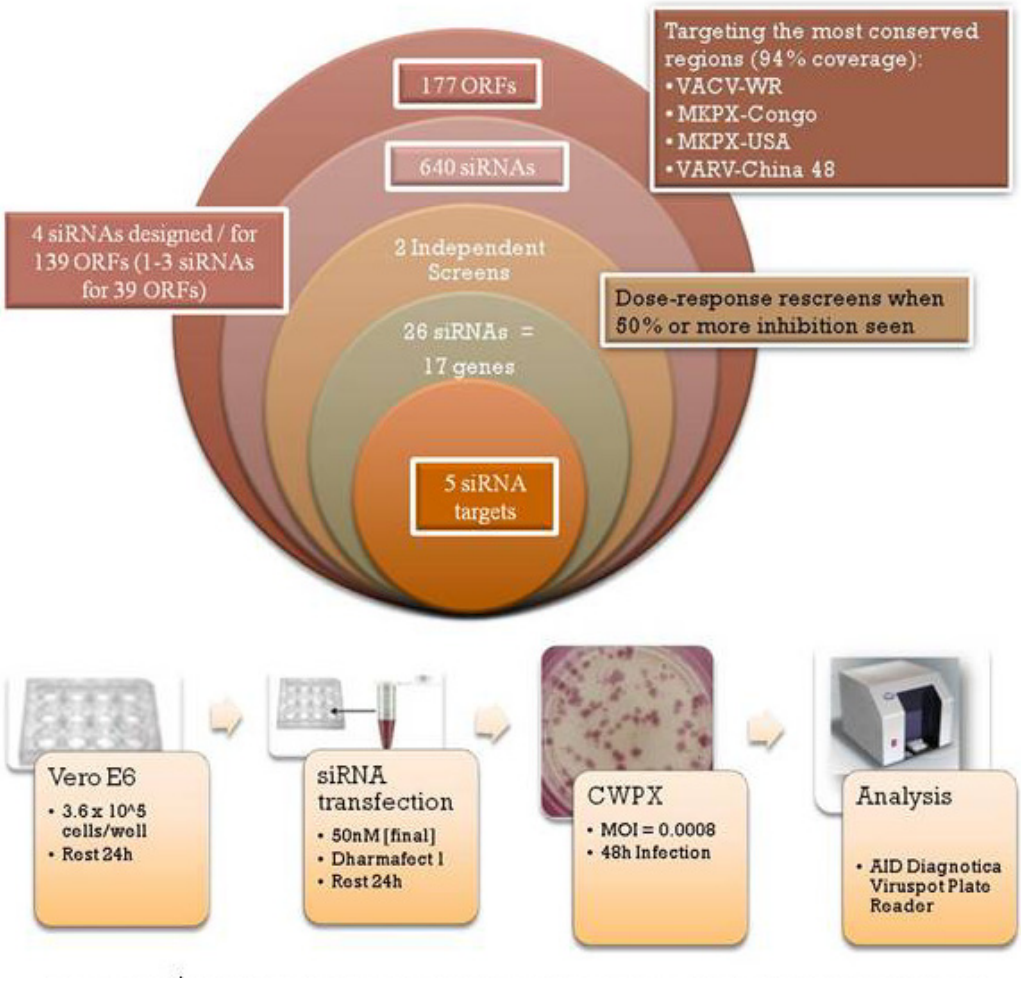


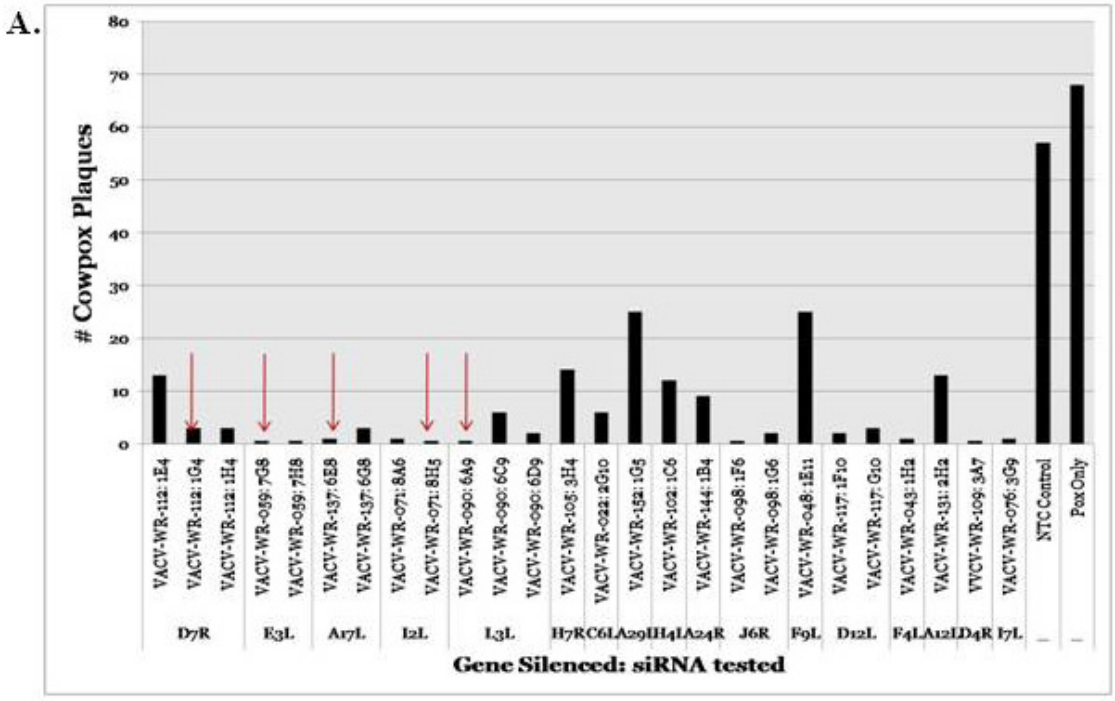
Figure 3.1 | Experimental protocol for screening the pox virus siRNA library and overall results.

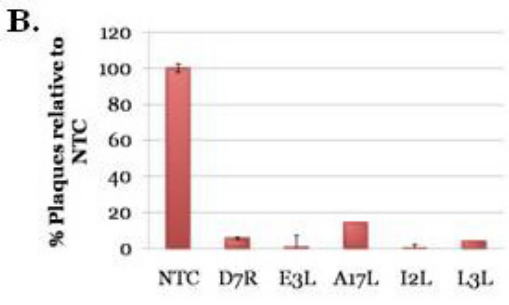
Table 3.1 | siRNA sequences and qPCR primers for siRNA library hits.

Gene CDC seq #	A17L VV_WR_137	D7R VV_WR_112	E3L VV_WR_059
siRNA Target Seq. (5' - 3')	AAGATGGAGGTATGATGCAAA	AAGTCACTGCCGTTCTAATGG	AAGCTCTGTACGATCTTCAAC
	AACAGCAGCAATCGTTTATGC	AACCACACGAGCTGACGTTAG	AAGAGTATTCGATAAGGCAGA
	AACATCGAAAGACAGGACTAT	AACGTTCCGTGTGTAATAACC	AACGTAGTGCTATGGTGTACA
	AACGATTATGGAGGAATGAAT	AATGCCGTATATAAGACGTAT	AATGAGTACTGCCAAATTACT
Sense (5' - 3')	AATTTGCATCATACCTCCATCCCTGTCTC	AACCATTAGAACGGCAGTGACCCTGTCTC	AAGTTGAAGATCGTACAGAGCCCTGTCTC
	AAGCATAAACGATTGCTGCTGCCTGTCTC	AACTAACGTCAGCTCGTGTGGCCTGTCTC	AATCTGCCTTATCGAATACTCCCTGTCTC
	AAATAGTCCTGTCTTTCGATGCCTGTCTC	AAGGTTATTACACACGGAACGCCTGTCTC	AATGTACACCATAGCACTACGCCTGTCTC
	AAATTCATTCCTCCATAATCGCCTGTCTC	AAATACGTCTTATATACGGCACCTGTCTC	AAAGTAATTTGGCAGTACTCACCTGTCTC
qPCR Fwd. Primer (5' - 3')	ATA TGC TTG ACG ACT TCT CTG CGG	AAC CAC ACG AGC TGA CGT TAG ACA	ATG TCT AAA ATC TAT ATC GAC GAG C
qPCR Rev. Primer (5' - 3')	GGC ATA AAC GAT TGC TGC TGT TCC	ATT TCG CCG AGA GGT AAT TCC ACG	TCA GAA TCT AAT GAT GAC GTA ACC
	I2L VV_WR_071	L3L VV_WR_090	
siRNA Target Seq. (5' - 3')	**XNBIO-1183, PLATE 8 (H5)	AAGGCGGCTAAACGTATTTCT	
		AAGCCTAAACCAAGACTACAG	
	siRNA # 159_1	AACCAAGACTACAGCCTAATC	
		AACCTGGTACACTGGTATATG	
Sense (5' - 3')	AAAGAAATACGTTTAGCCGCCCCTGTCTC		
	AACTGTAGTCTTGGTTTAGGCCCTGTCTC		
	AAGATTAGGCTGTAGTCTTGGCCTGTCTC		
	AACATATACCAGTGTACCAGGCCTGTCTC		
qPCR Fwd. Primer (5' - 3')	GGG TCT CCG GAA GAT GAT TTG ACA	TTC TAT CTA TTT GCG GCA ACC GGC	
qPCR Rev. Primer (5' - 3')	CCA ACA ACC GGT ATT ATT AGT TGA TGT GAC	GGC TAT TGT CTC ATG GCC GTG TAT	

**I2L siRNA sequence unavailable at time of printing. The official library designation for this siRNA is listed along with the plate number and well location. In addition, the siRNA name as designated in the siRNA library documentation is provided.

Figure 3.2 | Small-interfering RNA molecules targeting individual viral genes inhibit Cowpox virus multiplication. Vero E6 cells were transfected with poxvirus-specific siRNAs for 24 hours. After removal of transfection medium, the cells were infected with Cowpox virus. At 48 hours post-infection, cells were fixed and stained with crystal violet. **A**) Representative plaque counts obtained from *n*=5 independent screening experiments. Solid arrows indicate siRNA molecules selected for further study. **B**) Transfection of cells with representative poxvirus-specific siRNAs shown as percentage of siNTC control plaque numbers. Error bars represent the standard deviation of mean values from quadruplicate wells from *n*=3 experiments. **C**) Plaque images of cells transfected with siRNAs, including non-target control (NTC), 48 hours after infection.


A.
Cowpox Plaques
80
70
60
50
40
30
20
10
0
VACV-WR-112: 1E4
VACV-WR-112: 1G4
VACV-WR-112: 1H4
VACV-WR-059: 7G8
VACV-WR-059: 7H8
VACV-WR-137: 6E8
VACV-WR-137: 6G8
VACV-WR-071: 8A6
VACV-WR-071: 8H5
VACV-WR-090: 6A9
VACV-WR-090: 6C9
VACV-WR-090: 6D9
VACV-WR-105: 3H4
VACV-WR-022: 2G10
VACV-WR-152: 1G5
VACV-WR-102: 1C6
VACV-WR-144: 1B4
VACV-WR-098: 1F6
VACV-WR-098: 1G6
VACV-WR-048: 1E11
VACV-WR-117: 1F10
VACV-WR-117: G10
VACV-WR-043: 1H2
VACV-WR-131: 2H2
VVCV-WR-109: 3A7
VACV-WR-076: 3G9
NTC Control
Pox Only
D7R
E3L
A17L
I2L
L3L
H7R
C6L
A29L
H4L
A24R
J6R
F9L
D12L
F4L
A12L
D4R
I7L
Gene Silenced: siRNA tested


B.
% Plaques relative to NTC
120
100
80
60
40
20
0
NTC
D7R
E3L
A17L
I2L
L3L

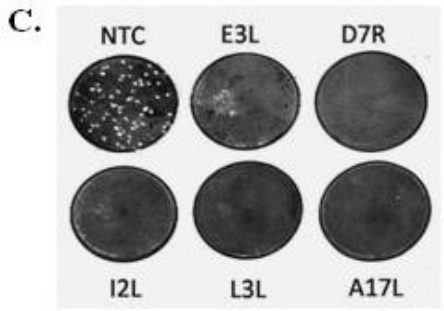

C.
NTC
E3L
D7R
I2L
L3L
A17L

Table 3.2 | siRNAs targeting a variety of poxvirus genes which when transfected into Vero E6 cells 24 hours prior to infection with CWPV or VACV inhibit viral multiplication by 50% or greater.

Vaccinia-Western Reserve*	VACV-COP[Δ]	Temporal Expression	PBR Database Functional Description[2]	% Reduction in Replicatior	Essential for Replication[#]	Confirmation By Deletion Mutagenesis	Ref.
022	C6L	E	Unknown	52	N	N	1
043	F4L	E	Ribonucleoside reductase small subunit	52	N	N	2,3,4,5
048	F9L	E[1]/L[5]	Disulfide bond formation pathway protein	56	N	Y	1,5
059	E3L	E/L	IFN resistance and PKR inhibitor (double-stranded RNA binding protein)	95	Y	Y	1,5
071	I2L	U	Unknown; involved in entry	99	Y	Y	1,6,7
076	I7L	L	Viral core cysteine proteinase	70	Y	Y	1,5,6,8
090	L3L	L	Internal virion protein required for transcription of vaccinia virus early genes	93	Y	Y	1,5,9,10
098	J6R	E	RNA polymerase 147 subunit	95	Y	N	2,5,11
102	H4L	L	RNA polymerase-associated transcription specificity factor RAP94	76	N	Y	2,5
105	H7R	E^/L[2,5]	Unknown	53	N	N	1,5
109	D4R	E	Uracil DNA glycosylase	69	N	Y	1,5,12
112	D7R	E	DNA-dependent RNA polymerase subunit	95	Y	Y	1,5,13,14
117	D12L	E	small subunit of 5' mRNA capping enzyme; early transcription termination factor; intermediate transcription initiation factor	91	Y	Y	1,15
131	A12L	E^/L[2,5]	Virion core protein Structural protein	81	Y	Y	1,2,5,16
137	A17L	L	IMV membrane protein required for morphogenesis	88	Y	Y	1,5,17
144	A24R	E	DNA-dependent RNA polymerase 132 subunit	78	Y	N	1,9,18
152	A29L	E	DNA-dependent RNA polymerase 35 subunit	56	N	N	1,5,19

*VACV-WR complete genome - GenBank accession: NC_006998
[Δ]VACV-COP genome map - GenBank accession: M35027
[#]Replication is inhibited by 70% or greater.
[^]Expression pattern confirmed personally through qPCR analysis
[1]Expression pattern confirmed personally through qPCR analysis along with the Poxvirus Bioinformatics Resource Center.
[2]Poxvirus Bioinformatics Resource Center: www.poxvirus.org

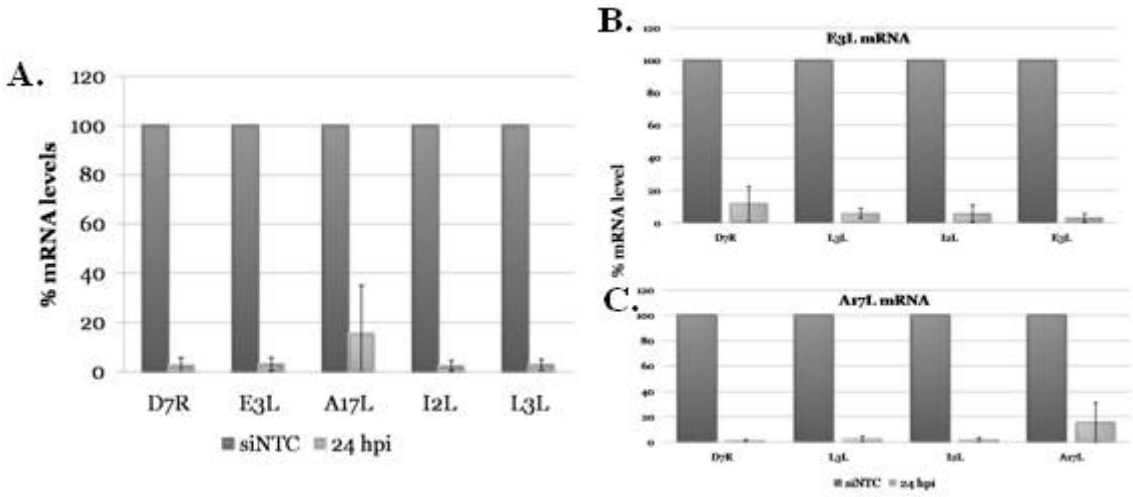


Figure 3.3 | Downregulation of poxvirus mRNA levels using RNAi. Vero E6 cells were transfected for 24h with viral gene-specific siRNA molecules followed by infection with CWPV, MOI= for 24h. The mRNA levels of each gene were measured by qPCR. **A)** Gene-specific downregulation was standardized against respective gene levels in samples transfected with the negative control, siNTC. **B)** Levels of E3L or **C)** A17L mRNA were determined in cells transfected with either D7R, L3L, or I2L-specific siRNA molecules in comparison to cells transfected with siNTC or the siRNA specific for either E3L or A17L, respectively. Each data point is the average of results from triplicate wells and represent *n*=2 independent experiments.

