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## Antiviral chemotherapeutic agents against equine herpesvirus type 1: the mechanism of antiviral effects of porphyrin derivatives

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ANTIVIRAL CHEMOTHERAPEUTIC AGENTS AGAINST  
EQUINE HERPESVIRUS TYPE 1:  
THE MECHANISM OF ANTIVIRAL EFFECTS OF PORPHYRIN DERIVATIVES

A Dissertation

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Agricultural and Mechanical College  
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Doctor of Philosophy

in

The Interdepartmental Program in  
Veterinary Medical Sciences through the  
Department of Pathobiological  
Sciences

by

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## DEDICATION

This work is in memory of my grandmother, Anna Evtihievna Vedenina-Juravel, a strong, enthusiastic, inspiring, brave, and kind lady that shaped my life, personality, and heart. She was always drawn to her home, vast rose garden, and orchid in the hard working city of Dneprodzerjansk, Ukraine. I can still taste the raspberries, mulberries, plums, and young potatoes of her garden. She would let me sleep late and offer delicious treats when I woke up, inspiring me to work very hard without ever directing to do so or scolding. Her home was a haven for my sister, my niece, and I, a peaceful retreat, a place to be truly yourself, and a place to try your very best. Our beloved grandma passed away in June of 2002. I did not have a chance to be there for her in the last years, while she patiently waited for me to come back during all those years. I want everyone reading this work to know about the great lady that had only three years of elementary education and managed to make the world a better place for hundreds of people of many generations, while never compromising the quest for kindness and honesty. Grandma, I will always love you.

I would also like to mention the lady who will always be a part of my life, but who I have never had the great honor of meeting, except through the eyes and hearts of her husband and two sons, Yvonne Louise Desiree Quist-Geerinck. Her husband and children were her life. She worked very hard her entire life, never asked anything for herself, and always placed others first, no matter what. She raised a son, Sebastiaan Sam Quist, who gave me the strength to try and passion to learn, showed me how to be true to myself and honest with others no matter the challenges, and gave me my family, reuniting every generation. She fought hard and brave until cancer took her away from us in October of 2001. I will forever admire her and cherish her memory.

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## LIST OF ABBREVIATIONS

=C-	methine bridges
°C	degree Celsius
•OH	hydroxyl radicals
<sup>1</sup> O <sub>2</sub>	singlet oxygen
3-OS HS	3-O-sulfated heparan sulfate
AD	anno domini
ANP	acyclic nucleoside phosphate
ATP	adenosine triphosphate
AZT	3'-azido-3'-deoxythymidine
BAC	bacterial artificial chromosomes
BC	before Christ
BHV-1	Bovine Herpes Virus Type 1
BIOLF-62	9,((2-hydroxy-1-(hydroxymethyl)ethoxyl) methoxyl) guanine
BIOMMED	Division of Biotechnology & Molecular Medicine
BTLA	B and T lymphocyte attenuator
C <sub>4</sub> H <sub>5</sub> N	pyrrole ring
CC50	50% cytotoxic concentration
CD111	cluster of differentiation 111
CHO	chinese hamster ovary cells
cm	centimeter
CMV	Cytomegalovirus
CO <sub>2</sub>	carbon dioxide
CPE	cytopathic effect
Cu(II)TPPS4	Cu(II)-5,10,15,20-tetrakis(4-[p-sulfobenzyl] sulfoamidonylphenyl) porphyrin
DMEM	Dulbecco modified Eagle's minimum essential medium
DMSO	dimethyl sulfoxide, (CH <sub>3</sub> ) <sub>2</sub> SO
DNA	deoxyribonucleic acid
ED	equine dermal cells
EDTA	ethyl diamine tetraacetate
EFT <sub>r</sub>	equine fetal trachea cells
EHM	Equine Herpesvirus 1 Myeloencephalopathy
EHV-1	Equine Herpesvirus-1
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
Eq	equine
ER	endoplasmic reticulum
FACS	Fluorescent-Activated Cell Sorting
FBS	fetal bovine serum
Fe(III)TPPS4	Fe(III)-5,10,15,20-tetrakis(4-[p-sulfobenzyl] sulfoamidonylphenyl) porphyrin
g24hr	every 24 hours

GAG	glycosaminoglycans
gB	glycoprotein B
gE	glycoprotein E
GFP	green fluorescence protein
gH	glycoprotein H
gP	glycoprotein
H2TPP	5,10,15,20-tetrakisphenylporphyrin
H2TPPS4	5,10,15,20-(4-chlorosulfonylphenyl)porphyrin
HCF	host cell factor
HCl	hydrochloric acid
HCMV-IE	human cytomegalovirus immediate early promoter
HIgR	Herpesvirus Immunoglobulin-like receptor
HIV	human immunodeficiency
HIV	human immunodeficiency virus
hr	hour
HS	heparan sulfate
HSGAG	heparan sulfate glycosaminoglycans
HSV	Herpes Simplex Virus
HveA	herpesvirus entry mediator A
HveB	herpesvirus entry mediator B
HveC	herpesvirus entry mediator C
HveM	herpesvirus entry mediator
ICTV	International Committee on the Taxonomy of Viruses
Ig	immunoglobulin
kbp	kilobase pairs
kDa	kilodalton
g	kilogram
LSU	Louisiana State University
m	meter
M	Molarity
M.O.I.	multiplicity of infection
MAb	monoclonal antibody
ml	milliliter
mm	millimeter
MW	molecular weight
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
nm	nanometers
NPC	nuclear pore complex
NSAIDs	non-steroidal anti-inflammatory drugs
O <sub>2</sub> <sup>-</sup>	superoxide ion
ORF	open reading frame
<i>Ori</i>	origin of replication

PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDT	photodynamic therapy
PDT	porphyrin based detection treatment
PEG	polyethelene glycol
PFU	plaque forming units
PH	pleckstrin homology domain
PILR	paired immunoglobulin-like type 2 receptor
PRR1	poliovirus related receptor type 1
PRV	Porcine Rhinotracheitis Virus
q12hr	every 12 hours, twice a day
RBCoV	Respiratory Bovine Coronavirus
RGR	relative growth rate
RK-13	rabbit kidney cells type 13
ROS	reactive oxygen species
TCID50	tissue culture infective dose 50
TGN	trans-Golgi network
TI	therapeutic index
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor family
TNFRSF14	tumor necrosis factor receptor superfamily, member 14
TPP	<i>meso</i> -tetraphenylporphirine
TPPS4	(sulfonated tetraphenyl porphyrin
TRAF	TNFR-associated factor
U <sub>L</sub>	unique long
URTD	upper respiratory tract disease
U <sub>s</sub>	unique short
UV	ultraviolet
VHS	virion host shutoff protein
VZV	Varicella Zoster Virus
$\alpha$ -TIF	$\alpha$ -gene transactivating factor

## **ABSTRACT**

Equine Herpesvirus-1 (EHV-1) is an important ubiquitous enzootic equine pathogen, causing significant economic losses to the horse industry. Despite extensive vaccination protocols, EHV-1 continues to be a major cause of epidemic abortion, perinatal mortality, respiratory disease and neurologic disease. EHV-1 infections are usually dealt with by using management practices that limit spread of the disease and secondary complications, providing symptomatic relief to infected horses, but no specific treatment is available. New therapeutic or virucidal agents could have great utility in slowing both the progression and spread of the disease in an epidemic situation.

A number of porphyrins and their derivatives have been tested to have activity against HIV, vaccinia, and coronavirus. Porphyrin based compounds were suggested to inhibit virus infection by reducing the fusogenic potential of the virus (Vzorov et al., 2002). However, the mechanism of action of porphyrin-based compounds is not well understood. While current antiherpetic agents target viral DNA replication, interference with the upstream replicative events such as fusion would not adversely affect the host cell metabolism, and makes them important targets for chemotherapeutic intervention of virus dissemination.

We screened a number of porphyrin and platinum compounds for EHV-1 antiviral activity by testing their ability to interfere with EHV-1 infection of rabbit kidney and equine cell cultures during the entry and post entry events of the viral life cycle in order to determine if compounds act at the level of binding, penetration, replication, or egress. We identified Cu (III) tetrasulfonated phenylporphyrin and Fe (II) tetrasulfonated

phenylporphyrin as lead candidate antiviral compounds on the basis of their *in vitro* efficacy, cytotoxicity and therapeutic index.

These compounds exhibited high antiviral potency during virus-to-cell fusion events, as well as no apparent cytotoxicity in cell culture assays at EHV-1 inhibitory concentrations. Specifically, selected porphyrin compounds inhibited free virus, gB-mediated virus entry, reduced the extent of virus spread, and cell-to-cell fusion in the virus-free cell fusion system. The EHV-1 antiviral properties and other pharmacological characteristics make porphyrins auspicious candidates for the treatment of EHV-1 infections and may promote understanding of membrane fusion events of EHV-1 life cycle.

## **CHAPTER I. INTRODUCTION**

### **STATEMENT OF PROBLEM**

Equine Herpesvirus-1 (EHV-1) is an important ubiquitous enzootic equine pathogen, causing significant economic losses to the large (hundred billion dollars) horse industry. Despite extensive vaccination protocols, EHV-1 continues to be a major cause of epidemic abortion, perinatal mortality, respiratory disease and occasionally neurological disease in horses. Furthermore, with increasing interactions within the equine industry, the potential losses during EHV-1 outbreaks are no longer confined to individual farms. In devastating recent outbreaks, an unusually high number of horses exhibited the neurological form of the disease, EHV-1 Myeloencephalopathy (EHM). There is a growing concern in the U.S. horse industry over the increased number of neurologic cases of EHV-1 reported in recent years, as well as the occurrence of several high-profile outbreaks. The Center for Emerging Issues, part of the Department of Agriculture's Animal and Plant Health Inspection Service, recently labeled the neurologic form of EHV-1 as a potentially emerging disease.

There is a pressing need for potent antiviral compounds to treat both disseminated as well as neurological forms of infections. EHV-1 infections are usually dealt with using management practices that limit spread of the disease, providing symptomatic relief to infected horses, including the use of steroids, non-steroidal anti-inflammatory agents, antibiotics for secondary bacterial infections as well as other compounds used to treat EHV-1-associated neurological symptoms like paresis, paralysis, distention of the urinary bladder, stiffness of pelvis or ocular damage, but no specific treatment is available. The current antiviral treatments options are based on extrapolation from established regimens



for nucleoside analogs such as acyclovir used for treatment of human infections with HSV-1 and VZV and have not been shown to change the outcome of EHV-1 infections significantly. There are no controlled studies reporting on the efficacy of the known antiherpetic agents in the treatment or prevention of EHM, nor describing their pharmacokinetics, bioavailability, and safety in horses. New therapeutic or virucidal agents could have great utility in slowing both the progression and spread of the disease in an epidemic situation.

While, current antiherpetic agents target viral DNA replication, fusion events of virus entry are the upstream replicative event, interference with which does not adversely affect the host cell metabolism, and makes them important targets for chemotherapeutic intervention of virus dissemination. The molecular events of the membrane fusion at the onset of herpesvirus infection are currently under extensive investigation in multiple laboratories and are still undetermined. Multiple viral surface glycoproteins are known to participate in the fusion events that may occur via endocytosis as well as pH-independent membrane fusion. Preliminary data indicates that the porphyrin based compounds inhibit virus infection by reducing the fusogenic potential of the virus (Dairou et al., 2004). Substantial work performed by Dr. Marzilli of the LSU Department of Chemistry and his collaborators at Emory University, Atlanta, GA has shown that specific porphyrin-based compounds exhibited strong and specific antiviral activities against different viruses such as HIV. Additional preliminary work in our laboratory has confirmed the antiviral properties of porphyrin compounds against coronaviruses, vaccinia, HSV-1 as well as EHV-1. The molecular basis for these antiviral activities is not known and it is the subject of the proposed investigations.

## **HYPOTHESIS**

The central hypothesis of the investigations was that select porphyrin compounds can specifically inhibit membrane fusion phenomena required for virus entry and virus spread through specific interactions with one or more viral glycoproteins required for membrane fusion.

## **STATEMENT OF RESEARCH OBJECTIVES**

The overall objective of the current investigation was to determine what specific stage of EHV-1 infection and viral components are affected by the porphyrin compounds.

### **Objective I**

To conduct screening tests to determine preliminary antiviral and cytotoxic effects of the available chemical compounds, including porphyrin, phthalocyanine, and platinum derivatives.

#### **Specific Aims**

1. To screen available chemical compounds for EHV-1 antiviral activity and to identify lead candidate compounds on the basis of their low *in vitro* toxicity and antiviral potency.
2. To elucidate the mechanism of action of the antiviral compounds by localizing the effect of the compounds to the specific stages of virus life cycle such as attachment, virus to cell fusion, replication, assembly, or cell to cell spread.

### **Objective II**

To conduct definitive tests to measure the antiviral effects of porphyrins and to unequivocally identify potential antiviral substances and the stage and components of EHV-1 viral infection that they affect.

## **Specific Aims**

1. To quantify the EHV-1 antiviral activity and cytotoxicity of the lead candidate antiviral compounds by determining the 50% effective concentration, 50% cytotoxic concentration, and therapeutic index of the compounds.
2. To assay the effects of antiviral compounds on virus entry, infectious virus production and plaque formation.
3. To determine whether selected porphyrin compounds specifically inhibit gB-mediated virus entry and virus spread.

## **LITERATURE REVIEW**

### **Historical Perspective**

*In medicine one must pay attention not to plausible theorizing, but to experience and reason together.*

~Hippocrates

*If your horse says no, you either asked the wrong question, or asked the question wrong.*

~Pat Parelli

### **Origins of Herpes**

More than 25 centuries have passed since investigations of herpesviruses have begun. Over the centuries, the inquiry has progressed from the basic classification of human cutaneous lesions and the individual accounts of animal diseases to be attributed to herpesviruses later, to the description of epidemiology of herpes infections, the discovery of etiological agents responsible for the disease, and ultimately, to the molecular characterization of the *Herpesviridae* family.

The first written documentation of the herpesvirus infection is dating back to ancient Greece. The word 'herpes' comes from the Greek word *herpein*, used by

Hippocrates (460 BC-377 BC) to describe lesions that creep or crawl, referring to the sequential appearance and local extensions of lesions in human HSV-1 infection (Wildy, 1973). Description of lesions resembling human herpes was also mentioned in Sumerian Tablet dated to 3<sup>rd</sup> millennium BC and the Egyptian Ebers Papyrus, around 1500 BC (Roizman and Whitley, 2001). Then, from Avicenna (880-1036 AD) and Gulielmus Salicetto (13<sup>th</sup> century) until the beginning of the 19<sup>th</sup> century, the term 'herpes' have changed to 'formica' (Latin: ant), 'furfur' (Latin: bran, scales) and, then, back to 'herpes' (Beswick, 1962). While historical account of human herpesvirus infections are dated far back, the clinical conditions caused by Equine Herpes Virus -1 and other animal herpesviruses have only been described over the past four centuries. Nonetheless, understanding the nature of the members of *Herpesviridae* family took centuries and paved the way to the discovery and our current knowledge of EHV-1.

### **Horses in Human History**

Until about 1 million years ago, there were artiodactyls, the one-toed horses of *Equus* species including the domestic horse, *Equus caballus*, all over Africa, Asia, Europe, North America, and South America, in enormous migrating herds that must easily have equaled the great North American bison herds, or the huge wildebeest migrations in Africa. In the late Pleistocene there was a set of devastating extinctions that killed off most of the large mammals in North and South America. All the horses of North and South America died out, along with the mammoths and saber-tooth tigers. These extinctions seem to have been caused by a combination of climatic changes and overhunting by humans, who had just reached the New World. To date, only two truly "wild" groups survived Przewalski's horse and the Tarpan. The Tarpan became extinct in the 19th century and Przewalski's,

discovered in 1879 in Mongolia, is endangered and until recently was considered extinct in the wild (Galosi et al., 2001).

Humans of Paleolithic period left visual record of their fascination with horses and other large herbivorous mammals, whose images were predominant over other animals. One of the most common subjects of Ice Age art is the horse; the wild animal that was sometimes used for food and whose speed and grace was admired by early humans. The earliest known cave wall art is in Chauvet Cave at Vallon-Pont d'Arc near Avignon, France. It is dated to be about 32,000 years old and depicted large elaborate scenes of horses (Bednarik, 1998; Clottes, 2002). Horses were immortalized in sculptures, as in 35,000 year old small ivory horse carving found at Hohle Fels Cave in southwestern Germany. The earliest domestication of horses is most likely to have occurred in the 8000 BC to 5000 BC window. The earliest evidence of domestication is in Ukraine at approximately 4,000 BC as seen by the fenced enclosures and the specific wear of the equine teeth consistent with a bit use. The earliest uncontroversial evidence of horses used as trained animals are the chariot burials of the Sintashta-Petrovka culture of Southern Ural Mountains in northern Kazakhstan, dating to around 2000 BC. The horse first appears in written history in 1700 BC, while the Trojan War of 1218 BC and Hippocrates 460-377 BC were yet to come. In about 1000 BC riding was initiated in the civilized lands of the Near East, and ever since, the horses have carried our civilization on their backs.

### **Earliest Identification of the Diseases Attributed to the Pathogen**

Horses were the foundation of veterinary medicine. In the 19<sup>th</sup> century practice, the horse continued to be the main focus of veterinary work in peace and war. As the cities grew, increase in the number of horses made the manure disposal too difficult, and thus,

the horse has lost to bicycles in major cities and to the railroads for distance travel. Then, with invention of internal combustion engine, the generated “horsepower” no longer required veterinary services. Horses still held their own on the farm, for local transport, and for sport for those who could afford them.

Herpes viruses of humans and horses co-evolved with their hosts. While modern EHV-1 causes respiratory disease, epidemic abortion as well as perinatal mortality, and occasionally neurological disease in horses, it should be considered that the historic accounts of human and equine clinical conditions now attributed to the herpesviruses, could actually have had other etiology: viral, bacterial, fungal, or even now an extinct pathogen.