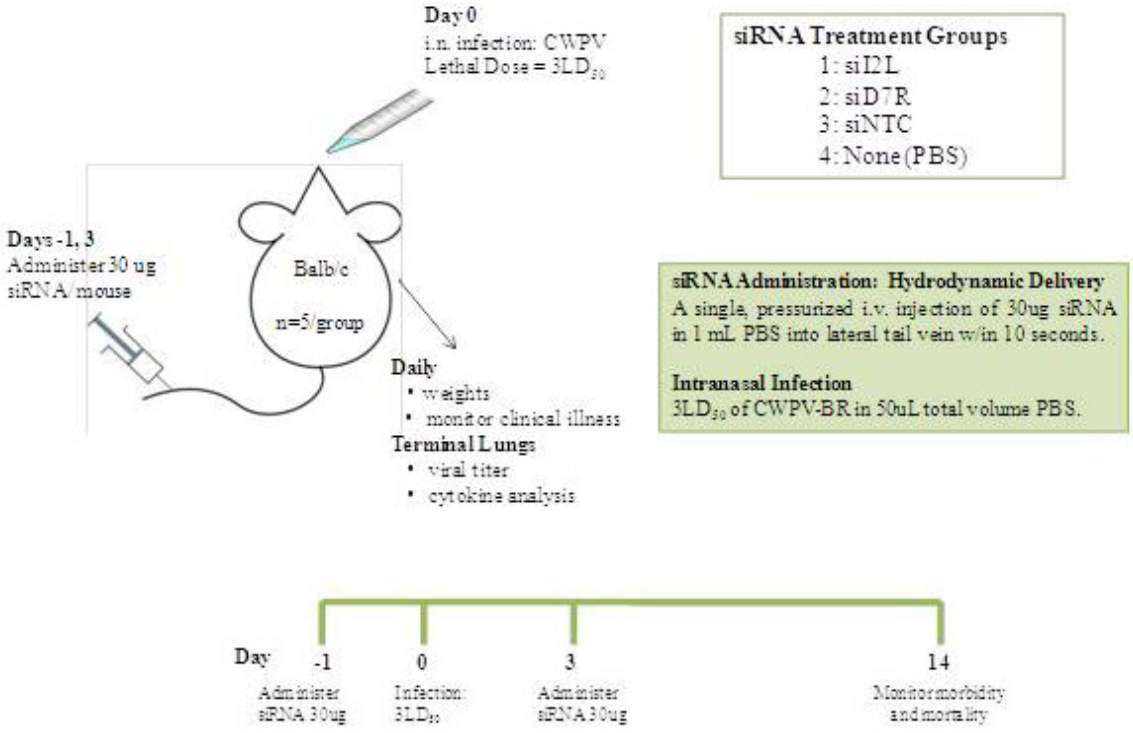
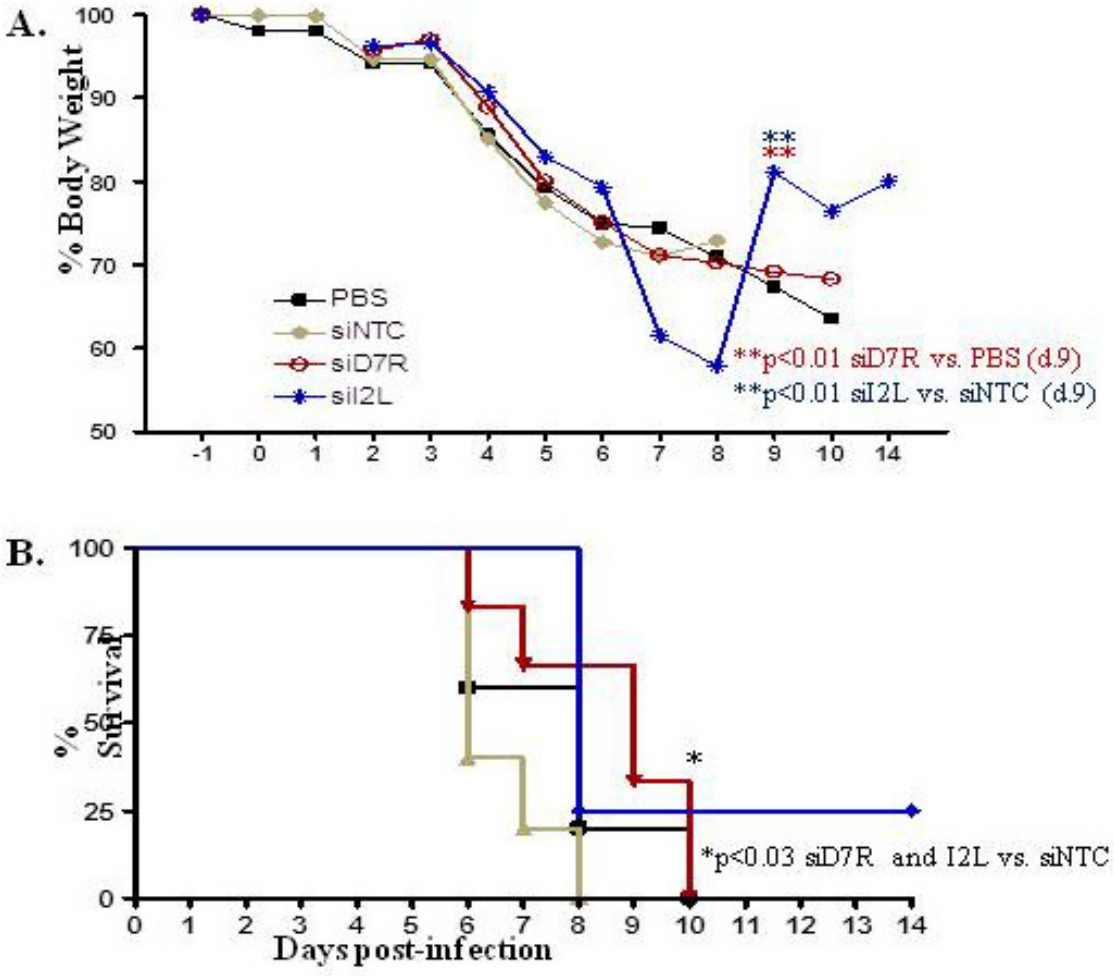


Figure 3.4 | siRNA in vivo experimental layout.

Figure 3.5 | Poxvirus-specific siRNA treatment partially protects mice against lethal challenge with $3LD_{50}$ of CWPV. BALBc mice (five per group) were treated with siD7R, siI2L or siNTC and challenged with CWPV. The percent of initial body weight **A**) and survival post-challenge **B**) are shown. **A**) Groups differ for weight loss, **$p<0.01$ (Two-Way Anova). The single surviving mouse from the siI2L-treated group was recovering at experiment termination. **B**) siD7R and siI2L groups differ from the siNTC group for survival, *$p<0.03$ by survival curve comparison.


A.
% Body Weight
100
90
80
70
60
50
-1
0
1
2
3
4
5
6
7
8
9
10
14
PBS
siNTC
siD7R
siI2L
**
**
**p<0.01 siD7R vs. PBS (d.9)
**p<0.01 siI2L vs. siNTC (d.9)
B.
% Survival
100
75
50
25
0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days post-infection
*
*p<0.03 siD7R and I2L vs. siNTC

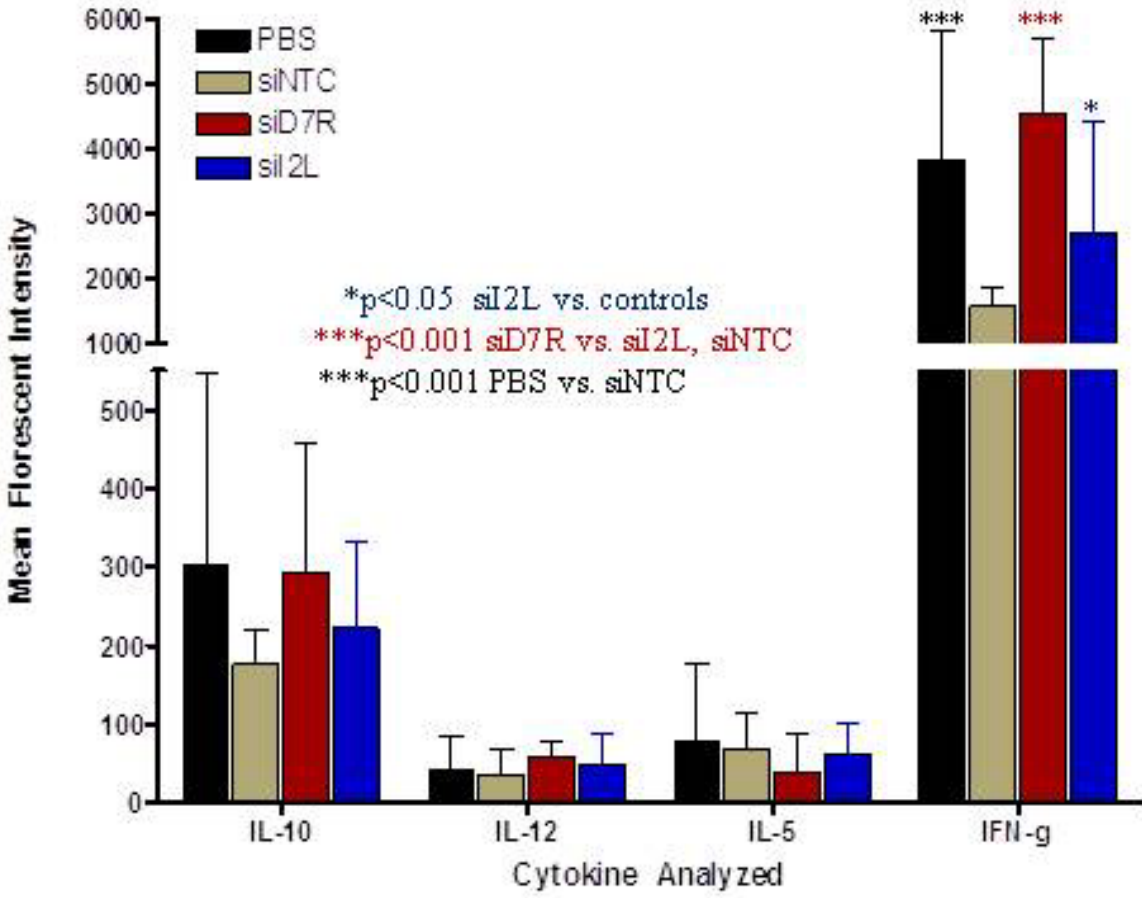


Figure 3.6 | Lung cytokine profiles after infection with CWPV 24 hours post-siRNA administration. Upon euthanasia, lungs were individually collected and homogenized with dounce homogenizer in PBS supplemented with 1% antibiotic/anti-mycotic. Supernatants were clarified via centrifugation. Lung cytokine profiles were assayed from 50µl of clarified supernatant using a Mouse Th1/Th2 6-plex kit and analyzed on a Luminex 200 system. IL-2 and IL-4 results were below the limit of detection. p-values determined by a two-way analysis of variance followed by Bonferroni's test.

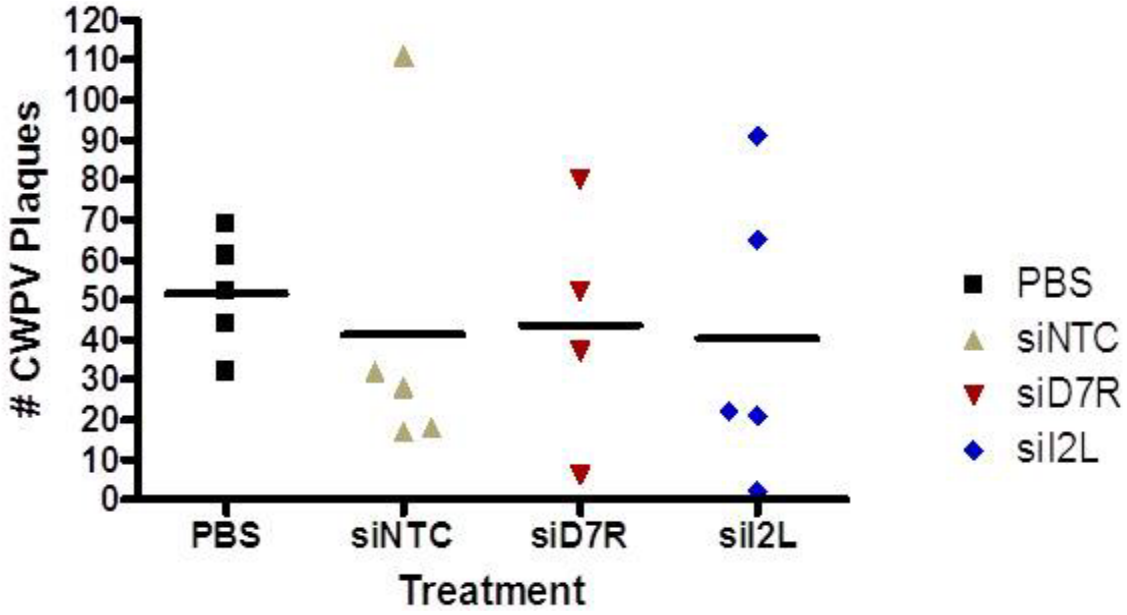
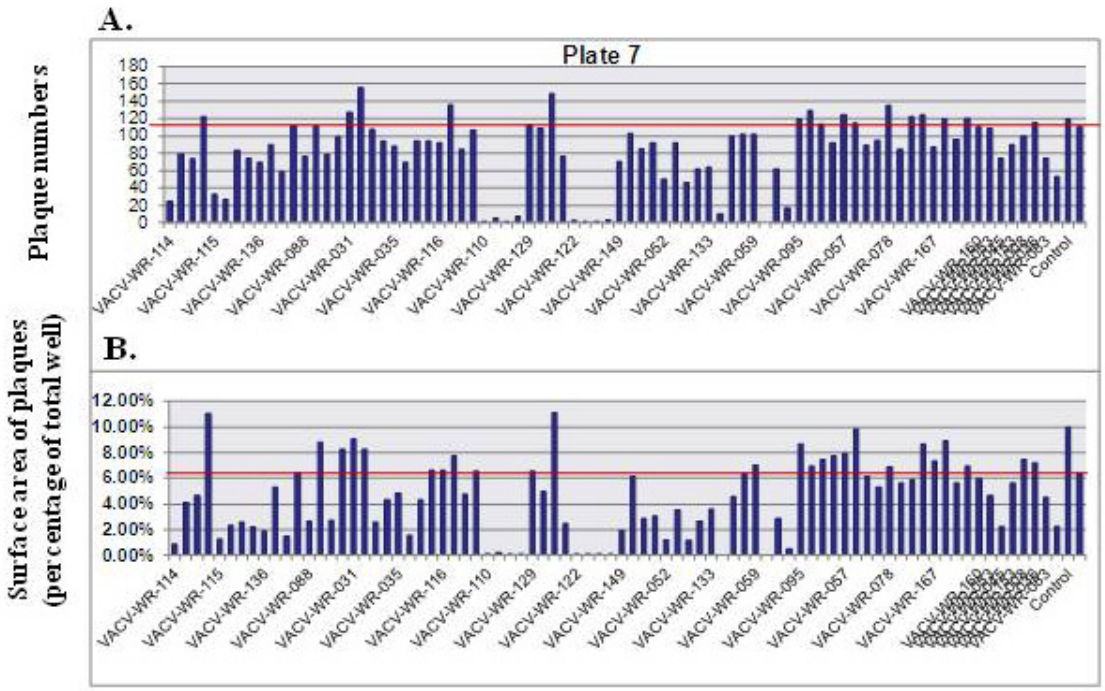


Figure 3.7 | Lung viral burden (1x10^5 dilution shown). Upon euthanasia, lungs were individually collected and homogenized with dounce homogenizer in PBS supplemented with 1% antibiotic/anti-mycotic. Supernatants were clarified via centrifugation. and supernatants titered on Vero E6 cells. Plaques were counted and imaged as previously described.

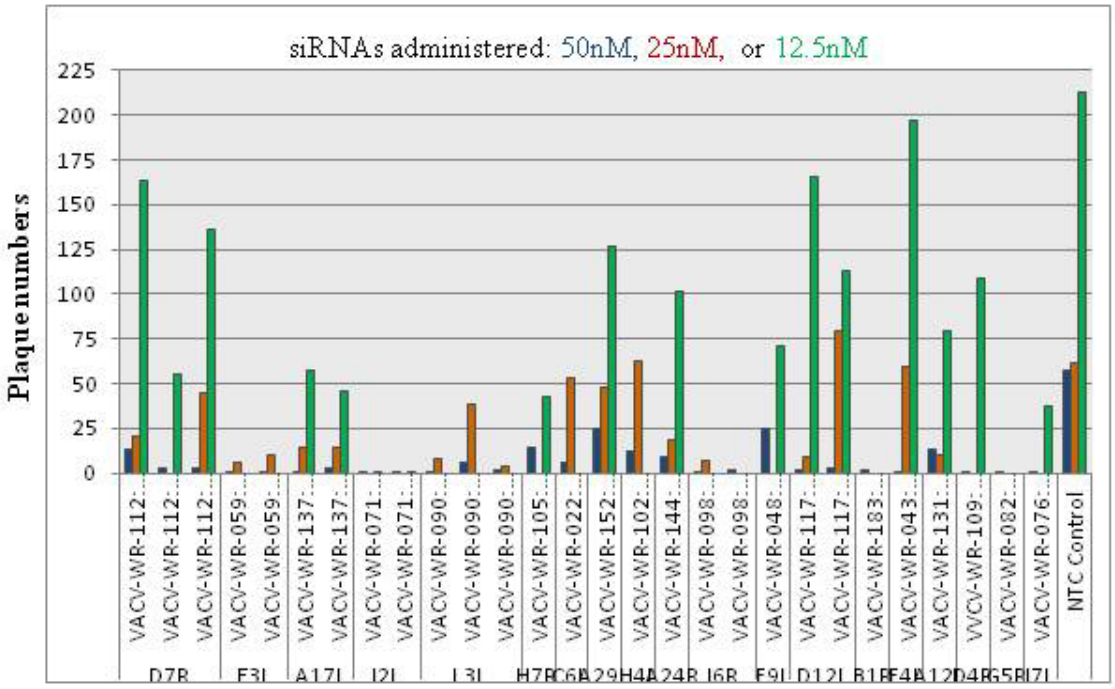
Supplementary Table 3.1 | Criteria for humane euthanasia of mice post-CWPV infection.

Clinical presentation/symptoms	Points *
weight loss (>35%)	3
emaciation	1
dyspnea	1
hunched back	1
cyanosis	1
failure to respond to external stimuli	3
lesions on the tail	1

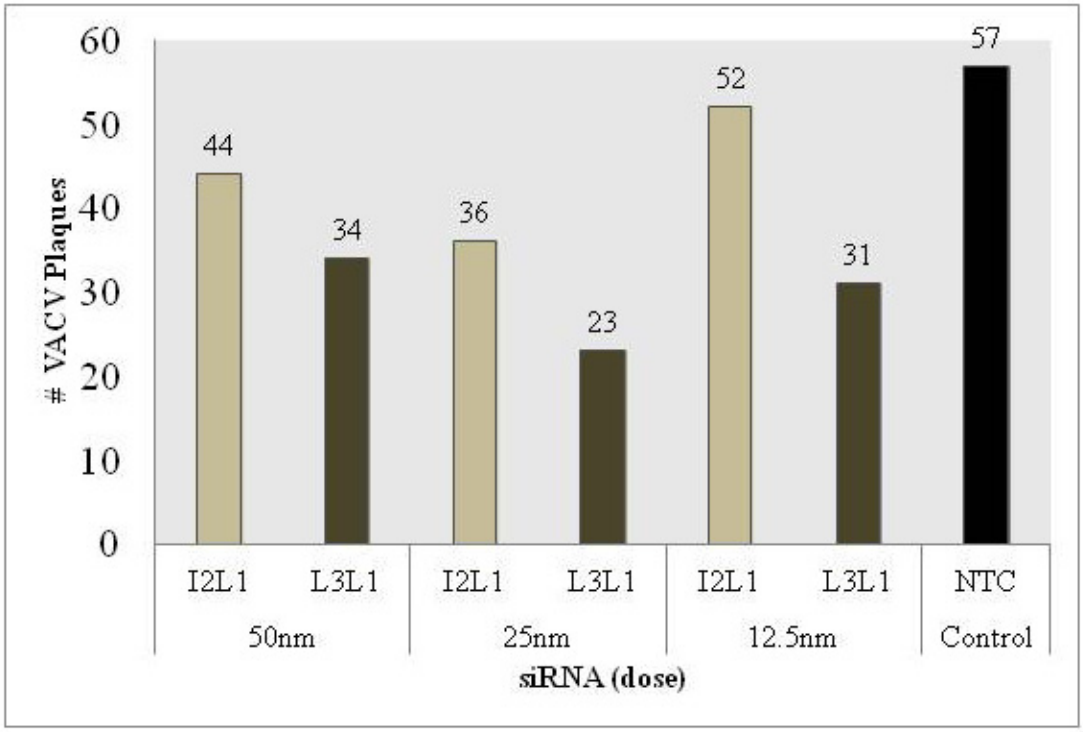
* Any animal that presents with 3 or more points will be humanely euthanized.



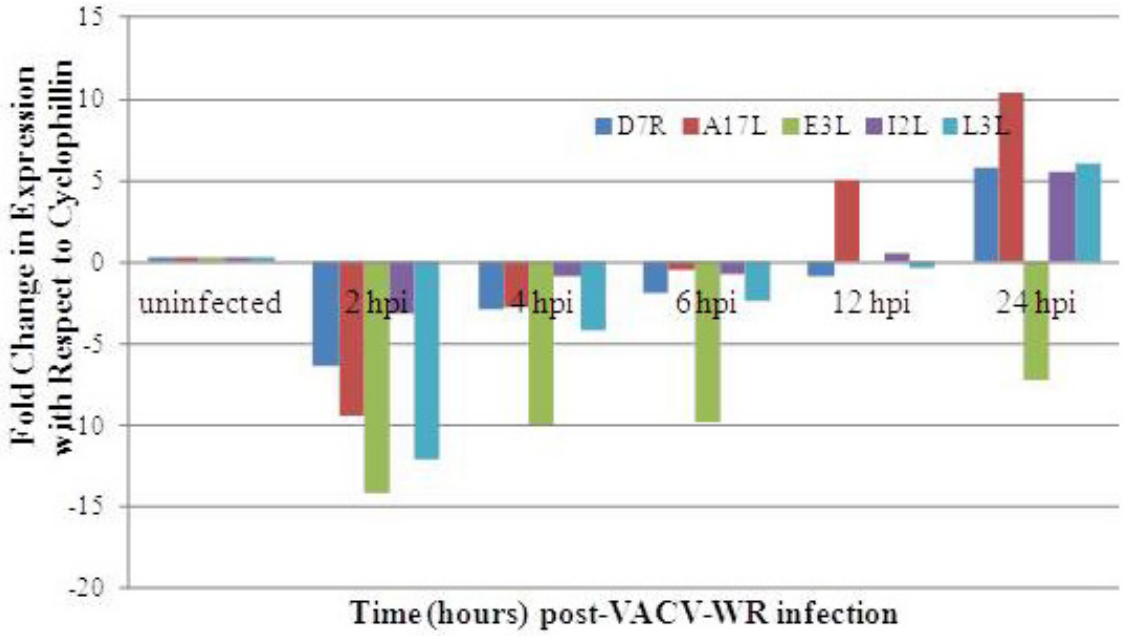
Supplementary Figure 3.1 | Representative data from primary screen. Vero E6 cells were transfected with pox-specific or control siRNAs as described. Twenty-four hours later, the cells were infected with cowpox virus. Forty-eight hours post-infection, plates were stained and plaques quantified as described. **A**) Total cowpox plaques per well were counted and compared to wells transfected with a NTC siRNA. **B**) Surface area of plaques as a percentage of total well coverage was measured and compared to control. The siRNA library was composed of 8 96-well plates. **A** and **B** represent data gathered from a single 96-well plate screened.



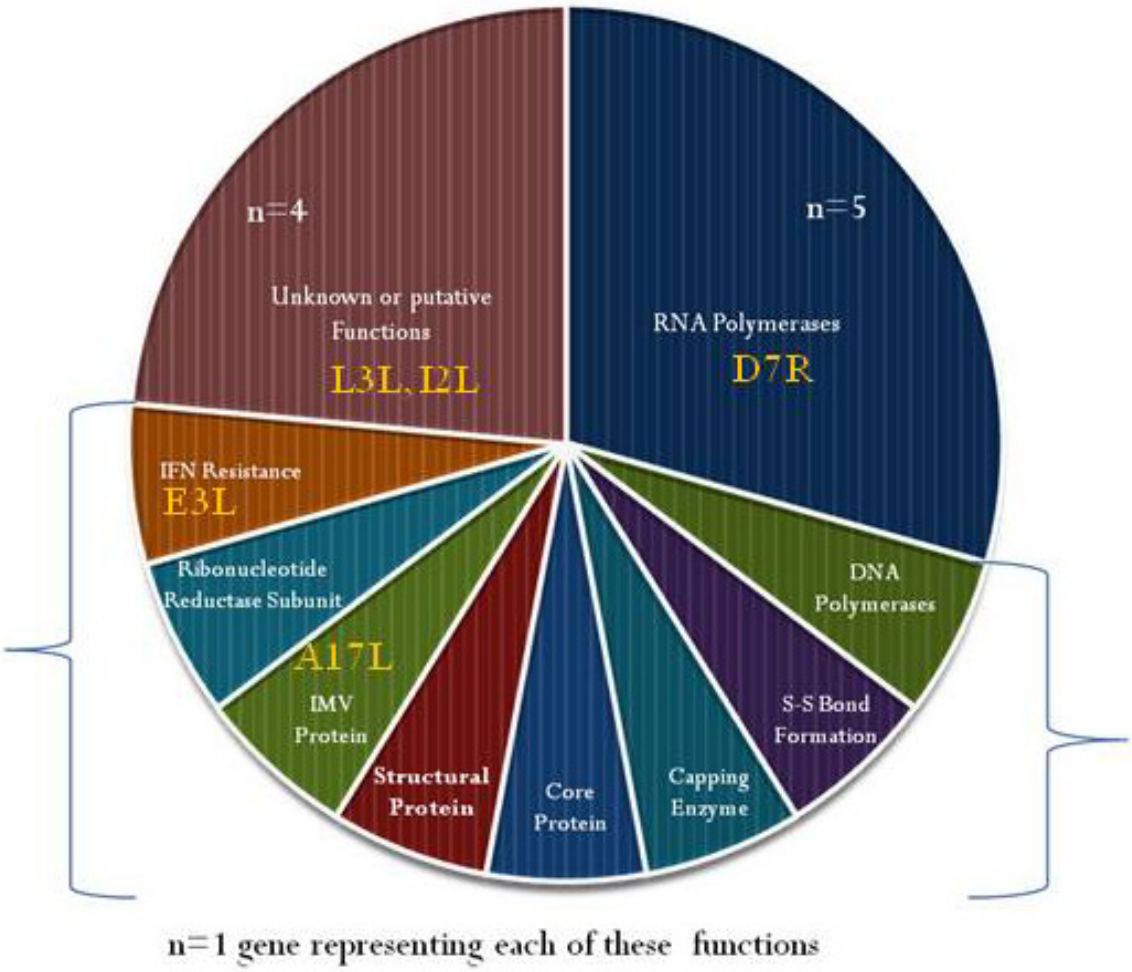
Supplementary Figure 3.2| Rescreen of siRNA hits from primary library screen. Vero E6 cells were transfected with pox-specific siRNAs at 50, 25 and 12.5nM final concentration and infected with Cowpox virus (strain Brighton Red) at MOI of 0.006. Replication was measured using a plaque reduction assay 48 h post-infection. Virus replication is compared to the negative control, NTC siRNA plaque counts. Each data point is the average of duplicate wells in *n* = 3 independent experiments.



Supplementary Figure 3.3 | The effect of two doses of siRNA at varying concentrations necessary to achieve effective gene silencing upon VACV infection. Vero cells were plated at 2.2 x 10^5 cells/well and transfected with the indicated siRNA at 12.5. 25, 50 or 100nM for 24h, then infected with VACV-WR (MOI=0.008). At 2hpi, cells were rinsed with PBS and transfected with a 2nd dose of siRNA at indicated concentrations. After 48 hours, cells were fixed and stained with crystal violet. Each data point is the average of results from duplicate wells and represent n=2 independent experiments.