In North America, the record of animal diseases was seldom mentioned in the literature of the colonial period (Berjeau, 1864; Leonard, 1979; Robertson, 1890; Rockwell, 1868). Noah Webster in his “Brief History of Pestilential Diseases”, published at Hartford in 1799, and George Fleming, prominent British veterinarian, in his “Animal Plagues”, published at London in 1871, left the best and most complete descriptions of animal plaques that occurred in America prior to 1800 (Flemming, 1871). Horses commonly had conditions called colds, with clogged sinuses, copious defluxions that could have been easily attributed to respiratory disease caused by EHV-1 and secondary bacterial infections. These were treated by steaming the upper respiratory tract by means of a nose bag with boiling water as an early advent of modern nebulization. The respiratory tract was often subject to inflammation in the form of bronchitis, congestion of the lungs, or pneumonia. In “The Illustrated Horse Doctor”, Edward Mayhew attributed many such cases to neglect, for example, letting an animal stand for hours in cold, wet weather after

subjecting the animal to hard labor (Mayhew, 1861). Herpes virus reactivation from latency upon stress and immune suppression could have easily been to blame. Aside of continuous steaming, a poultice was held with bandage around the throat. A waterproof jacket applied to the thorax, and a flannel kept moist in cold water was wrapped around the chest. Diethyl ether, a general anesthetic and analgesic and laudanum, an opium tincture, medications were also used. In pneumonia, the standard treatment was steam and dosing with solution of diethyl ether, aconite (a local topical analgesic in neuralgia, sedative in acute laryngitis, antipyretic, negative chronotrope) and belladonna (atropine, also an antidote of aconitum), the latter two being toxic hallucinogenic compounds at higher doses. Brain disorders, such as EHV-1 myeloencephalopathy, must have been truly frightening to horse owners, with phrenitis (old term for encephalitis) often leading to violent behavior and was said to be the result of an injury caused by the carter flailing the head with the butt of his whip. An infectious abortion of mares was a veritable scourge to horse breeders. In 1889-90, the virulence reached its climax in North America. In Illinois, nearly 2,500 foals were lost to EHV-1 with about 5,000 surviving. In some towns the losses reached 75% (Williams, 1889).

### **EHV-1 Discoveries**

The discovery of etiological agents responsible for disease goes alongside the development of technology (Table I-1). In the 1890s, advances in microbiology, pathology, and immunology have impacted the veterinary medicine. The first virus was discovered in 1882, the agent of tobacco mosaic disease by Dmitry Iosifovich Ivanovsky (1864-1920), a Russian-Ukrainian scientist considered the father of Virology (Ivanovsky, 1882). However, the viral etiology of the epizootic abortion of mares as differentiated from

bacterial abortion was not determined until 1936 by William Wallace Dimock and Phil Edwards at the Kentucky Agriculture Experimental Station, Lexington (Dimock and Edwards, 1933; Dimock and Edwards, 1936). They described the gross pathological changes of aborted fetuses, including intranuclear inclusion bodies in the fetal lungs and livers, and gave clinical observations of viral abortions (Dimock et al., 1942). Later, the equine abortion virus was cultivated in laboratory animals and tissue culture (Anderson and Goodpasture, 1942; Doll et al., 1953; Randall et al., 1953), and a more extensive pathological findings were made by Drs. Westerfield and Dimock (Westerfield and Dimock, 1946). Only, in 1963, the virus was shown by electron microscopy to be a member of the herpes group (Plummer and Waterson, 1963).

### **History of Antiherpetics**

The first successful demonstration of antiviral therapy for a herpesvirus infection is attributed to the reduction in mortality in HSV encephalitis achieved with vidarabine therapy (Whitley et al., 1977). This therapy introduced an entirely new era of antivirals, but was associated with toxicity and difficulties in drug administration. For mucocutaneous and visceral HSV infections, the real advance was the discovery of acyclovir and the demonstration of its mechanism of action, attributed to Dr. Gertrude Elion, a Nobel Prize winner in medicine for the discovery of modern antivirals (de Miranda et al., 1982; Elion, 1982, 1983). Her approach was revolutionary, she used her understanding of the structure of nucleic acids to synthesize molecules to specific targets, rather than simply screening randomly chosen molecules, method still often relied on today. In fact, it was researchers trained by Elion who first saw the anti-HIV potential of 3'-azido-3'-deoxythymidine (AZT), at the time an unused anti-cancer drug from the 1960s (Sepkowitz, 2001).



Acyclovir was the first selective inhibitor of herpes replication. Having one of the most remarkable safety profiles of any therapeutic agent, acyclovir became the treatment of choice for all HSV and VZV infections. Twenty years after the success of acyclovir, it is now a generic medication and remains the foundation of antiherpetic therapeutics. Other medications have been licensed, the most widely known are valaciclovir and famciclovir (Crumpacker, 1996; De Clercq, 1993; Stein, 1997).

### **History of Porphyrins**

The answer to the ancient questions of why the grass is green and the blood is red was given by Hans Fischer, for which he earned the Nobel Prize for Chemistry in 1930, “for his researches into the constitution of *haemin* and chlorophyll and especially for his synthesis of haemin” (Fischer, 1999). Both *haemin* (the oxidized ferric form of heme, also called hematin) and chlorophyll are members of a family of vivid compounds, the modified tetrapyrroles or porphyrins, which were dubbed the “pigments of life”(Battersby, 2000; Battersby et al., 1980) and which Nature uses in numerous roles in the biosphere among the broad array of its varied life forms. The origin of word porphyrin comes from Greek word for purple, porphuros. Hemin crystals were discovered in 1853 (Olbrycht, 1953).

Porphyrias are a group of inherited and acquired metabolic disorders of heme biosynthetic pathway enzymes, leading to overproduction and accumulation of porphyrin derivatives that manifest in skin, neurologic, cardiovascular, and digestive disturbances accompanied by extremely severe pain. The severe forms of condition has been suggested as an explanation for vampire and werewolf legends, due to photosensitization, sensitivity to sulfur containing garlic, increased hairgrowth, and other symptoms.

**Table I-1. Historical landmarks of understanding the nature of *Herpesviridae* and EHV-1 infection**

Date	Event	Reference
484 - 425 BC	First accounts of a herpesvirus when Herodotus drew an association between cutaneous eruptions and fever caused by HSV-1	(Roizman and Whitley, 2001)
25 BC- 50 AD	Celsius described an actual herpetic lesion caused by VZV	(Roizman and Whitley, 2001)
129- 200 AD	Galen recognized that recurrent HSV lesions develop at the same anatomical location, regional distribution now understood to be due to the infection of the nerve innervating the specific area	(Roizman and Whitley, 2001)
1847	First infectious disease control technique in human hospital, a hand scrub between patients	(Best and Neuhauser, 2004)
1882	Discovery of the first virus, tobacco mosaic virus, by Ivanovsky	(Ivanovsky, 1882)
1883	The most enlightening description of HSV recurrences was published by Unna	(Unna, 1883)
1893	Although previously well characterized, the HSV transmissibility was first recognized	(Whitley et al., 1998)
1910	Isolation of human and veterinary patients with contagious diseases became standard as well as the use of sterile instruments, gowns, masks, and gloves for surgical procedures in large university human hospitals	
1930s	The milestone of HSV biology, an observation was made by Andrews and Carmichael that recurrent infections occurred only in adults who carried neutralizing antibodies, an occurrence in sharp contrast to the behavior of other known infectious agents of that time.	(Andrews and Carmichael, 1930)
1936	Viral etiology of the epizootic abortion of mares was differentiated from bacterial abortion	(Dimock and Edwards, 1933)
1938- 1939	Doerr and Burnet and Williams understood the true nature of HSV latency and reactivation	(Roizman and Knipe, 2001)
1940	Description of gross pathological changes of aborted fetuses, including intranuclear inclusion bodies in the fetal lungs and livers, and clinical observations of viral abortions	(Dimock, 1940)
1941	First report of HSV-1 infection in the brain	(Smith et al., 1941)
1942	Equine abortion virus was cultured in laboratory animals and tissue culture	(Anderson and Goodpasture, 1942)
1950	Complement-fixation test for equine virus abortion	(Doll and Hull, 1950)
1953	Adaptation of equine abortion virus in Syrian hamsters	(Doll et al., 1953)
1954	Early steps to recognition of equine rhinopneumonitis and abortion as clinical conditions caused by a single agent.	(Doll et al., 1954)
1956	Agglutination of horse erythrocytes by tissue extracts from hamsters infected with equine abortion virus	(McCollum et al., 1956)
1953 and 1963	EHV-1 was isolated as two presumably serologically distinct herpesviruses EHV-1, as equine abortion in 1953 and rhinopneumonitis virus, EHV-2, isolated from the respiratory tract of a colt with a runny nose and a cough in 1963	(Doll et al., 1953; Plummer and Waterson, 1963)
1961	Electron microscopic study of equine abortion virus 13 years post HSV-1 visualization in 1948	(Tajima et al., 1961)
1963	Virus was shown by electron microscopy to be a member of the herpes group	(Plummer and Waterson, 1963)
1969	EHV-1 and EHV-4 recognized as separate strains	(Plummer et al., 1969)
1974	Isolation of latent herpes viruses in horses	(Iurov and Sologub, 1974)
1992	EHV-1 genome cloned	(Telford et al., 1992)
1959	Live-attenuated vaccine developed	(Doll et al., 1959)
1961	Preventative vaccination against EHV abortion introduced	(Doll, 1961)
2002	Myeloencephalopathy outbreaks	(Stierstorfer et al., 2002)
2002	Acyclovir used as a treatment for EHV-1 infections	(Wilkins, 2003)
2003	Preliminary results indicated EHV-1 myeloencephalopathy is due to atypical strain of EHV-1	(Goehring et al., 2006) (Allen, 2006)

## **Taxonomy of EHV-1**

### **Method of Classification and Properties of Subfamilies**

As discussed earlier, although the diseases caused by herpesviruses have been known for centuries, the discovery of etiological agents responsible goes alongside the development of technology. HSV-1 has been isolated in 1940, but the big development occurred between 1950 and 1956 with the application of tissue culture to isolate the other members of the *Herpesviridae* family. Identifying these new and apparently related viruses was what led to a scientific desire for them to be classified. Current classification of *Herpesviridae* family came into being in 1981.