Supplementary Figure 3.4 | Kinetics of poxvirus gene expression. Orthopoxvirus ORFs were assayed for expression kinetics via qPCR analysis. Vero E6 cells plated and upon reaching confluency, infected with VACV-WR, MOI =1. At baseline time 0, 2, 4, 6, 12and 24hpi total RNA was purified from infected wells. mRNA levels of each gene listed were measured using real-time qPCR. Data were normalized to cellular cyclophillin levels. Each data point is the average of triplicate wells and represent n=2 independent experiments.



Supplementary Figure 3.5 | Functional groups of open reading frames identified as essential for orthopoxvirus replication in vitro.

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CHAPTER 4

SCREENING A SMALL COMPOUND ARRAY IDENTIFIES METHOTREXATE, RALTITREXED, HOMOHARRINGTONINE AND IDARUBICIN AS COMPOUNDS WITH ANTI-ORTHOPOXVIRUS EFFICACY[1]

[1] Cadet, VE, Michel, F, Gao, X, Hogan, RJ. To be submitted to *Antiviral Research*.

Abstract

Poxvirus zoonoses have been well documented in recent years. Cowpox infected cats, rodents and cattle, in close contact with humans, have been reported to transmit the virus through open wounds, large respiratory droplets and fomites. The emerging infectious pathogen monkeypox has been noted to undergo serial human transmission. Vaccinia virus has been shown time and again to be transmissible through direct physical and indirect contact from recent vaccinees to unvaccinated individuals along with transmission from infected cattle to humans. Typically, disease course is mild and self-limiting; however severe generalized infections have been described in patients with atopic dermatitis or immunosuppression. With the waning of specific immunity in the general population combined with the severity of complications occurring post orthopoxvirus infections in the immunocompromised, the need for continued research and development of therapies has been underscored. To address the deficiency of FDA-approved poxvirus treatments, a small molecule library was screened for the purpose of illuminating compounds which inhibit poxvirus multiplication. We have identified four compounds, each currently in use as chemotherapeutics and/ or immunosuppressive drugs, which protect *in vitro* at concentrations as low as 0.04 micromolar. The four compounds identified, methotrexate, idarubicin, homoharringtonine, and raltitrexed, were further examined in a dose-response manner in Type II pneumocytes and methotrexate was chosen for *in vivo* drug efficacy analysis. These results identify methotrexate as a candidate from which derivatives can be studied for potential administration to protect against human orthopoxvirus infections. They also highlight several other potential compounds which can be further explored for in vivo protective efficacy in future studies.

Introduction

Although smallpox was eradicated in 1980, orthopoxvirus zoonoses have been well documented in recent years. For example, human cowpox (CWPV) infections have been noted as recently as 2011 via contact with infected dairy cattle along with domestic pet cats and small rodents, primarily [1-3]. Significant economic losses have been encountered in several parts of the world due to reemerging buffalopox infections in buffalo, cattle, and humans [4-6]. Monkeypox (MPXV), another orthopoxvirus, spread to a cluster of patients in the midwestern U.S. in 2003, the first outbreak of its kind in the western hemisphere, thus highlighting the risk of transmission from infected pets to humans [7-10]. The prototypic member of the genus, vaccinia virus (VACV), is the live vaccine agent utilized to confer protection against variola virus (VARV), the causative agent of smallpox disease. It has been repeatedly shown to be transmissible through direct physical and indirect fomite contact from vaccinated to unvaccinated individuals [11-13]. Given that much of the general population in the U.S. has not been vaccinated nor possesses immunity to poxviruses, together with the severity of complications that occur following VACV infection of immunocompromised persons, combined with the orthopoxvirus zoonoses experienced in at least four of the seven continents of the world, the need for continued research and development of therapies for poxviruses has been underscored. Presently, there is a significant lack of antiviral therapies licensed to treat any of the myriad of poxvirus infections.

To directly address the deficiency in FDA-approved poxvirus treatments, the NIH Clinical Collection (NCC), a small molecule array composed of compounds which have almost all completed phases I-III trials, was acquired [14]. All compounds were selected

for inclusion in the array based upon favorable attributes such as purity, solubility, commercial availability, along with extensive safety and bioavailability profiles. We screened the library *in vitro* for individual compound ability to inhibit CWPV and VACV replication in Vero cells, a vaccine-approved cell line. From this screen, four drugs currently in use as chemotherapeutics and/or immunosuppressives were identified: methotrexate (MTX), idarubicin (IDA), homoharringtonine (HHT), and raltitrexed (RTX).

Methotrexate (aminomethylpteroylglutamic acid) is an antimetabolite nucleoside analogue compound introduced for clinical use in the 1950s [15]. Historically, it has been used to treat a broad range of neoplastic disorders including acute lymphoblastic leukemia, non-Hodgkin's lymphoma, bladder carcinoma, breast cancer and testicular tumor [16-19]. Recent literature reports that it is also effective in treating some autoimmune diseases [20], ectopic pregnancy and the induction of medical necessary abortions [19]. MTX directly competes with folic acid (FA), an essential co-factor for the enzyme dihydrofolate reductase (DHFR) involved in the *de novo* synthesis of DNA nucleotides, purines and pyrimidines. The inhibition of DHFR leads to imbalanced nucleotide pools, disrupting DNA synthesis [15, 19, 21].

Methotrexate antagonist leucovorin (folinic acid, FNA) is the active metabolite of folic acid. Leucovorin is used principally as its calcium salt and is an antidote to folic acid antagonists which block the conversion of folic acid to folinic acid. FNA has been shown to bypass the MTX-induced inhibition of DHFR as it directly provides the necessary tetrahydrofolates (THF) intracellularly. The protective effects of exogenous FNA against MTX-induced chromosomal damage has been noted in various studies upon

administration of FNA from 0.5-6 hours post-MTX treatment [15, 19, 21-23]. Further, post-treatment with FNA ameliorates the toxicity of MTX without compromising its therapeutic efficacy [23]. VACV has been shown to be inhibited to varying degrees in cells treated with MTX. [24]

Raltitrexed, a second antifolate compound identified, is a compound used in cancer chemotherapy and is chemically similar to folic acid (FA). It inhibits thymidylate synthase. (TS) and the formation of precursor pyrimidine nucleotides is hindered, ultimately, preventing DNA and RNA synthesis [17, 25]. HHT is a small molecule plant alkaloid, derived from the Cephalotuxus fortuneii tree that is used in the treatment of both acute and chronic myeloid leukemia. Upon administration, HHT inhibits protein synthesis by binding to ribosomes and inhibiting polypeptide chain elongation. Its cytotoxicity is cell-cycle specific, primarily affecting cells in G1 and G2 phases in a manner directly proportional to the extent of inhibition of protein synthesis. This inhibition leads to an upregulation of genes associated with apoptosis and the inhibition of angiogenesis, all in a dose and time dependent manner [26, 27]. IDA (4-demethoxydaunorubicin)] is an antileukemic compound in a family called antitumor antibiotics. It is a DNA-intercalating analog of daunorubicin. Mechanistically, it inserts itself into DNA, thereby preventing unwinding. The resulting inhibitory effect on nucleic acid synthesis is due to its interaction with the cellular enzyme topoisomerase II. [15, 26, 28]

We report that in vitro screening of the NCC has identified four compounds as potent inhibitors of VACV and CWPV replication. After validation, MTX was chosen for further analysis of drug efficacy in a Balb/c model of CWPV infection *in vivo.*

*S*ubsequent dose-response studies were carried out in Type II pneumocysts. We have also extended the work of previously published studies using MTX to protect against VACV [24, 29-32] by providing means to counter the induced toxicity observed upon compound administration [18, 19, 22, 23]. These results identify a candidate from which derivatives can be studied for potential administration to protect against human orthopoxvirus infections. They also highlight several other potential compounds which can be further explored for in vivo protective efficacy in future studies.

Materials and Methods

Cell culture and viruses. Vero E6 (Vero, ATCC: CRL-1586) and A549 (ATCC: CCL-185) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) at 37°C, 5% CO_2. Mouse lung epithelial (MLE)-15 cells were kindly provided by Dr. Kimberly Klonowski (University of Georgia) and propagated in Hites medium (RPMI-1640 supplemented with the following: insulin/ transferrin/ selenite, human transferrin, hydrocortisone, 200mM L-Glutamine, β-estradiol, 1% HEPES, and 4% fetal bovine serum), pH 7.4 at 37°C, 5% CO_2. Cells used in all experiments were determined to be mycoplasma-free and grown without antimicrobial supplementation. CWPV-Brighton Red (BR) and VACV-Western Reserve (WR) were the generous gift of Dr. Bernie Moss (NIH). All viruses were propagated in Vero cells as previously described [33].

Screen of the NIH Clinical Collection and primary hit validation. Two independent screens of n=446 compounds were carried out (Biofocus DPI, Inc: NCC-002) (**Figure 4.1**). Vero cells were plated at $1.0x10^4$ cells/well in standard 96-well plates at a volume of 150µl DMEM supplemented with 5% FBS. Upon cells reaching ~80% confluency, each compound was administered at a final concentration of 10µM. 24 hours post-treatment, cells were infected with CWPV and incubated for 48 hours prior to being fixed with ice-cold methanol and stained with with 0.13% crystal violet containing a final concentration of 5% methanol and 10% formaldehyde v/v. Treatment efficacy was assessed by measuring crystal violet absorbance at optical density of 530 nm (OD_{530}). Final absorbance per well was calculated in comparison to infected, non-treated control wells. Compounds identified were rescreened in Vero cells at concentrations ranging from 20uM to 0.04µM and 4 hits identified. Subsequent type II pneumocyte drug validation was carried out at 5, 1, 0.2 and 0.04µM concentrations in A549 and MLE-15 cells. They were plated at $4.0x10^5$ cells/well in standard 12-well plates in 1mL DMEM supplemented with 5% FBS or Hites media, as indicated, and treated with the compounds at varying concentrations. 48 hours post-infection with CWPV, monolayers were fixed and stained as described above. Plaques were counted using a dissecting microscope.

Chemicals. MTX [PubChem Compound ID (PCCID): 126941] and HHT (PCCID: 285033) were purchased from Tocris Bioscience: Cat. No. 1230 and 1416, respectively. IDA (PCCID: 42890), RTX (PCCID: 104758) and FNA (PCCID: 143) were purchased from Sigma-Aldrich: Prod. No. I1656, R9156 and F7878, respectively (**Fig 4.2**).

Compounds were reconstituted in H_20, PBS, or PBS/DMSO according to manufacturers' recommendations.

Cell viability. The viability of Vero cells plated at $1.0x10^4$ and $2.0x10^4$ cells/well and treated with the various compounds at previously determined optimal concentrations was assessed using quantitative colorimetric measurement of lactate dehydrogenase (LDH) release upon cell lysis of treated cells according to the manufacturer's instructions (Promega: G1780).

Mouse infection and treatment protocol. Female BALB/c mice (National Cancer Institute) were maintained in microisolator housing with food and water provided ad libitum. All mice were specific-pathogen free and aged 8-10 weeks at time of experiment. In three independent experiments (n=4-7 mice/group), mice were administered 5mg/kg/day, 20mg/kg/day or 40mg/kg/day of MTX in 100-200µl PBS with or without the addition of FNA within 1 or 6 hours post-MTX administration via intraperitoneal injection for rescue of MTX-induced toxicity or at the same time give FA supplementation. Control mice were administered PBS, PBS/DMSO, FNA, or FNA in combination with MTX. Experimental layouts can be seen in **Supplementary Figures 4.1** and **4.2.** Under light anesthesia (2,2,2-tribromoethanol by intraperitoneal injection) mice were inoculated intranasally with 3 times the previously determined 50% lethal dose (LD_{50}) of CWPV diluted in 50µl PBS (final administered dose: $2.16x10^5$ PFU) or PBS on day 0. Mice were weighed daily, observed for signs of illness and euthanized upon reaching 30% weight loss and/or an apparent clinical score of

3 (**Supplementary Table 3.1**). All studies were carried out in accordance of the University of Georgia Institutional Animal Care and Use Committee.

Lung analyses. Upon euthanasia, lungs were individually collected and homogenized with a glass dounce homogenizer in PBS supplemented with 1% antibiotic/anti-mycotic [(Cellgro: 30-004-CI) a combination of 10,000 units/ml penicillin G, 10 mg/ml streptomycin sulfate and 25 µg/ml amphotericin B with 8.5 g/L NaCI]. Supernatants were clarified via centrifugation at 13,000 rpm for 20 minutes and titered on Vero cells. Plaques were counted and imaged as previously described. Lung cytokine profiles were assayed from 50µl of clarified supernatant using the Mouse Th1/Th2 6-plex kit (Invitrogen: LMC0002) and analyzed on a Luminex 200 system.

Statistical Analysis. Data were analyzed using GraphPad Prism 5.01. One-way Anova with Dunnett's Multiple Comparison Test was used to identify significant differences in weight. Newman-Keuls Multiple Comparison test of survival differences was used to test for significance in length of survival (days). Two-way Anova with a Bonferroni post-test was used to test for significant differences in lung cytokine values. Differences with $p < 0.05$ were considered significant.

Results

Screening and identification of hit compounds. The NCC compound library was designed by the NIH to include drug-like chemicals which had not been represented in prior arrayed collections. There are 446 compounds distributed among six 96-well plates, each supplied in a 50µl aliquot of a 10mM solution in 100% DMSO. The majority of included molecules has been through clinical trial phases I-III and have extensive bioavailability data publicly available. In addition, most have well-characterized safety profiles. The collection targets fourteen major systems and/or disease states (**Figure 4.1A**). [14]

A summary of the library screen and hit validation can be found in **Figure 4.1B.** For each of the two independent screens, $4x10^4$ Vero cells were seeded in 96-well format and individual compounds administered at a final concentration of 10 µM. Twenty-four hours post-treatment, cells were infected with CPXV and incubated for 48 hours prior to being fixed and stained. Treatment efficacy was determined by measuring crystal violet absorbance at OD_{530}. Absorbance values were normalized to those measured in infected wells that had not received any drug treatment. Based on these results, MTX, RTX, IDA and HHT were selected for further studies (**Figure 4.2**). Examination of the chemical structures of the hits show that methotrexate and raltitrexed are very similar in structural composition. Idarubicin and homoharringtonine have similar structural properties as well (**Figure 4.3**). Their inclusion as hits also corresponded to inhibition of viral multiplication by 50% or more in the plaque reduction assays (**Figure 4.4A and B**).

Hit validation To confirm the hits identified in the primary screen, the 4 compounds were purchased from various manufacturers as listed. Their efficacy was verified when

compounds were rescreened in Vero cells at concentrations ranging from 20uM to 0.04μM (data not shown). In order to determine the optimal concentration for each compound in Vero cells, the reduction in plaques post-CWPV infection was monitored along with visual inspection of the cellular monolayer post-infection (**Figure 4.4**). The following concentrations were found to inhibit CWPV efficiently while inducing minimal cytopathic effects (CPE): MTX: 0.04uM, IDA: 0.2uM, HHT: 0.2uM and RTX: 5uM. At the highest concentration assayed, MTX showed little compound-induced CPE, while IDA and HHT were both highly toxic to Vero cells at 1 and 5uM. RTX administration provided the least drug-induced CPE upon visual inspection, however, even at 5uM, CWPV multiplication inhibition was not as complete as when cells were treated with the same concentration of MTX (**Figure 4.4A**). Vero cells were imaged 48 hours post CWPV infection in treated cells (**Figure 4.4B**).