Equine herpes virus 1 (EHV-1) is a member of family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* as classified by the Herpesvirus Study Group of the International Committee on the Taxonomy of Viruses (ICTV) (Fauquet and Mayo, 2001). Herpesviruses are highly disseminated in nature and most animal species have at least one herpesvirus (Table I-2). Of nearly 160 herpesviruses that have been characterized, seven herpesviruses have been isolated so far from horses and are divided into two subfamilies: the *alphaherpesvirinae* (equine abortion virus (EHV-1), equine arterivirus (EHV-3), equine rhinopneumonitis virus (EHV-4), EHV-8, and EHV-9) and the *gammaherpesvirinae* (EHV-2, EHV-5). In each subfamily the viruses belong to the same genus, *varicellovirus* (EHV-1,-3,-4,-8, and -9) and *radinivirus* (EHV-2 and -5) respectively.

*Herpesviridae* classification scheme is used to depict evolutionary relatedness and to predict biological properties of newly identified members in relation to well known ones. As such, human herpesvirus 1 is considered a model for properties of the viruses that

belong to the *Alphaherpesvirinae* subfamily including EHV-1, although it belongs to a different genus than the EHV-1. On the other hand, Pseudorabies Virus (PRV) of swine and Varicella Zoster Virus (HSV-3, VZV; human chicken pox virus) are the other two well studied viruses that are members of the *Varicellovirus* genus and are often used for extrapolation of some of EHV-1 properties yet unknown.

Memberships in the *Herpesviridae* family are assigned based on the architecture of the virion and include large, dsDNA genome, enveloped viruses. The family is subdivided into three subfamilies – the *Alphaherpesvirinae*, the *Betaherpesvirinae*, the *Gammapherpesvirinae*, and *Ictalurivirus* – on the basis of biological properties (Roizman et al., 1981). The subfamilies are further subdivided into genera based on DNA sequence homology, similarities in genome sequence arrangement, and relatedness of important viral proteins.

Members of *Alphaherpesvirinae* subfamily are associated with variable host range, relatively short reproductive cycle, rapid spread in culture with efficient destruction of infected cells (cytolytic), and capacity to establish latent infections primarily, but not exclusively in the sensory ganglia, and therefore, significant primary and recurrent infection. The subfamily contains the genera *Iltovirus*, *Mardivirus*, *Simplexvirus*, and *Varicellovirus*.

By comparison, members of *Betaherpesvirinae* are characterized by restricted host range, long reproductive cycle, and slow infectious cycle with cytomegalia in cell culture. The virus can be maintained in latent form in secretory glands, lymphoreticular cells, kidneys, and other tissues. The subfamily consists of the genera *Cytomegalovirus*, *Muromegalovirus*, and *Roseolovirus* (van Regenmortel et al., 2000).

**Table I-2. Select members of the *Herpesviridae* family. Well studied or clinically significant viruses are represented.**

Subfamily	Genus	Species	Vernacular Name/Disease Condition
<i>Alphaherpesvirinae</i>	<i>Iltovirus</i>	<i>Gallid herpesvirus 1</i>	
	<i>Mardivirus</i>	<i>Gallid herpesvirus 2</i>	Marek's disease virus type 1
		<i>Gallid herpesvirus 3</i>	Marek's disease virus type 2
		<i>Meleagrid herpesvirus 1</i>	Turkey herpesvirus
	<i>Simplexvirus</i>	<i>Ateline herpesvirus 1</i>	
		<i>Bovine herpesvirus 2</i>	bovine mammillitis and pseudo-lumpy skin disease
		<i>Cercopithecine herpesvirus 1</i>	also known as Herpes B virus, causes a Herpes simplex-like disease in Macaques
		<b><i>Human herpesvirus 1</i></b>	(Herpes simplex virus type 1) oral and/or genital herpes (predominantly orofacial)
		<i>Human herpesvirus 2</i>	(Herpes simplex virus type 2) oral and/or genital herpes (predominantly genital)
	<i>Varicellovirus</i>	<i>Bovine herpesvirus 1</i>	infectious bovine rhinotracheitis, vaginitis, balanoposthitis, and abortion in cattle
		<i>Bovine herpesvirus 5</i>	encephalitis in cattle
		<b><i>Suid herpesvirus 1</i></b>	(pseudorabies virus) PRV
		<b><i>Equid herpesvirus 1</i></b>	Equine rhinotracheitis virus, abortion in horses
		<b><i>Equid herpesvirus 3</i></b>	coital exanthema in horses
		<b><i>Equid herpesvirus 4</i></b>	rhinopneumonitis in horses
		<b><i>Equid herpesvirus 8</i></b>	
		<b><i>Equid herpesvirus 9</i></b>	
		<i>Canid herpesvirus 1</i>	severe hemorrhagic disease in puppies
		<i>Caprine herpesvirus 1</i>	conjunctivitis and respiratory disease in goats
		<i>Felid herpesvirus 1</i>	viral rhinotracheitis and keratitis in cats
		<i>Duck herpesvirus 1</i>	duck plague
		<b><i>Human herpesvirus 3</i></b>	Varicella-zoster virus, chickenpox and shingles
		<i>Phocid herpesvirus 1</i>	
	<i>Mardivirus</i>	<i>Gallid herpesvirus 2</i>	Marek's disease
	<i>Iltovirus</i>	<i>Gallid herpesvirus 1</i>	infectious laryngotracheitis in birds
<i>Betaherpesvirinae</i>	<i>Cytomegalovirus</i>	<i>Human herpesvirus 5</i>	(Human cytomegalovirus) CMV: causes infectious mononucleosis, retinitis, etc.
	<i>Roseolovirus</i>	<i>Human herpesvirus 6</i>	Human B-cell lymphotropic virus or roseolovirus: causes "sixth disease" (known as roseola infantum or exanthem subitum)
		<i>Human herpesvirus 7</i>	closely related to HHV-6; causes roughly the same symptoms
		<i>Suid herpesvirus 2</i>	inclusion body rhinitis in swine
<i>Gammaprotervirinae (lymphoproliferative)</i>	<i>Lymphocryptovirus</i>	<i>Human herpesvirus 4</i>	(Epstein-Barr virus) lymphocryptovirus: causes infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma
	<b><i>Rhadinovirus</i></b>	<i>Alcelaphine herpesvirus 1</i>	wildebeest herpesvirus
		<i>Bovine herpesvirus 4</i>	bovine malignant catarrhal fever
		<b><i>Equid herpesvirus 2</i></b>	equine cytomegalovirus infection
		<b><i>Equid herpesvirus 5</i></b>	
		<i>Human herpesvirus 8</i>	Kaposi's sarcoma-associated herpesvirus – KSHV, primary effusion lymphoma and some types of multicentric Castleman's disease
<i>Ictalurivirinae</i>	<i>Ictalurivirus</i>	<i>Ictalurid herpesvirus 1</i>	Channel catfish virus
		<i>Acipenserid herpesviruses</i>	1, 2, & 3

Gammaherpesviruses infect T or B lymphocytes, produce no infectious progeny, and are implicated in neoplastic transformation of lymphocytes. The host range of members of *Gammaherpesvirinae* subfamily is limited to the taxonomic family or order of their natural host. Latent virus is frequently demonstrated in lymphoid tissues. Subfamily includes *Lymphocryptovirus* and *Rhadinovirus* genera (van Regenmortel et al., 2000).

At first, EHV-1 was isolated as two presumably serologically distinct herpesviruses EHV-1, an equine abortion or rhinopneumonitis virus (e. g. (Doll et al., 1953)) and EHV-2, isolated from the respiratory tract of a colt with a runny nose and a cough (Plummer and Waterson, 1963). The type 1 thought 4 virus designations were proposed by Plummer in 1969 (Plummer et al., 1969). First report of HSV-1 infection in the brain dates to 1940s (Smith et al., 1941).

### **Evolutionary Relatedness and Coevolution with the Host**

The herpesviruses are believed to be of ancient origin, at least 300 million years old (Davidson, 1993). They have coevolved with their hosts, mainly mammals, birds, and fish, with possible multiphyletic origins and lateral transfers. Evolution occurred via three mechanisms - cumulative point mutations, gene duplication, and gene capture (McGeoch et al., 2006). Another ancestral relationship of herpesviruses links them to bacteriophages via similarities of their capsid proteins and assembly mechanisms (Steven et al., 2005; Steven and Spear, 1997). Phylogenetic reconstruction of herpesvirus evolution is generally based on nucleotide or amino acid sequence comparisons of specific proteins and varies depending on the particular protein chosen for analysis (Figure I-1). The analysis of DNA polymerase, major capsid protein, DNA-packaging tegument protein and glycoprotein B sequences revealed that the genome closest to a consensus herpesvirus genome is that of

human herpesvirus 6, suggesting that this genome is closest to a progenitor herpesvirus (Karlin et al., 1994). Chicken and other avian species were found to be more natural or more ancient hosts of herpesviruses.

Originally, by DNA-DNA hybridization studies, the EHV-1 and EHV-3 types exhibit only 2 to 5% homology. As much or more genetic relatedness was reported between herpesviruses of other animal species, 8-10% base sequence homology between HSV-1 and PRV (Ludwig et al., 1972) and 14% between HSV-1 and BHV-1 (Sterz et al., 1973). The DNA homology among alphaherpesviruses supports the hypothesis that the alpha class is of relatively recent ancestry (Alba et al., 2001; Karlin et al., 1994). The equid herpesvirus 1 has the most random herpesvirus genome and stands out as the consensus alphaherpesvirus, suggesting it may approximate an ancestral alphaherpesvirus. The most similarity among alphaherpesviruses is between EHV-1 and PRV-1. There is also great similarity between VZV, HSV-1 and EHV-1 (Karlin et al., 1994; Telford et al., 1992). Other studies suggest that closely related EHV-1 and EHV-4 are evolutionary nearest to BHV-1, then VZV, then, HSV-1 and HSV-2, and at last GHV-2 (Alba et al., 2001).

Complete genomes are often a mosaic, where genes were acquired from different sources and have undergone lateral transfer, transposition, and recombination events in the course of evolution. When genomic comparison was followed by protein sequence alignments, again the sequence closest to the consensus sequence of *Herpesviridae* was HHV-6, with EHV-1 identified as being second to dominate the hierarchy. Similarities among EHV-1 and HHV-6 include the fact that both viruses are lymphotropic and can infect T-cells but are also associated with infection of monocytes, epithelial cells and the central nervous system.

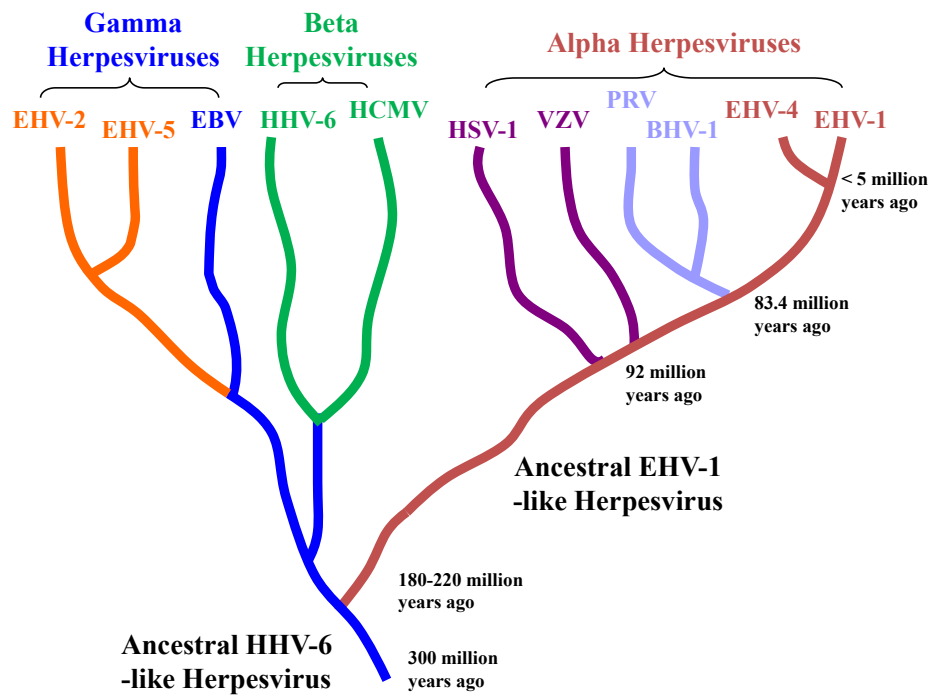


Figure I-1. Evolutionary relatedness of herpesviruses.



It could be speculated that herpesvirus ancestors inhabited the avian species first, and later, underwent transfer from birds to mammals, and then, potentially from humans to horses and back to humans. The estimates of the time of virus transfer between host species indicate a considerable antiquity, close to the end of the Cretaceous period. The three subfamilies are estimated to have arisen 180 to 220 million years ago (McGeoch et al., 1995), before major mammal evolution and therefore became a diverse group of viruses. Within the subfamilies, the phylogenetic tree patterns reflect patterns in the tree lineages of mammalian hosts, suggesting coevolution (cospeciation) of the host and virus lineages, and thus enabling to estimate the division between EHV-1 and EHV-4 to be less than 5 million years ago, separation from PRV and BHV-1 at 83.4 million years ago when artiodactyls (“even-toed”) and perissodactyls (“odd-hoofed” mammals) have evolved, and from VZV and HSV-1 more than 92 million years ago when primates and ungulates (hoofed animals) advanced (Karlin et al., 1994; McGeoch and Cook, 1994; McGeoch et al., 1995; McGeoch et al., 2000). Molecular resource partitioning principle is that coexisting life forms strive to establish independent niche with respect to resources, to avoid competition for host resources. Therefore, avoidance of competition between ancestral viruses, led to spatial and/or temporal isolation aka distinct host specificity, cellular tropism, sites of reactivation, and latency.

### **Clinical Isolates and Laboratory Strains**

EHV-1 field isolates were obtained by culture of diagnostic material (nasal swab or blood or tissue samples) on Rabbit kidney (RK-13), equine dermal (NBL-6) cell lines, or primary equine (equine embryonic lung or equine fetal kidney) cell types (Table I-3). Strains can be adapted to grow on bovine and hamster cells with mutation in gC (Sugahara

et al., 1997). The majority of EHV-1 field isolates are archive material held at the Animal Health Trust (United Kingdom isolates) and the Gluck Equine Research Center (U.S. and Canadian isolates). Additional isolates, or purified DNA from isolates, originating from outbreaks in other countries are maintained by G. Fortier (Laboratoire Frank Duncombe, France), M. Studdert (University of Melbourne, Australia), S. Raidal (Murdoch University, Australia), C. van Maanen (Animal Health Service, The Netherlands), C. Galosi (Cátedra de Virología, UNLP, Argentina), and K. van der Meulen (University of Ghent, Belgium).

Neuropathogenic strain AB4 and nonneuropathogenic strain V592 and other strains were compared on a DNA level to identify a variation in a single amino acid that is strongly associated with neurologic versus nonneurologic disease outbreaks. The amino acid is located within a highly conserved region of the genome that encodes a key viral enzyme, the DNA polymerase gene, which is conserved in all herpesviruses (Nugent et al., 2006). Mutation did not alter the kinetics of viral replication, and therefore, had minimal effect on viral shedding that is important for horizontal spread in a population (Goodman et al., 2007).

**Table I-3. Known EHV-1 parental strains.**

<b>Virus</b>	<b>Isolation, year &amp; place</b>	<b>Passage, cells &amp; #</b>	<b>Reference</b>
Kentucky A	1953, Kentucky	Hamster 400, LM	
Kentucky D	1952, Kentucky	Hamster 94	PubMed 13944111
Ab4	1980, England	ED 12	PubMed 1318606
V592	England		PubMed: 10805982
Ab1	England		
HSV25A	Australia		PMID: 9870583
Kentucky T431	1980, Kentucky	ED 4	
Karpas	1966, France	EK	PubMed 5909615
438/77	1981, Australia		PubMed 6270790
HH-1	1967, Hokkaido		
2104	1986, Hokkaido	FHK3	*

\* EQ-Primary equine kidney cells, ED – equine dermal cells, FHK – fetal horse kidney cells, BK – primary bovine kidney cells, MDBK – Madin-Darby bovine kidney cells, LM – mouse L cells

## **Architecture of Herpes Virion**

EHV-1 has been studied in the laboratory for more than 50 years (Girard et al., 1963; Reczko et al., 1965; Reczko and Mayr, 1963) and its properties have been well characterized either directly or by extrapolation of the findings with HSV-1, VZV, or of its closest relatives, PRV and BHV-1. *Alphaherpesvirinae* family is usually represented by Herpes simplex virus type 1 (HSV-1). However, HSV-1 is a *Simplexvirus* genus member. *Varicellovirus* genus of the *Alphaherpesvirinae* is typified by Varicella Zoster Virus (VZV). In subsequent review of the architecture and life cycle of EHV-1, the findings that are true for all alphaherpesviruses and those specific to the varicellovirus genus or EHV-1 will be described and noted accordingly. However, the review is broadened to include the most detailed knowledge of herpesvirus virology, even if certain aspects have not been confirmed specifically for EHV-1. Therefore, a collective knowledge of herpes virology researchers is reviewed below as related to Equine Herpes Virus 1.

EHV-1 virion consists of four structural units: DNA core, capsid, tegument, and the envelope (Figure I-2). All herpesviruses have the same basic structure, a rigid icosahedral capsid surrounded by a membrane envelope. The capsid is encircled by the tegument proteins and contains the virus DNA in a highly condensed form in which it is protected from mechanical and other damage.