Identification of compounds *in vitro* typically precedes *in vivo* testing. A Balb/c mouse model of intranasal CWPV infection would be utilized, therefore, cells typically found in the lung epithelium are a good *in vitro* model for determining the efficacy of each compound at the *in vivo* site of infection. Each of the four compounds were titrated to reasonably nontoxic doses in Type II pneumocytes, A549 (**Figure 4.4C**) and MLE-15 cells (**Figure 4.4D**). The compounds had varying efficacy at inhibiting CWPV multiplication in these cell lines when compared to Vero cells. At the Vero cell optimal compound concentrations, HHT and RTX proved slight toxic to A549 cells and severely cytotoxic in MLE-15 cells. IDA at 0.2uM was cytotxic in A549 but not MLE-15 cells. MTX, administered at 0.4uM, was both effective at viral inhibition and non-toxic in all three cell lines assayed.

Vero E6 cell viability when treated with NCC hit compounds as measured from LDH release. To assess the viability of Vero cells when treated with the various compounds, cells were plated at $1.0x10^4$ and $2.0x10^4$ cells/well and cell viability was measured using quantitative colorimetric measurement of lactate dehydrogenase (LDH) released into the supernatant (**Figure 4.5A**) and upon cell lysis (**Figure 4.5B**). An increase in LDH secretion would indicate a potentially cytotoxic effect of the compound on the integrity of the cell membrane. A maximum-LDH release positive control was provided by the manufacturer. As shown in **Figure 4.5A**,.there was no difference in absorbance values measured at optical density 490nm in the supernatant of cells treated with the compounds when compared to non-treated cells after 24 hours. LDH normally builds up in the cell to a small extent. An increase over physiological levels can also indicate a potential toxicity being exerted on the cell. Levels of LDH released upon lysis of the cell monolayer 24hours post treatment was similar to that observed in untreated cell levels when Vero cells were administered each of the compounds at the determined optimal concentration (**Figure 4.5B**). The exception was upon administration of MTX at 0.2uM, a dose that effectively inhibits viral replication via plaque assay (**Figure 4.4A**). At this concentration, Vero cells released LDH at approximately twice the levels of untreated cells (**Figure 4.5B**), indicating that MTX at a this concentration is likely accelerating cell death.

Methotrexate efficacy in a mouse model of poxvirus infection. To assess whether MTX, a compound effective at reducing viral replication *in vitro* had a protective effect *in vivo*, we used an established murine model of CWPV infection. Mice were administered MTX

in PBS/DMSO at 5mg/kg/day, or 20mg/kg/day in 200µl PBS via intraperitoneal injection on days -1, 2 and 5 post-infection with CWPV-BR ($3LD_{50}$). Control mice were administered PBS/DMSO (*n*=5 mice per group). Animals were monitored for morbidity and mortality until 30% of initial body weight was lost, at which point they would be euthanized, or upon regaining weight to within starting range (**Supplementary Figure 4.1**). MTX was unable to protect the mice from weight loss at the doses administered (**Figure 4.6A**). Despite the lack of protection from weight loss, MTX administration of 20mg/kg on the aforementioned schedule allowed those mice to survive an additional day in comparison to control mice (**Figure 4.6B**). Lungs were isolated from mice that succumbed to infection or were euthanized due to weight loss and homogenized in PBS, clarified via centrifugation and supernatants titered on Vero E6 cells. Plaques were counted and imaged as previously described. Lung viral burdens varied for all treatment groups. Mice that received MTX 5mg/kg exhibited the highest viral burdens, as evidenced by plaque numbers (**Figure 4.6C**). Treating mice with MTX 20mg/kg beginning 1 day pre-infection and three days post-infection proved efficacious at limiting viral replication in the lungs in comparison to treating with 5mg/kg or delaying MTX 20mg/kg administration until 2 days post-infection (**Figure 4.6C**).

Lung cytokine profiles are skewed to a Th2 response. Reports in the literature suggest that treatment with MTX results in marked suppression of IFN-g production. As CWPV induces a robust Th1 response mediated by IFNg production, experiments were undertaken to assess cytokine levels in the lungs isolated upon death. Lung cytokine profiles were assayed from 50µl of clarified supernatant (described above) using a Mouse

Th1/Th2 6-plex kit and analyzed on a Luminex 200 system. (**Figure 4.7A**) IL-10, IL-12 and IL-5 expression are measured. Mice receiving MTX 5mg/kg exhibited the highest level of IL-10 secretion, though all groups treated with MTX prior to infection were found to produce a high level of IL-10. Moderate levels of IL-5 were produced by mice administered the lowest dose of MTX (5mg/kg) as well as the PBS controls (**Figure 4.7A**). IFN-g, IL-2 and IL-4 expression was measured as well and IL-2 and IL-4 results were below the limit of detection (data not shown). IFNg, on the other hand, was strongly induced in control mice administered PBS prior to infection, while all mice that received MTX secreted low amounts of IFNg, approximately 6-fold lower (**Figure 4.7B**).

Folinic acid does not enhance MTX-mediated survival of mice from CWPV infection. In order to evaluate the ability of FNA to rescue MTX-induced cellular responses and prolong survival based on MTX-mediated protection of mice from CWPV infection, a second Balb/c mouse experiment was carried out. Mice (n=5-6/group) were treated with MTX pre-CWPV infection (3LD50) intranasally on the schedule shown in **Supplementary Figure 4.2**. Average weight by treatment group (**Figure 4.8A**) and percent survival (days) post-treatment and infection. (**Figure 4.8B**). were measured. In comparison to all other infected treatment groups, MTX 5mg/kg without FNA rescue protected mice from weight loss post-CWPV infection ($p<0.0012$). This protection correlated with survival of 40% in the MTX 5mg/kg treated mice, a level statistically significant in comparison to all other infected and treated groups ($p<0.05$). All surviving mice were recovering at experiment termination.

FNA rescue restores INFg secretion in the lungs of MTX-treated mice. Mice administered MTX without FNA supplementation and infected with CWPV experienced a depressed Th1 response (**Figure 4.7A,B**). To see if FNA supplementation restored a functional antiviral immune response to CWPV infection, lung cytokine profiles were assayed from 50µl of clarified supernatant (described above) using a Mouse Th1/Th2 6-plex kit and analyzed on a Luminex 200 system. (**Figure 4.9**) IL-10, IL-12 and IL-5 expression are measured. Mice receiving MTX 20mg/kg exhibited the highest level of IL-10 secretion, though all groups treated with MTX prior to infection were found to produce a high level of IL-10. The lowest levels were seen in the control groups and low dose MTX group of mice (**Figure 4.9A**). Moderate levels of IL-12 were produced by mice administered the lowest dose of MTX (5mg/kg), while IL-5 secretion was apparent in all treatment groups with mice administered MTX 5mg/kg +FNA exhibiting the highest levels (**Figure 4.9A**). IFN-g, IL-2 and IL-4 expression was measured as well and IL-2 and IL-4 results were below the limit of detection (data not shown). IFNg, expression was suppressed when mice were administered MTX alone and restored with FNA supplementation (**Figure 4.9B**).

Supplementation with FNA reduces lung viral burden in mice treated with 20mg/kg of MTX. Upon *e*uthanasia, lungs were individually collected and homogenized with dounce homogenizer in PBS supplemented with 1% antibiotic/ antiycotic. Supernatants were clarified via centrifugation and titered on Vero E6 cells. Plaques were counted and imaged as previously described. The administration of FNA had the effect of inhibiting viral replication in the lungs in comparison to all other treatment groups (**Figure 4.10**).

Mice treated with 5mg/kg of MTX had similar levels of virus in the lungs as PBS controls despite supplementation with FNA or experiencing a 40% survival rate.

Discussion

Screening a chemical library boasting a collection of compounds that have mostly completed clinical trials and are readily attainable with well-known attributes can have many benefits. Screening the NCC is a direct method of addressing the lack of FDA-approved drugs available to treat poxvirus infections. From this screen, four FDA-approved drugs currently in use as chemotherapeutics and/or immunosuppressives were identified as protecting Vero cells from lethal CWPV challenge: methotrexate (MTX), Idarubicin (IDA), Homoharringtonine (HHT), and Raltitrexed (RTX). Dose-response rescreens validated each of the compound's efficacy in Vero cells, a vaccine-approved cell line, along with in Type II pneumocytes. Results obtained using MTX were consistent in each cell line in terms of reducing viral multiplication and not inducing CPE, a finding confirmed upon LDH release analysis. Based on these results, MTX was chosen for *in vivo* drug efficacy analysis.

We used an established Balb/c model of CWPV infection to assess if MTX had a protective effect *in vivo*. Despite the protection it provides in vitro, MTX was unable to protect the mice from weight loss at the doses administered although administration of 20mg/kg beginning one day prior to infection allowed those mice to survive an additional day in comparison to control mice. This dose of MTX also proved efficacious at limiting viral replication in the lungs in comparison to the alternate treatment schedules.

The typical response to CWPV infection is the induction of a robust Th1 response mediated by IFNg production. Published reports describe treatment with MTX resulting in marked suppression of IFNg production, thus inhibiting appropriate antiviral responses. Assessment of lung cytokine profiles confirmed this to be the case upon CWPV infection as all MTX treatment groups experienced an induction in IL-10 and IL-5 along with a 6-fold depression in IFNg secretion in comparison to control animals. This skewed immune response is likely responsible for the increased lung viral burdens seen.

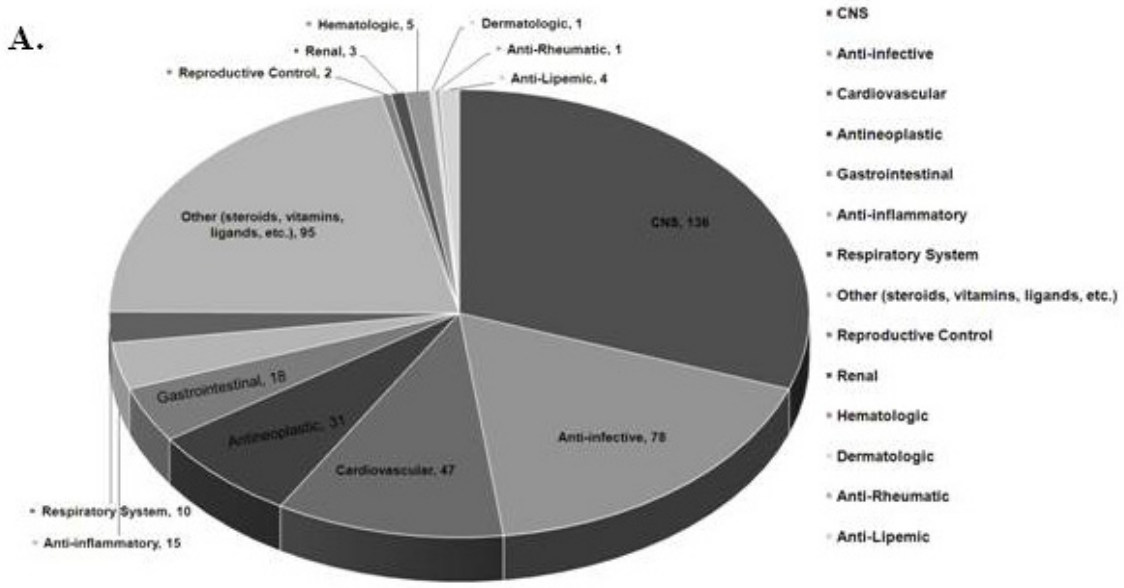
Methotrexate antagonist FNA is used principally as an antidote to folic acid antagonist by directly providing the necessary tetrahydrofolates (THF) intracellularly. Based on various studies examining efficacy of administration of FNA from 0.5-6 hours post-MTX treatment [15, 19, 21-23], we attempted to rescue MTX-induced toxicity by administering FNA within an hour of MTX dosing. We again assessed lung cytokine levels to see if FNA could restore a Th1 antiviral response. We report that IFNg, expression was restored with FNA supplementation in MTX-treated mice. This increase in IFNg correlates to a decrease in viral viral replication in the lungs in mice supplemented with FNA in comparison to all other treatment groups. Interestingly, this restoration of the appropriate antiviral response to CWPV infection did not correspond with a decrease in weight loss or increase in survival. The treatment group that did exhibit 40% survival and maintained weight to a better degree than any other treatment group were administered the low dose of MTX, 5mg/kg.

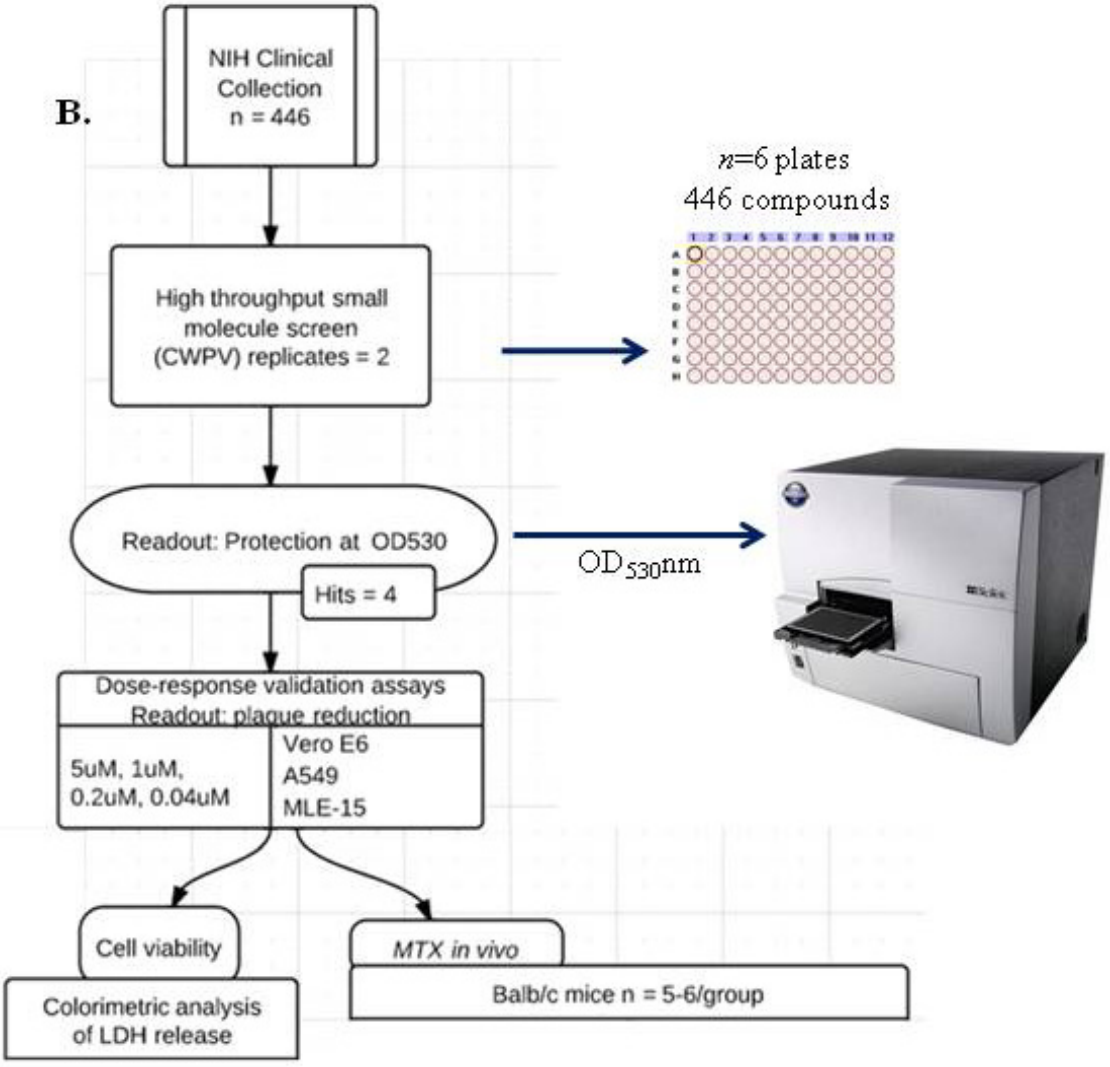
Ultimately, these results identify MTX, a compound with anti-poxvirus capability. Further studies will be directed at optimizing delivery of MTX along with timing of FNA rescue in an attempt to augment the antiviral immune response to achieve a decrease in

viral replication and increase in survival. In addition, MTX is a candidate from which derivatives can be studied for potential administration to protect against human orthopoxvirus infections. Results obtained from the compound screen also highlight several other potential compounds which can be further explored for in vivo protective efficacy in future studies.