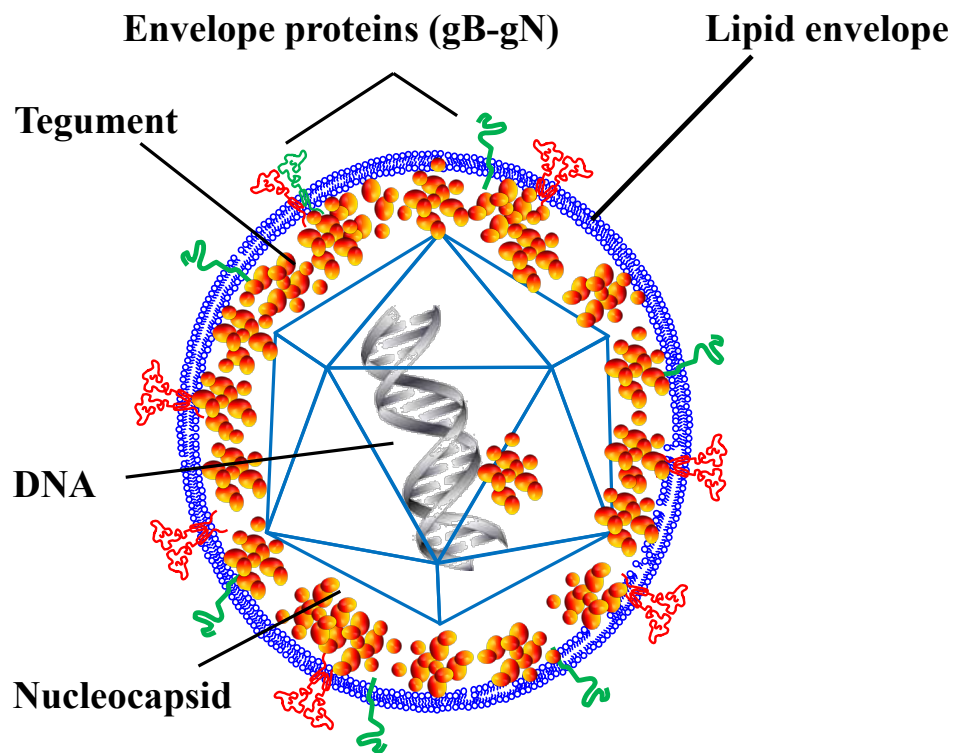
### **The Envelope**

Equine herpesvirus 1 virions are about 120-200 nm in diameter, slightly pleomorphic, spherical and are enveloped in a cell-derived bilipid membrane. Outer covering of the virion has a typical trilaminar appearance (Epstein, 1962), and appears as if an arrangement of patches of altered cellular membranes (Armstrong et al., 1961; Falke et

al., 1959; Morgan et al., 1968). Projected from the surface of the envelope are roughly 800 distinct spikes of viral glycoproteins (Table I-4) dispersed over the entire surface varied in length, spacing, and in the angles at which they emerge from the membrane (Abodeely et al., 1970; Abodeely et al., 1971; Grunewald et al., 2003; Klingeborn and Pertoft, 1972), in clusters, and/or in transmembrane contact with tegument proteins (Grunewald et al., 2003). Their distribution was nonrandom, suggesting functional clustering. Glycoprotein B, for example, contributes about 5% of the envelope spikes. At least 11 membrane proteins are present on the surface of the virion envelope (Roizman and Knipe, 2001). The copy number of individual proteins can exceed 1,000 per virion (Spear, 2004; Steven and Spear, 1997). These proteins are the target for neutralizing antibodies and therefore, the potential targets for viral vaccines.

Herpesvirus envelope proteins play essential roles in the initial stages of virus infection, i.e., virion attachment and penetration (Spear and Longnecker, 2003) to select cell types. Additionally, they are also important in mediating proper assembly and egress of virus particles in the late phase of the replicative cycle (Mettenleiter, 2004), and their function will be discussed in further detail in the review of EHV-1 life cycle.

Lipid rafts, specific microdomains on the surface of cellular membranes were found to play a critical role in virus replication because of localizing and concentrating viral components in such microdomains for entry, assembly, and budding of the virus. Rapid redistribution of cell-surface anchored viral protein such as gB within membrane lipid rafts would bring protein species in the proximity of each other to form multimers and to interact with cellular receptors during early stages of virus infection, serving as a platform for cell signaling and entry (Bender et al., 2003).



**Figure I-2. EHV-1 virion.** Herpesvirus virion consists of a core containing a linear, double stranded DNA, an icosadeltahedral capsid, approximately 100-110 nm in diameter, comprised of 162 capsomeres with a fissure running down the long axis as well as an amorphous, sometime asymmetric material that surrounds the capsid, designated as the tegument, and an envelope containing viral glycoprotein spikes on its surface. The arrow shows a transmembrane contact between a glycoprotein and the tegument.

**Table I-4. *Herpesviridae* glycoproteins.**

Characteristics	EHV-1 ORF	HSV-1	PRV	VZV ORF
Envelope glycoprotein (gK)	6	UL53	gp04	5/gp06
Envelope protein (gN)	10	UL49.5	gp08	9A/gp10.5
Tegument/type 2 membrane protein, required for gB fusion	15	UL45	-	±
Envelope glycoprotein (gC) ; role in cell entry	16/gp13	UL44	gp30/gIII	14/gp16/gpV
Probable integral membrane protein, along with US3 role in disrupting the nuclear lamina during egress	17	UL43	gp29	15/gp17
Membrane-associated phosphoprotein	26	UL34	gp20	24
Envelope glycoprotein (gB); role in cell entry	33/gp14	UL27	gp14/gII	31/gp33/gII
Envelope glycoprotein (gH) complexes with gL; role in cell entry	39	UL22	gp36	37/gp39
Integral membrane protein ; role in virion egress, multiple membrane spanning protein	41	UL20	gp38	39/gp41
Envelope glycoprotein (gM) type 3	52	UL10	gp48	50/gp51
Envelope glycoprotein (gL)	62	UL1	gp56	60/gp61
Envelope glycoprotein (gG)	70	US4	gp67	-
Envelope glycoprotein (gp300)	71	US5	-	-
Envelope glycoprotein (gD)	72	US6	gp68	-
Envelope glycoprotein (gI) type 1	73	US7	gp69	67/gpIV/gp68
Envelope glycoprotein (gE) type 1	74	US8	gp70	68/gpI/gp69

## **The Tegument**

The tegument is a term originally introduced by Roizman and Furlong, to describe the amorphous protein structures contained between the capsid and the envelope that serves as a delivery compartment for proteins that are required early in the course of infection. These structures had no distinctive features in electron-microscopic sections but appeared to be fibrous on negative staining (Morgan et al., 1959). Subsequently, immunoelectron microscopy indicated that the tegument is an ordered structure with structural polarity, but is devoid of a unique geometrical organization (Stefan et al., 1997). Tegument surrounds the nucleocapsid and consists of proteinaceous globular material which may be variable in amount, thus leading to variation in virion size. The tegument occupied about two-thirds of the volume enclosed within the membrane, and the capsid occupied about one-third (Grunewald et al., 2003). Innermost portion of the tegument located adjacent to the capsid exhibits icosahedral symmetry, resulting from the interaction of a large tegument protein, UL36, with the pentons (VP5) of the capsid (Machtiger et al., 1980; McNabb and Courtney, 1992a; Newcomb et al., 1996; Zhou et al., 1999). The outermost part interacts with virus envelope membrane, sometimes in transmembrane contact with envelope glycoproteins.

The tegument of HSV-1 and EHV-1 contains about 20 proteins, including VP1/2 (UL36), VP11/12 (UL46), VP13/14 (UL47), VP16 (UL48,  $\alpha$ -*trans*-inducing factor, alpha-TIF), VP22(UL49), ICP0, ICP4, and the virion host shutoff protein (UL41) as well as the products of the genes US2, US3, US10, US11, UL11, UL13, UL14, UL16, UL17, UL21, UL37, UL51, and UL56 shown in Table I-5 (Copeland et al., 2008; Mettenleiter, 2002b; Mettenleiter, 2004; Schimmer and Neubauer, 2003).

PRV tegument proteins UL36 and UL37 have been shown to physically interact and UL36 possibly interacts with the major capsid protein (Klupp et al., 2002). UL36 is present in the deepest layers of the tegument and attaches to the major capsid protein (VP5) on capsid vertexes (McNabb and Courtney, 1992b; Zhou et al., 1999). UL48 located within both the inner and outer tegument layers (Schmitz et al., 1995), has been shown to interact with cytoplasmic tail of gH (Gross et al., 2003). In PRV, there is evidence of UL49 interaction with gE/I and M (Fuchs et al., 2002). New tegument protein interactions which could be potential targets for future antivirals are UL11-UL16, UL36-UL48, UL46-UL48, UL47-UL48, and UL48-UL49 (Vittone et al., 2005).

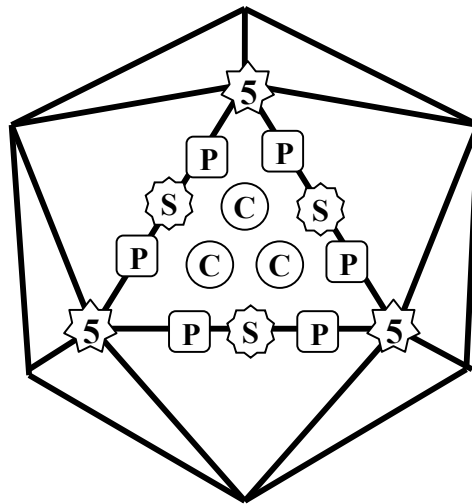
**Table I-5. Tegument proteins of *Herpesviridae*.**

Characteristics	EHV-1 ORF	HSV-1	VZV
Latency associated transcript, EICP0	63	ICP0	
	64	ICP4	
	3?	UL56	2?
	8	UL51	7
Tegument protein	11	VP22(UL49)	9
Tegument phosphoprotein; transactivator of immediate-early genes	12	alphaTIF (Vmw65) VP16 (UL48)	10
Tegument protein	13	VP13/14 (UL47)	11
Tegument protein	14	VP11/12 (UL46)	12
Tegument/envelope protein	15	UL45	±
Tegument protein ; virion host shutoff protein	19	VHS(UL41)	17
Tegument protein	<b>23</b>	UL37	21
Large tegument protein	<b>24</b>	VP1/2 (UL36)	22
Tegument protein	40	UL21	38
ORF 45	45	UL17	43
Tegument protein	46	UL16	44
ORF 48	48	UL14	46
Tegument protein, probable serine-threonine protein kinase	49	UL13	47
Myristylated tegument protein, role in virion envelopment	51	UL11	49
ORF 68	68	US2	-
Serine-threonine protein kinase	69	US3	66
Tegument protein, type 2 membrane protein	76	US9?	65
Virion protein	66	US10	64
		US11	



## The Nucleocapsid

The morphologically distinctive herpesvirus capsid has been well studied in order to understand the dynamics of virus assembly (Baker et al., 1990; Newcomb et al., 2003). A thick-walled, spherical, ~ 120 nm in diameter nucleocapsid is isometric and composed of 162 capsomers arranged in 5:3:2 axis of symmetry, triangulation class T = 16 icosahedral symmetry as shown in Figure I-3 (Caspar and Klug, 1962; Wildy et al., 1960). Twelve capsomers at the vertices are "pentons". The remainder 150 capsomers are "hexons", 9.5x12.5 nm in longitudinal section with a channel of 4 nm in diameter running from the surface along their long axis (Watson et al., 1963). Hexons are located at the edges and faces of the capsid and fall into three classes (P – peripentonal, E- edge, and C-center), depending on their positions on the surface lattice (Steven et al., 1986). EHV-1 hexons are composed of six molecules of major capsid protein (hexamers of VP9 (148 kDa)) and six of VP26 (UL35, 12kDa), which occupy the top of each hexon (Newcomb et al., 1989) (Table I-6).