Figure 4.1 | Small molecule screen summary. A) NIH Clinical Collection by Therapeutic Indication. The NCC contains 446 compounds arrayed in six 96-well plates, each supplied in a 50µl aliquot of a 10mM solution in 100% DMSO. The collection is a group of molecules targeting 14 major systems and/or disease states. Three categories, CNS, anti-infectives, and cardiovascular, represent 58.5% of all drugs in the collection. *Figure adapted from Chart of Collection Annotated by Therapeutic Indication (ref. Panchenko DA. 2008).* **B**) Library screen summary. For each of the two independent screens of n=446 compounds, $4x10^4$ Vero E6 cells were plated in standard 96-well plates in 150uL DMEM supplemented with 5% FBS until ~80% confluency, at which point the compound was administered at a final concentration of 10µM. 24 hours post-treatment, cells were infected with CWPV and incubated for 48 hours prior to being fixed and stained. Treatment efficacy was determined by measuring crystal violet absorbance at OD_{530}.

A.


Hematologic, 5
Dermatologic, 1
Renal, 3
Anti-Rheumatic, 1
Reproductive Control, 2
Anti-Lipemic, 4
Other (steroids, vitamins, ligands, etc.), 95
CNS, 136
Gastrointestinal, 16
Antineoplastic, 31
Anti-infective, 78
Cardiovascular, 47
Respiratory System, 10
Anti-inflammatory, 15
CNS
Anti-infective
Cardiovascular
Antineoplastic
Gastrointestinal
Anti-inflammatory
Respiratory System
Other (steroids, vitamins, ligands, etc.)
Reproductive Control
Renal
Hematologic
Dermatologic
Anti-Rheumatic
Anti-Lipemic


B.
NIH Clinical Collection n = 446
n=6 plates
446 compounds
High throughput small molecule screen (CWPV) replicates = 2
Readout: Protection at OD530
Hits = 4
OD530nm
Dose-response validation assays
Readout: plaque reduction
5uM, 1uM, 0.2uM, 0.04uM
Vero E6
A549
MLE-15
Cell viability
Colorimetric analysis of LDH release
MTX in vivo
Balb/c mice n = 5-6/group

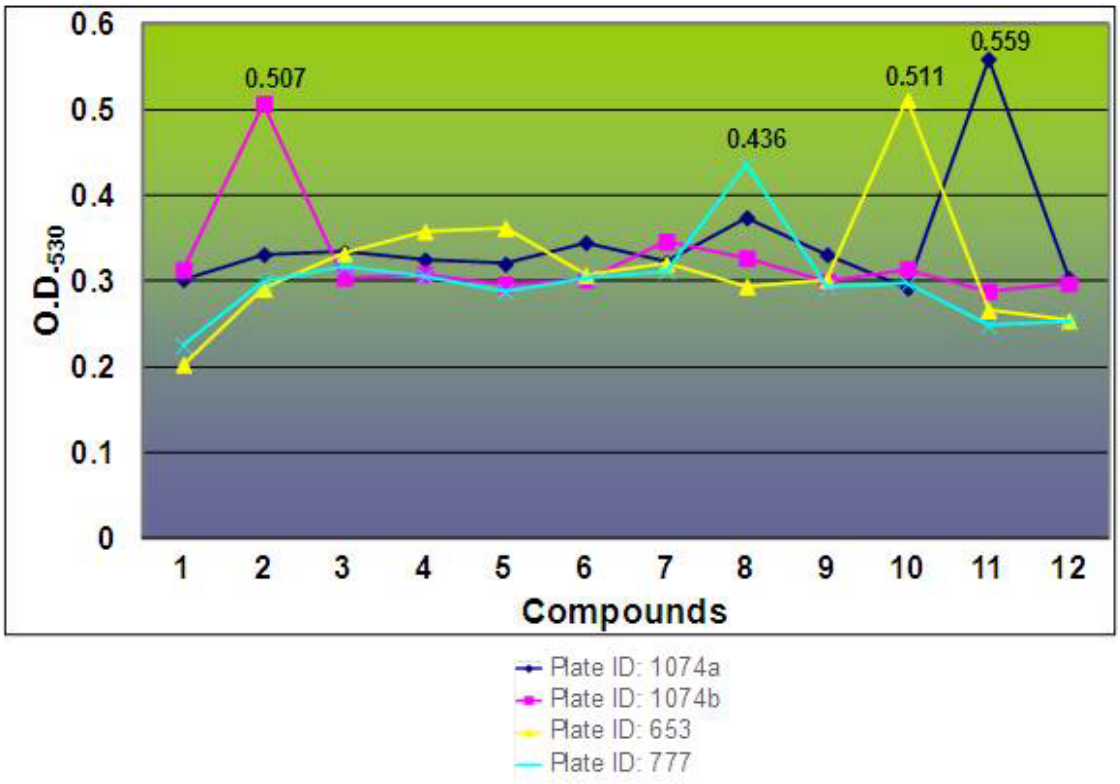


Figure 4.2. NCC library screen absorbance data to determine treatment efficacy in Vero E6 cells. 446 compounds from the NIH Clinical Collection were screened in Vero cells as described above. Once cells were fixed and stained with crystal violet, treatment efficacy was determined by measuring absorbance at optical density (O.D.) 530. Protection correlated to an increase in O.D. as compared to other compounds in the screen. The four hits identified in the bulk screen are highlighted here among *n*=48 total drugs shown as a sample of the entire set.

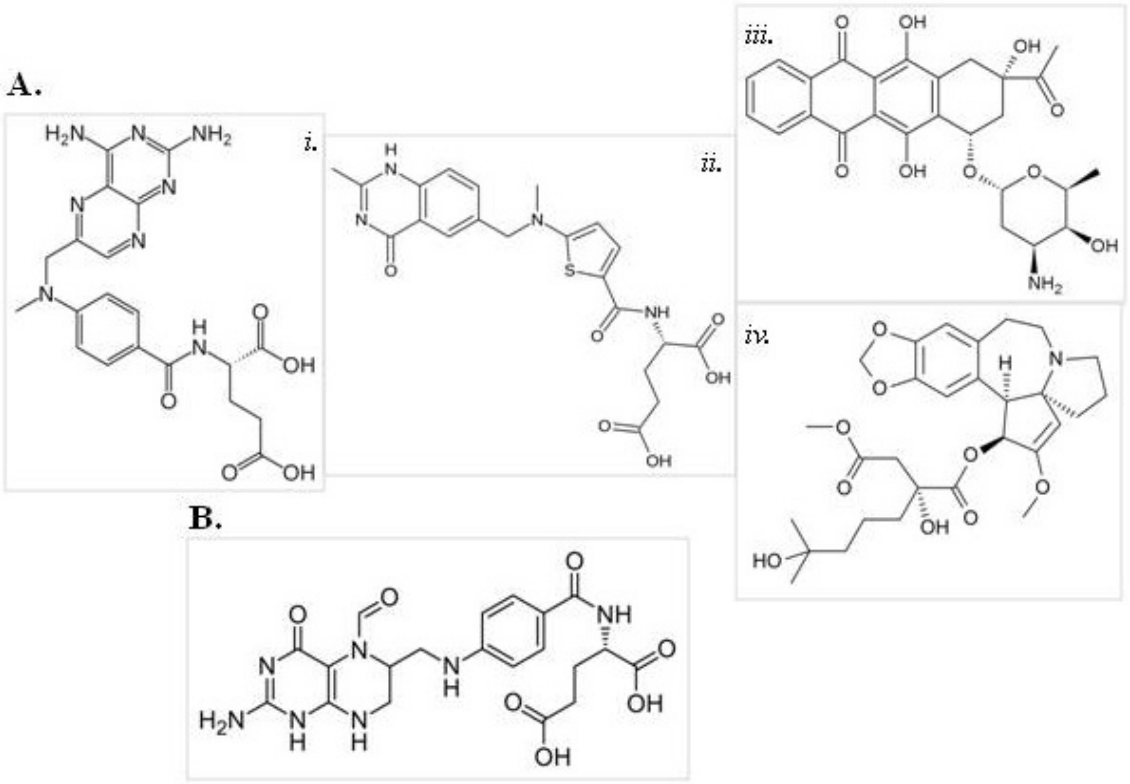
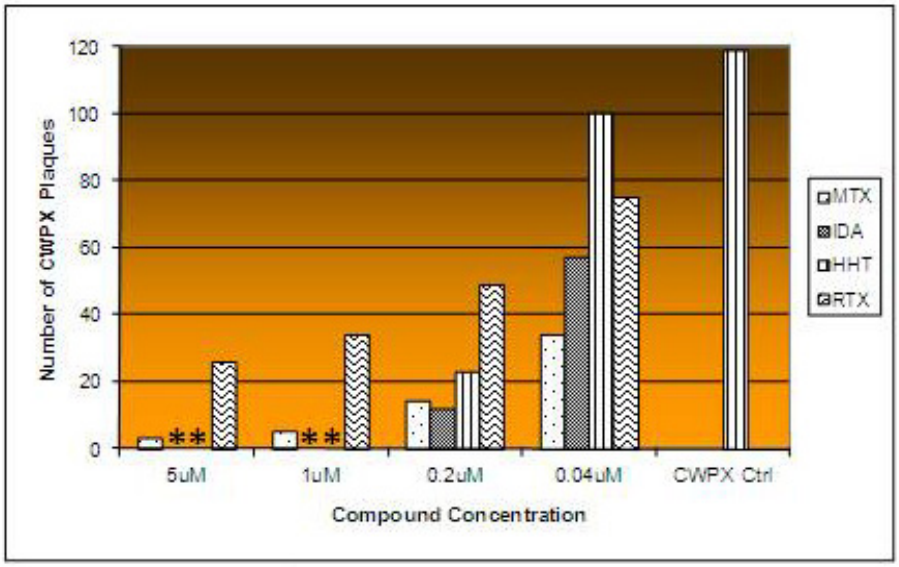


Figure 4.3 | Chemical structures. **A**) NCC hit compounds. *i*. Methotrexate, *ii*. Ratitrexed, *iii*. Idarubicin and *iv*. Homoharringtonine. **B**) Methotrexate toxicity antidote, folinic acid.

Figure 4.4 | Inhibition of CWPV multiplication by various chemical compounds in a dose-response manner. Vero cells were plated at $4x10^5$ cells/well in 12-well plates. **A**) Compounds which inhibited viral multiplication in the bulk screen as determined by $O.D._{530}$ values were rescreened at 5, 1, 0.2 and 0.04 μM concentrations and 4 hits identified. MTX, IDA, HHT and RTX were titrated to reasonably nontoxic doses over a range of 5 to 0.04 μM concentrations in **B**)Vero E6, **C**) A549 and **D**) MLE-15 cells to determine the lowest dose necessary to effectively inhibit poxviral replication in vitro. Plaques were quantified visually using a dissecting microscope. Representative plaque images obtained from n=3 independent experiments.

A.



*Obvious compound-induced CPE

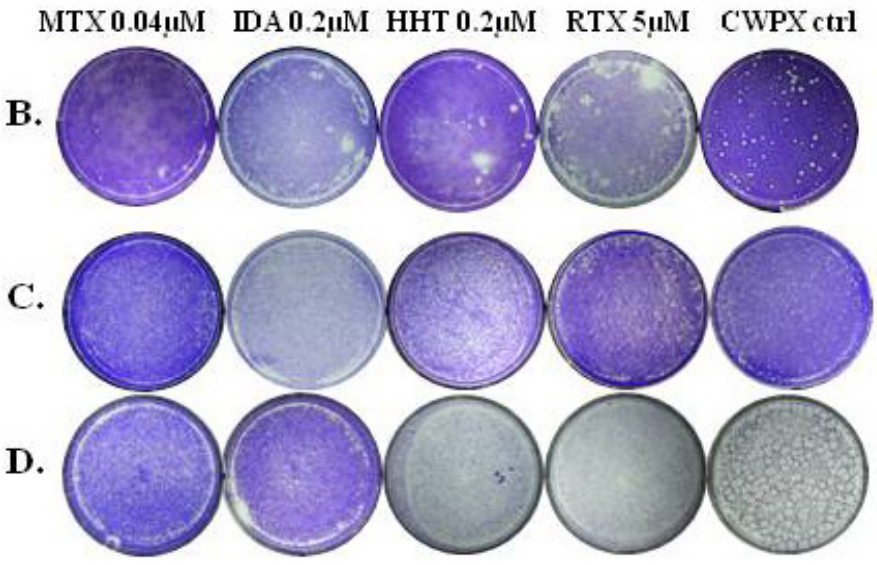


Figure 4.5 | Cytotoxicity of NCC hit compounds in Vero E6 cells as measured from LDH release. Vero cells were plated at $1x10^4$ and $2x10^4$ cells/well in 96-well plates. Upon reaching confluency, compounds were administered at the indicated concentrations. LDH release as a measure of cellular cytotoxicity due to compound treatment was assayed. A maximum-LDH release positive control provided by the manufacturer. **A**) Absorbance values measured at optical density 490nm. **B**) LDH released upon cell lysis 24hours post treatment.