**Figure I-3. Types of capsomers present on the T-16 capsid surface lattice. P – peripentonal hexon, E- edge hexon, C-center hexon, 5 penton.**

Eleven of the 12 pentons are pentamers of VP9, while the 12<sup>th</sup> is a unique vertex, a portal, cylindrical structure composed of twelve UL6, and contains an axial channel through which DNA is introduced into the capsid. By further extrapolation from HSV-1, portal protein UL6 interacts with the DNA cleavage and packaging proteins (putative terminase subunits) UL15 and UL28. Capsomers are connected in groups of three by 320 triplexes, heterotrimers formed from two copies of VP23 (34kDa) protein and a single copy of VP19c (50kDa) that lie above capsid floor (Okoye et al., 2006; Trus et al., 1996). Scaffolding protein UL26.5 participates in capsid formation but thereafter is lost and is not found in the mature capsid or virion. EHV-1 capsids also contain several other proteins, including, VP19 (59 kDa, -360 copies), VP23 (36 kDa, -660 copies), and VP26 (12 kDa, -1,300 copies) (Newcomb et al., 1989; Perdue et al., 1975). The number of polypeptide species contained in the virions generally varies from 35 to 45. Virus capsid assembly is under the control of tightly regulated program that includes the involvement of viral scaffolding proteins and cellular chaperonins, maturational proteolysis, and conformational changes on an epic scale.

**Table I-6. Protein components of capsid shell.**

<b>Components of capsid shell required for capsid assembly</b>	<b>EHV-1</b>	<b>HSV-1</b>
Major capsid protein	ORF 42 (VP9)	VP5 (UL19)
Assemblin (protease), capsid assembly protein /capsid scaffold protein (Peptidase family S21)	ORF 35 (protease) and ORF 35.5 (capsid assembly protein)	VP21 (UL26) and VP22a (UL26.5)
Form triplexes to connect capsomers		VP23 (UL18) and VP19c (UL38)
Portal protein		UL6
DNA packaging protein	ORF 27	UL33
		VP26 (UL35)
Terminase subunits, DNA cleavage and packaging proteins, form part of the terminase		UL15 and UL28
	VP19, VP9, VP23, VP26	VP24 (UL26)

## The Core

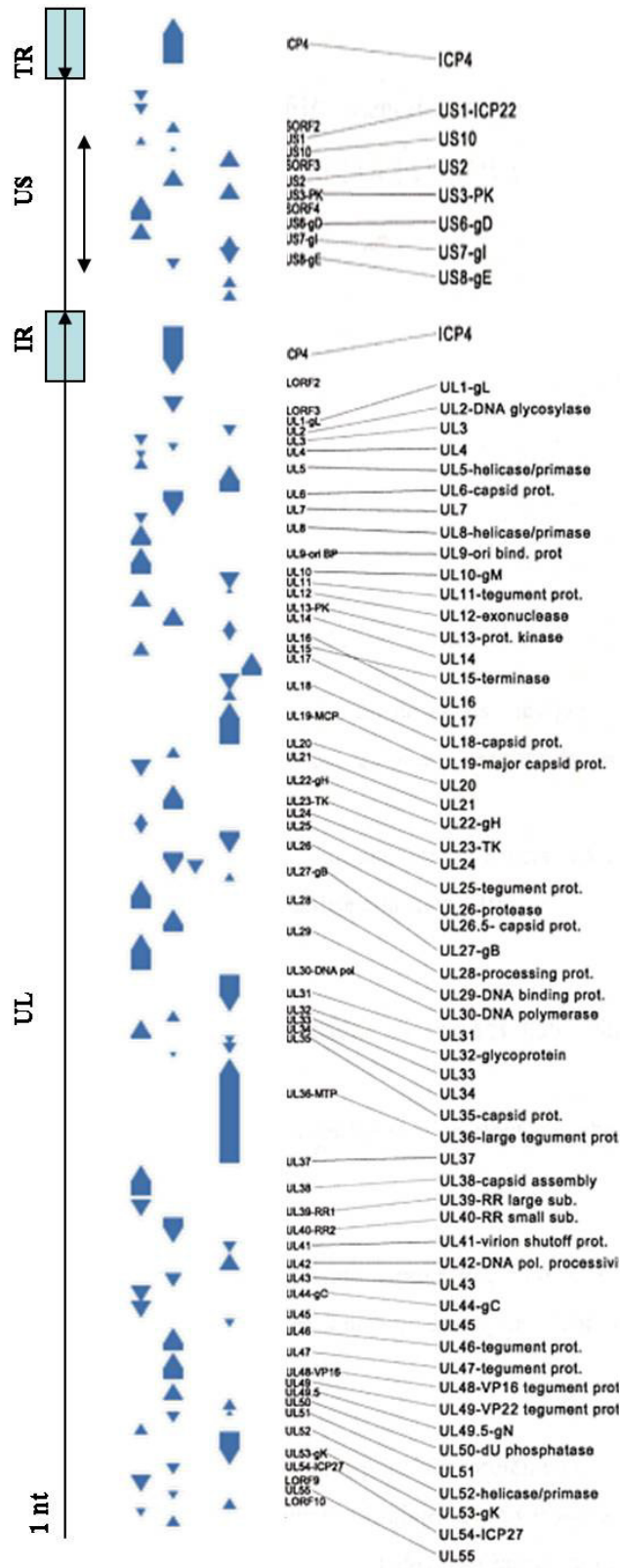
The core of mature virion consists of a fibrillar spool on which one molecule of linear double stranded DNA is wrapped in a form of a torus with regularly spaced ( $\sim 26$  Å for HSV-1) concentric layers (Furlong et al., 1972; Zhou et al., 1999), the arrangement similar to those in dsDNA bacteriophages such as  $\lambda$ , T4, and P22 (Brown, 2002; Prevelige and King, 1993). DNA is densely coiled in a “liquid crystalline” arrangement (Booy et al., 1991) with the ends of the proteinaceous fibers anchored to the underside of the capsid shell.

## The Viral Genome

EHV-1 has a double-stranded linear DNA genome of approximately 150 kbp ( $96 \times 10^6$  Da), has a base composition of 56.67% G + C, and contains 80 open reading frames able to encode proteins for the production of progeny genomes and mRNAs as shown in Table I-7 (Telford et al., 1992; Whalley et al., 1981). All herpesvirus genomes have a unique long ( $U_L$ ) and a unique short ( $U_S$ ) region, bounded by inverted repeats (Figure I-4). After infection of susceptible cells, the linear viral genomes of herpesviruses form circular molecules via ligation of the genomic ends.

**Table I-7. Genome characteristics of clinically important alphaherpesviruses**

Genome	Genus	Strain	Length, nt	G+C%	Protein	% coding
EHV-1	Varicello	Ab4	150,224	56	80	83
EHV-4	Varicello	NS805567	145,597	50	79	85
HSV-1	Simplex	17	152,261	68	77	79
HSV-2	Simplex	HG52	154,746	70	77	79
VZV (HSV-3)	Varicello	Dumas	124,884	46	73	89
SVV (CeHV-9)	Varicello	Delta	124,784	40	74	88
CeHV-1 (B Virus)	Simplex	E2490	156,789	74	75	76
PRV-1 (SuHV-1)	Varicello	Several	143,461	73	69	73
BHV-1	Varicello	several	135,301	72	70	84
BHV-5	Varicello	SV507/99	137,821	74	70	83
MDV (GHV-2)	Mardi	1	177,874	44	105	77



**Figure 1-4. Map of the EHV-1 genome.** (A) General structure of the genome. Note the unique long (UL) and unique short (US) coding regions and the repeats designated *a*, *b* and *c*. (B) Alignment of the open reading frames in a physical map that is collinear with the genome.