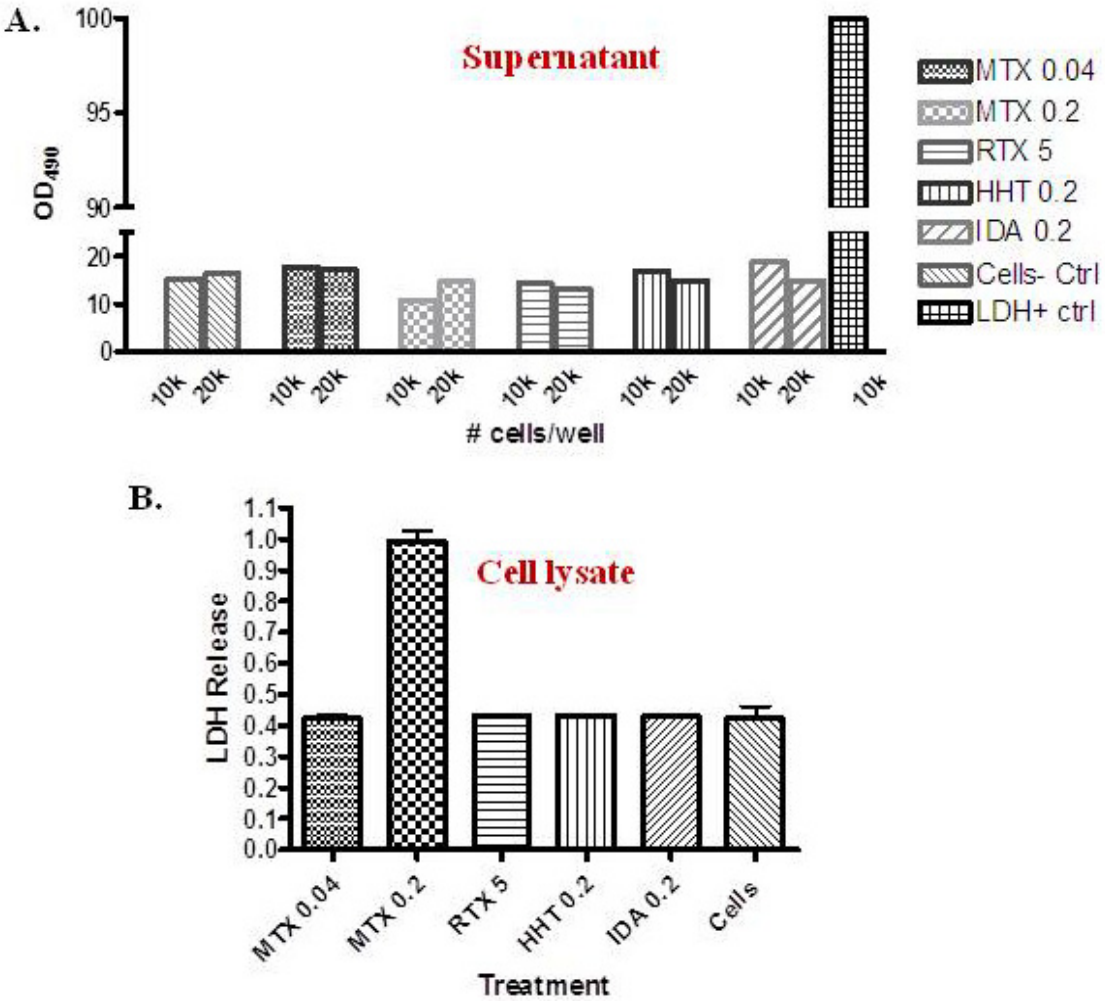
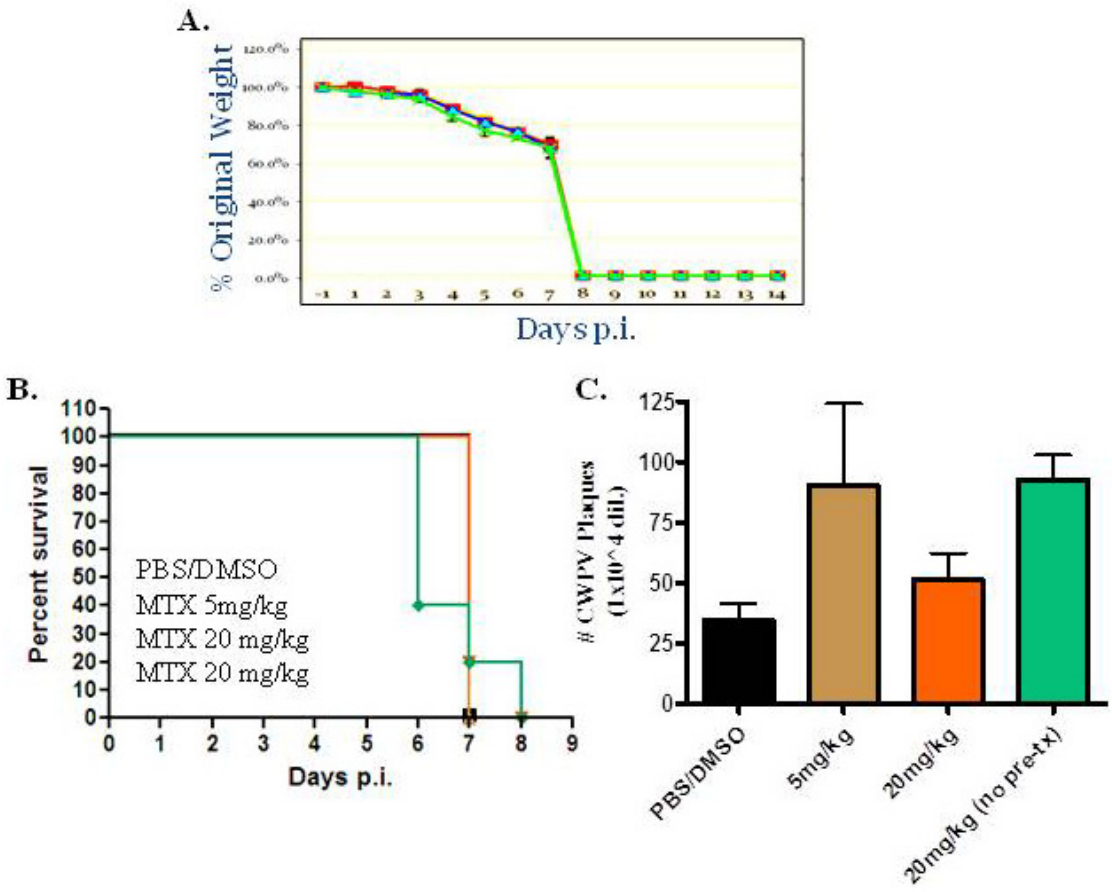

A.
Supernatant
100
95
90
20
10
0
OD490
10k
20k
cells/well
MTX 0.04
MTX 0.2
RTX 5
HHT 0.2
IDA 0.2
Cells- Ctrl
LDH+ ctrl
B.
Cell lysate
LDH Release
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
MTX 0.04
MTX 0.2
RTX 5
HHT 0.2
IDA 0.2
Cells
Treatment

Figure 4.6 | Weight loss, survival and lung viral burden in Balb/c mice treated with MTX. MTX was administered in 3 different treatment schedules to the indicated groups of n=5 mice. Group A, the control, received PBS on days -1, 2 and 5 post-infection with CWPX-BR. Groups B and C were treated with 5mg/kg (100ug) or 20mg/kg (500ug), respectively at days -1, 2, and 5 post-infection. Group D received 20mg/kg (500ug) of MTX on days 2 and 5 post-infection. Lungs were isolated from mice that succumbed to infection or were euthanized due to weight loss and homogenized in PBS, clarified via centrifugation and supernatants titered on Vero E6 cells. 1x10^4 dilution shown. Plaques were counted and imaged as previously described.


A.
% Original Weight
Days p.i.
B.
Percent survival
PBS/DMSO
MTX 5mg/kg
MTX 20 mg/kg
MTX 20 mg/kg
Days p.i.
C.
CWPV Plaques (1x10^4 dil.)
PBS/DMSO
5mg/kg
20mg/kg
20mg/kg (no pre-tx)

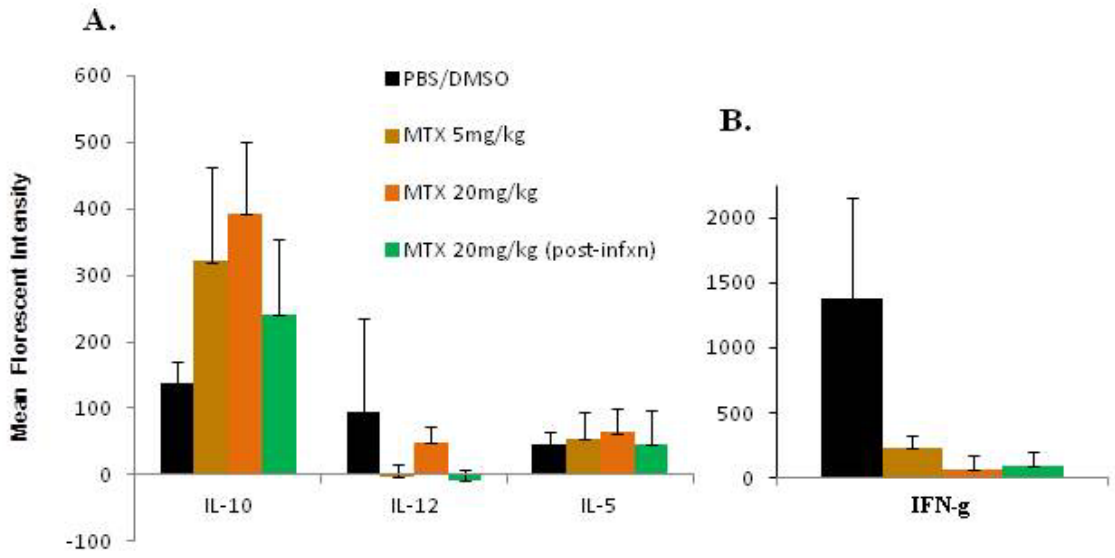
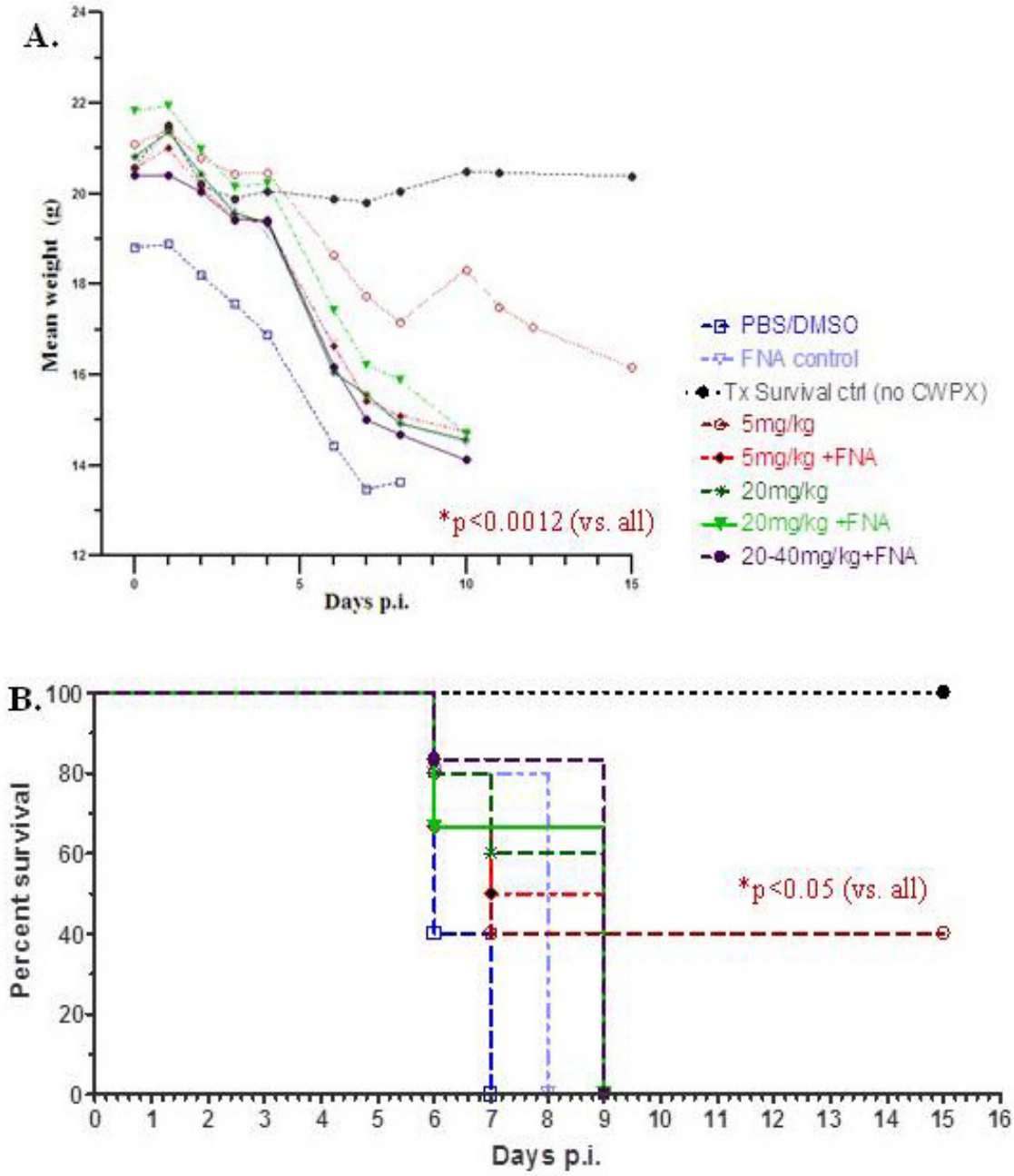


Figure 4.7 | Lung cytokine profiles after treatment with MTX and infection with CWPV. Upon euthanasia, lungs were individually collected and homogenized with dounce homogenizer in PBS supplemented with 1% antibiotic/anti-mycotic. Supernatants were clarified via centrifugation. Lung cytokine profiles were assayed from 50µl of clarified supernatant using a Mouse Th1/Th2 6-plex kit and analyzed on a Luminex 200 system. **A**) IL-10, IL-12 and IL-5 expression. **B**) IFN-g expression. IL-2 and IL-4 results were below the limit of detection (data not shown).

Figure 4.8 | Evaluation of FNA to rescue MTX-induced toxicity in a mouse model of poxvirus infection. In a second experiment, Balb/c mice (n=5-6/group) were treated with MTX pre-CWPV infection (3LD50) intranasally on the schedule shown in **Supplementary Figure 4.2**. **A**) Percent survival (days) post-treatment and infection. **B**) Average weight by treatment group.


A.
Mean weight (g)
Days p.i.
*p<0.0012 (vs. all)
PBS/DMSO
FNA control
Tx Survival ctrl (no CWPX)
5mg/kg
5mg/kg +FNA
20mg/kg
20mg/kg +FNA
20-40mg/kg+FNA
B.
Percent survival
Days p.i.
*p<0.05 (vs. all)

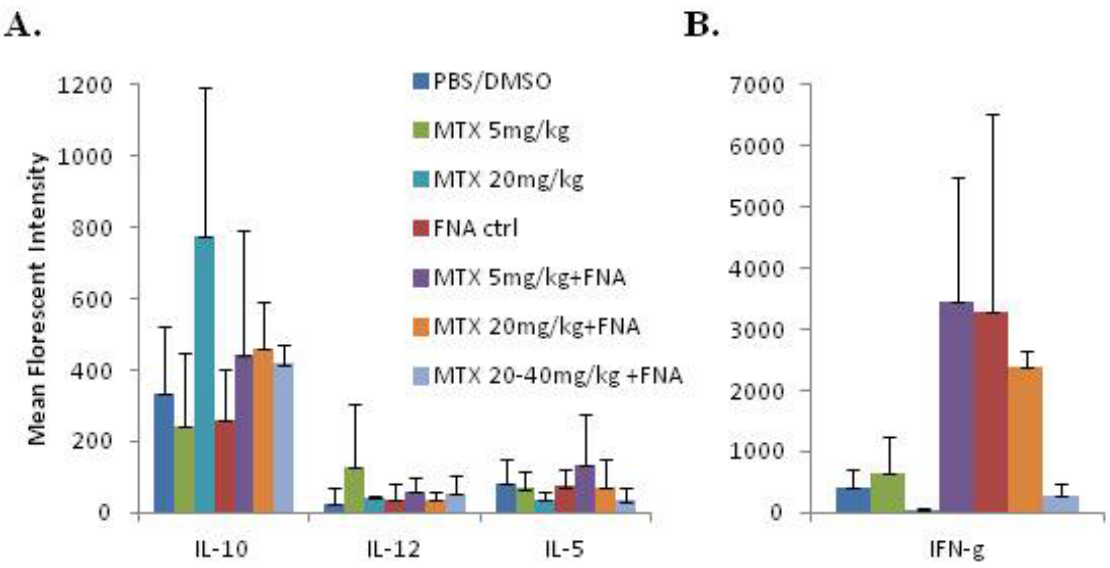


Figure 4.9 | Lung cytokine profiles after treatment with MTX and infection with CWPV. Upon euthanasia, lungs were individually collected and homogenized with dounce homogenizer in PBS supplemented with 1% antibiotic/anti-mycotic. Supernatants were clarified via centrifugation. Lung cytokine profiles were assayed from 50µl of clarified supernatant using a Mouse Th1/Th2 6-plex kit and analyzed on a Luminex 200 system. **A**) IL-10, IL-12 and IL-5 expression. **B**) IFN-g expression. IL-2 and IL-4 results were below the limit of detection (data not shown).

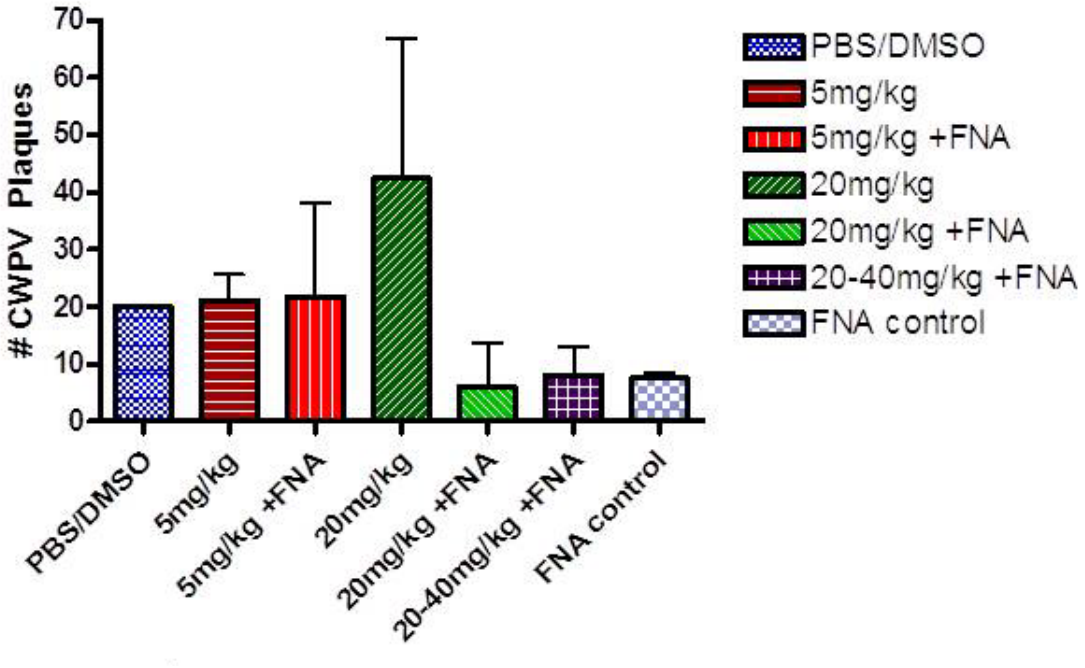
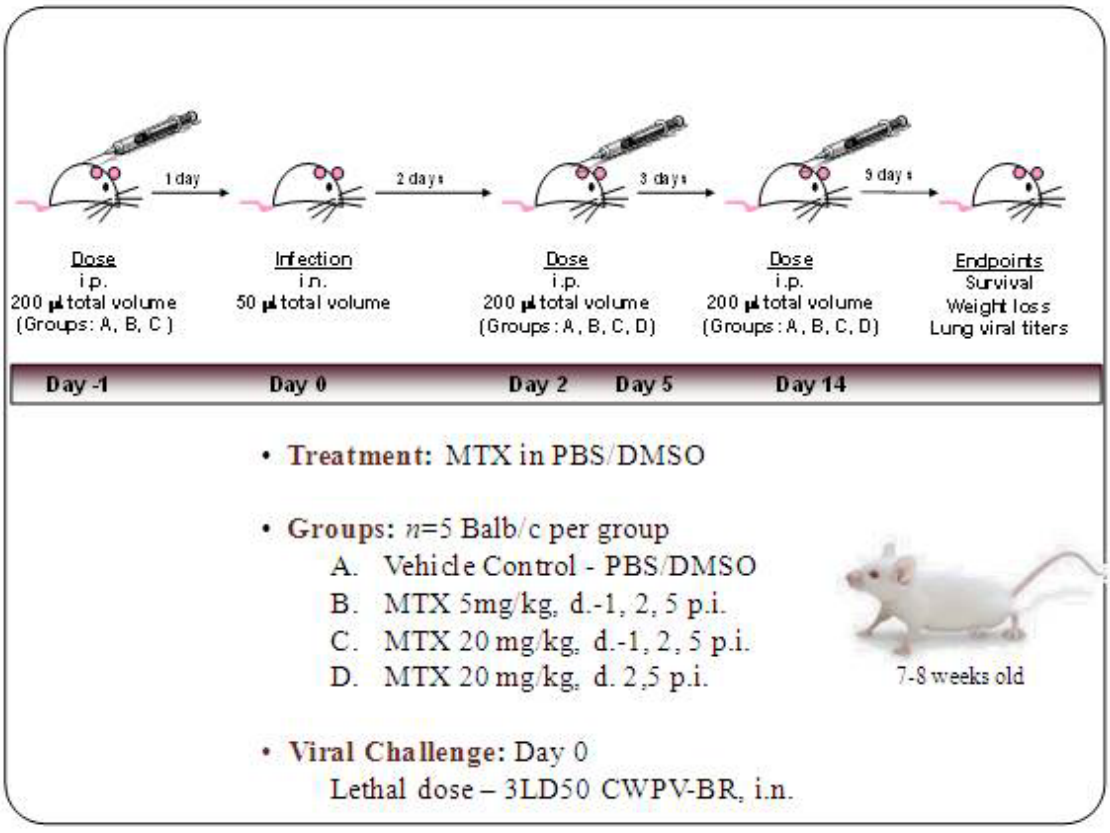


Figure 4.10 | Lung viral burden (1x10^4 dilution shown). Upon euthanasia, lungs were individually collected and homogenized with dounce homogenizer in PBS supplemented with 1% antibiotic/anti-mycotic. Supernatants were clarified via centrifugation and supernatants titered on Vero E6 cells. Plaques were counted and imaged as previously described.

Supplementary Figure 4.1 | Methotrexate efficacy in a mouse model of poxvirus infection. Balb/c mice were treated with MTX in 200uL total volume, i.p. according to the schedule shown. Two doses of MTX were utilized prior to infection: 5mg/kg (100ug) or 20mg/kg (500ug). Groups A, B and C were dosed 1 day prior to infection while Group D began MTX treatment 2 days p.i.


1 day
2 days
3 days
9 days
Dose
i.p.
200 µl total volume
(Groups: A, B, C)
Infection
i.n.
50 µl total volume
Dose
i.p.
200 µl total volume
(Groups: A, B, C, D)
Dose
i.p.
200 µl total volume
(Groups: A, B, C, D)
Endpoints
Survival
Weight loss
Lung viral titers
Day -1
Day 0
Day 2
Day 5
Day 14
• Treatment: MTX in PBS/DMSO
• Groups: n=5 Balb/c per group
A. Vehicle Control - PBS/DMSO
B. MTX 5mg/kg, d.-1, 2, 5 p.i.
C. MTX 20 mg/kg, d.-1, 2, 5 p.i.
D. MTX 20 mg/kg, d. 2,5 p.i.
7-8 weeks old
• Viral Challenge: Day 0
Lethal dose – 3LD50 CWPV-BR, i.n.

Supplementary Figure 4.2 | Methotrexate efficacy with folinic acid rescue in a mouse model of poxvirus infection. Balb/c mice (*n*=5-6/group) were treated with MTX in 200uL total volume, i.p. according to the schedule shown. FNA was given to MTX-only matched groups within an hour of MTX administration. Groups 4-7 received MTX prior to infection with CWPV ($3LD_{50}$) at either 5mg/kg (100ug) or 20mg/kg (500ug) on day -1, while for Group 8, MTX 20mg/kg (500ug) was given on days -7 and -4 and MTX 40mg/kg (1mg) on day -1 prior to infection with FNA within and hour. Control groups received PBS/DMSO (MTX vehicle control) or FNA prior to infection or MTX with FNA with no CWPV infection.

Group #	Treatment	Description	# mice/grp	Dosing Schedule	Virus (Y/N)
I	DMSO/PBS	MTX Vehicle Control	5	d.-1, 3, 6	Y
II	FNA 10mg/kg	FNA Control	5	d.-1, 3, 6	Y
III	MTX 20mg/kg	Tx Survival Control	3	d.-1, 3, 6	N
	+FNA			d.-1, 3, 6 *	
IV	MTX 5mg/kg	-FNA	5	d.-1, 3, 6	Y
V	MTX 5mg/kg	+FNA	6	d.-1, 3, 6	Y
VI	MTX 20mg/kg	-FNA	5	d.-1, 3, 6	Y
VII	MTX 20mg/kg	+FNA	6	d.-1, 3, 6	Y
VIII	MTX 20mg/kg	+FNA, MTX	6	d.-7, -4	Y
	MTX 40mg/kg	dose escalation		d.-1, 3, 6	

FNA

1 hour post-MTX administration

200uL total vol., i.p.

Daily

- weights
- monitor clinical illness

Terminal Lungs

- viral titer
- cytokine analysis

11-12 weeks old

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CHAPTER 5

CONCLUSIONS

Smallpox disease has been eradicated since 1980, however, poxvirus zoonoses have been well documented in recent years. Fear that VARV will be released in an act of bioterrorism combined with the transmissibility of VACV, as the live vaccine agent against smallpox directly challenges the lack of licensed antivirals to combat poxvirus infection. Given that much of the general worldwide population has not been vaccinated nor possess immunity to poxviruses, coupled with the severity of complications that typically occur following VACV infection of immunocompromised persons, the need for continued research and development of therapies for poxviruses has been underscored. These studies were undertaken with a two-fold aim.

First, identifying ORFs that are essential to OPV multiplication to identify essential viral factors. Work was initiated via a genome-wide library screen of individual short-interfering RNAs (siRNAs) targeting 177 conserved OPV genes. The development and screening of this cross-functional tool providing an extremely global view of several OPVs causing zoonoses today. From the primary screen, 26 siRNAs were identified targeting 17 ORFs which, when depleted, inhibit CWPV multiplication *in vitro*. Five genes encode proteins which are a component of RNA polymerases (A24R, A29R, D7R, H4L and J6R). Nine genes were found to encode the following functions: interferon resistance (E3L), a subunit of the ribonucleotide reductase (F4L), IMV membrane protein (A17L), structural protein (A12L), core protein (I7L), capping enzyme and transcription

factor (D12L), disulfide bond formation (F9L), a Uracil DNA glycosylase (D4R) and a virion protein and transcription factor (L3L). Viral genes which have no published function or a putatively described function that proved to be essential to viral multiplication are C6L, I2L, L3L and H7R. siRNAs targeting each of the genes listed show an antiviral potency in reducing CWPV or VACV replication in Vero cells by 52% - 99% in standard plaque reduction assays with a subset of them being validated and show a corresponding decrease in viral gene-specific mRNA levels (A17L, D7R, E3L, I2L and L3L).

Finally, experiments aimed at assessing in vivo efficacy of several of the targets in a murine model of poxvirus disease were carried out. Despite the robust multiplication inhibition seen in vitro, in vivo results indicate that upon prophylactic administration of siRNAs targeting poxvirus D7R and I2L genes, death in Balb/c mice is delayed, but not completely prevented. Several potential reasons for this difference exist. 1) Replication kinetics of CWPV in vitro suggest that peak expression of these viral transcripts occurs at approximately 24hpi. In our experiment, the animals received the siRNA at days -1 and 3 pi. Perhaps treating the mice at day 0 or 1 pi would have caused a more significant decrease in the gene transcript levels, and therefore viral levels, allowing the mice to recover. 2) siRNA molecules were intravenously administered via the mouse tail vein with the expectation that delivery would be systemic. It is possible that siRNA delivery efficiency was low at the site of infection. Future studies will examine the timing of siRNA administration along with a dose response curve to determine optimal concentration. Finally, an obstacle to overcome with siRNA administration is delivery. Results seen could also be due to the inefficient delivery of the siRNAs. Small molecule

inhibitors developed using these data, may prove to be more effective. Taken together, these results show the utility of the genome-wide siRNA screen for studying viral replication factors, along with identification of potential therapeutic targets for anti-orthopoxvirus therapies.

The second aim was to directly address the deficiency in FDA-approved poxvirus drugs. A small molecule array comprised of 486 compounds was acquired. By using a library composed of compounds which have almost all completed phases I-III trials, the results obtained had great potential to be expanded upon to directly address the lack of specifically approved drugs to combat OPVs. The drugs in the library were selected for inclusion based upon favorable attributes such as purity, solubility, commercial availability, and safety. This array, obtained from the NIH, was unique in itself in that it was the first time any of these drugs were found in such a collection.

The library was screened in vitro for individual compound ability to inhibit both CWPV and VACV replication. From this screen, four drugs currently in use as chemotherapeutics and/or immunosuppressives were identified: methotrexate (MTX), Idarubicin (IDA), Homoharringtonine (HHT), and Raltitrexed (RTX). The four compounds were further examined in a dose-response manner and methotrexate was chosen for in vivo drug efficacy analysis.

We used an established Balb/c model of CWPV infection to assess if MTX had a protective effect in vivo. Despite the protection it provides in vitro, MTX was unable to protect the mice from weight loss at the doses administered although administration of 20mg/kg beginning one day prior to infection allowed those mice to survive an additional day in comparison to control mice. This dose of MTX also proved efficacious at limiting

viral replication in the lungs in comparison to the alternate treatment schedules. Overall, however, assessment of lung cytokine profiles confirmed an immune response skewed towards Th2 expression which is likely responsible for the increased lung viral burdens seen.

An antagonist to methotrexate's toxicity, FNA is used principally as an antidote by directly providing the necessary tetrahydrofolates (THF) intracellularly. Based on this, we attempted to rescue MTX-induced toxicity by administering FNA within an hour of MTX dosing and again assessed lung cytokine levels to see if FNA could restore a Th1 antiviral response. We report that IFNg, expression was restored with FNA supplementation in MTX-treated mice. Although the increase in IFNg correlated to a decrease in viral replication in the lungs, this restoration of the appropriate antiviral response to CWPV infection did not correspond with a decrease in weight loss or increase in survival. The treatment group that did exhibit 40% survival and maintained weight to a better degree than any other treatment group were administered the low dose of MTX, 5mg/kg, without FNA supplementation.

While there are numerous published reports of both small molecules inhibiting orthopoxviruses (OPVs) along with RNA interference (RNAi) being used to identify viral genes which when blocked inhibit viral replication, none have taken the comprehensive approach discussed here. We were able to screen an entire OPV genome-wide library of siRNAs and identify multiple ORFs that can potentially be targets for small molecule inhibitors. In addition, screening the NCC allowed for identification of four compounds which directly protect the cells from both VACV and CWPV infections. We have also

extended the work of previously published studies using methotrexate to protect against VACV by providing means to counter the drug toxicity observed.