The EHV-1 genome is divided into segments of a long unique region, U<sub>L</sub> (112.9 kbp); an internal repeat, IR<sub>S</sub> (12.7 kbp); a unique short segment, U<sub>S</sub> (11.9 kbp); and a terminal repeat, TR<sub>S</sub> (12.7 kbp). In addition, U<sub>L</sub> is flanked by a short inverted repeat of 32 bp (TR<sub>L</sub>/IR<sub>L</sub>) (Telford et al., 1992; Yalamanchili and O'Callaghan, 1990). An organization of TR<sub>L</sub>-U<sub>L</sub>-IR<sub>L</sub>-IR<sub>S</sub>-U<sub>S</sub>-TR<sub>S</sub> is typical of an alphaherpesvirus. U<sub>S</sub> region is found in either possible orientation relative to the fixed U<sub>L</sub> segment. As a consequence, virus DNA consists of an equimolar mixture of the two isomeric forms (P and I). The same genome arrangement and isomerization are also reported for BHV-1 (Hammerschmidt et al., 1988), EHV-3 (Atherton et al., 1982; Sullivan et al., 1984), PRV (Stevely, 1977), and VZV (Davison, 1984).

### **EHV-1 Glycoproteins**

Through the use of multiple glycoproteins and other virus-specified proteins alphaherpesviruses have evolved mechanisms to deal with multiple membrane barriers during entry via fusion of the viral envelope with cellular membranes, intracellular virion morphogenesis and egress, cell-to-cell spread, and virus-induced cell fusion (Mettenleiter, 2002a; Mettenleiter, 2002b; Roizman and Knipe, 2001; Spear, 2004; Spear and Longnecker, 2003). The EHV-1 specifies at least 14 glycoproteins: gB/gp14 (Guo, 1990; Osterrieder et al., 1996; Pilling et al., 1994), gC/gp13 (Allen and Coogle, 1988; Allen and Yeargan, 1987; Matsumura et al., 1993), gD (Audonnet et al., 1990), gE (Audonnet et al., 1990), gG (Colle et al., 1992), gH (Robertson et al., 1991), gI (Audonnet et al., 1990), gK/UL4 (Zhao et al., 1992), gL (Telford et al., 1992), gM (Osterrieder et al., 1996; Pilling et al., 1994), gN(HSV-1 UL49.5), gp10, gp2, and gp21/22a, most of which share high homology to those of herpes simplex virus type 1 (HSV-1) as prototypic virus for studying

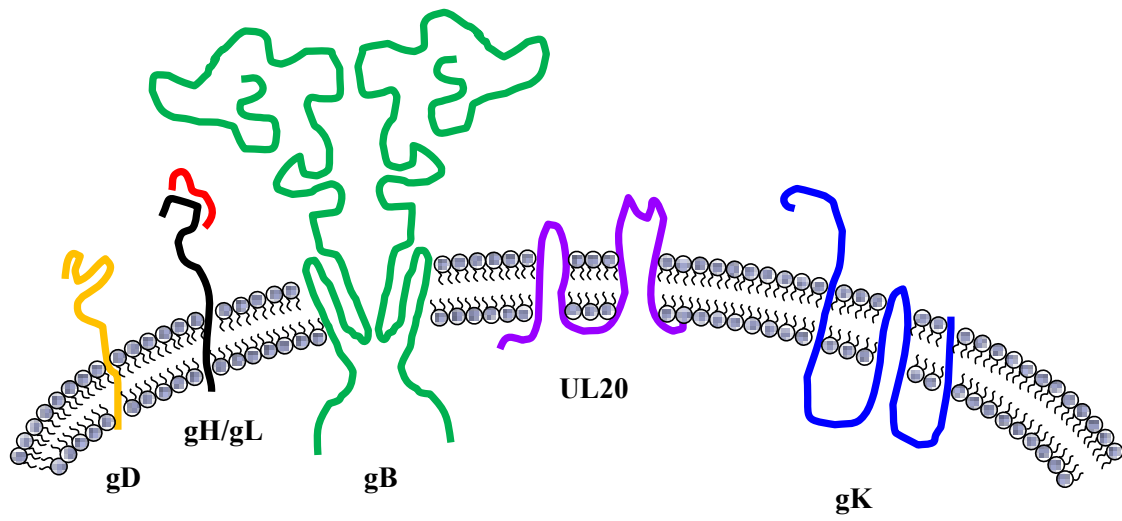
the *Alphaherpesvirinae* subfamily (Audonnet et al., 1990; Birch-Machin et al., 2000; Crabb et al., 1991; Elton et al., 1991; Flowers et al., 1995b; Kukreja et al., 1998a; Osterrieder et al., 1995; Stokes et al., 1996; Tewari et al., 1994; Wellington et al., 1996b; Whalley et al., 1995; Whalley et al., 1989). In addition to those glycoproteins similar to HSV-1, EHV-1 also possesses at least three unique glycoproteins, designated gp10, gp2, and gp21/22a (Allen and Coogle, 1988; Allen and Yeargan, 1987). The functions of several individual EHV-1 envelope proteins, such as glycoprotein B (gB), gC, gD, gE, and gM, have been analyzed in some detail. EHV-1 also encodes several non-glycosylated membrane associated proteins: UL20, ORF76/US9, ORF37/UL24, 17/UL43, and 26/UL34.

Viral glycoproteins have three parts: the external ectodomain, which interacts with the host; a transmembrane segment, typically a single alpha-helix, and the internal part, the endodomain (Figure I-5). All known glycoproteins that are capable of membrane fusion are trimers for at least part of the infectious cycle. Viral glycoproteins are also key determinants of membrane-associated events occurring during virion morphogenesis and egress from infected cells.

### **Glycoprotein B**

EHV-1 gB (gp14) is highly conserved with the gB (HSV, CMV, EBV) and gpII glycoproteins (PRV, VZV) of other herpesviruses, suggesting that this glycoprotein has a similar overall structure, synthesis, maturation, and function in each virus (Bell et al., 1990; Guo, 1990; Whalley et al., 1989).

The gene encoding gB includes a transcription unit composed of a CAT box, a TATA box, a ribosome-binding sequence, a polyadenylation signal and an open reading



**Figure I-5. *Herpesviridae* glycoproteins and their putative orientations in the cellular membrane.**

frame (ORF) 33 of 3.4 kb beta-gamma gene transcribed from left to right (Bell et al., 1990; Guo, 1990). The primary translation product is 980 amino acids, 118K molecule which is cotranslationally glycosylated to the large precursor molecule of 138K form by the addition of high mannose oligosaccharides. The 138K form is then proteolytically cleaved to two smaller forms, 77-75K and 55-53K, linked by a disulfide bond(s) to form a 145K complex. The 77-75K species contains both high mannose and hybrid oligosaccharides while the 55-53K form of gB contains some complex oligosaccharides (Sullivan et al., 1989). The amino acid sequence of gB has the characteristic features of membrane glycoprotein including a 20-aa signal sequence at the N-terminus, marking it for translocation into the lumen of the endoplasmic reticulum (ER) (McGeoch et al., 1987; Perlman and Halvorson, 1983; Wellington et al., 1996a), a 743-aa surface domain, a 40-aa membrane anchoring region, a 108-aa hydrophilic cytoplasmic domain at the C-terminus and eleven potential sites for N-linked glycosylation (Guo, 1990; Sullivan et al., 1989; Whalley et al., 1989). The cytoplasmic domain of gB is the longest among HSV-1 glycoproteins, implying a crucial role for this domain in gB-mediated functions. HSV-1 gB contains at least one Pleckstrin homology (PH) domain, a large family of cellular proteins implicated in lipid binding and signaling functions (DiNitto et al., 2003).

Alphaherpesvirus gB possess similar secondary and tertiary structures (Riggio et al., 1989). The glycoprotein has three-stranded coiled coils at the trimer axis, reminiscent of class I, and a long three-stranded beta-sheet with a structure similar to that of a class II motif but with different strand topology (Steven and Spear, 2006). Members of both classes accomplish fusion through a large-scale conformational change, triggered by a signal from a receptor-binding component.



EHV-1 gB is essential for virus growth and is required for direct cell-to-cell spread in vitro since gB-negative virus exhibits significant reduction in viral titers and no plaque formation when grown and titrated on noncomplementing cells (Neubauer et al., 1997b). gB monoclonal antibodies also inhibit cell-cell fusion via complement-dependent neutralizing antibody (Wellington et al., 1996c). There is a variety of evidence indicating that gB plays important roles in membrane fusion phenomena during virus entry and virus-induced cell fusion. Single amino acid substitutions and truncations of the carboxyl terminus of HSV-1 gB cause extensive virus-induced cell fusion (Baghian et al., 1993; Bzik et al., 1984b; Cai et al., 1988b; Gage et al., 1993). Transient co-expression of gB with gD, gH and GL causes cell-to-cell fusion, which is substantially increased by carboxyl terminal truncations of gB (Foster et al., 2001b; Haan et al., 2001; Klupp et al., 2000; Pertel, 2002). These results suggest a direct role for gB in membrane fusion and that perturbations of the carboxyl terminal domains of gB facilitate gB-mediated cell-to-cell fusion.

Alphaherpesviridae gB is viewed as the primary component of herpesvirus membrane fusion machinery. HSV-1 mutant viruses lacking gB are not able to enter into cells (Cai et al., 1987) due to a post-attachment defect that can be resolved by polyethelene glycol (PEG) mediated fusion of viral and cellular membranes (Cai et al., 1988a). Interesting is that attachment of the gB-negative EHV-1 virions to target cells is similar to that of the wild type virus (Neubauer et al., 1997b), but treatment with PEG, a fusogen, while enhances the virus production does it to a lesser extend than in a similar experiment with gB-negative PRV, suggesting that EHV-1 gB might not be as stringently required for virus penetration as are its homologs in other Alphaherpesvirinae (Neubauer et al., 1997b).

Glycoprotein B has been shown to interact with a paired immunoglobulin-like type 2 receptor (PILP) alpha to gain entry into the cells devoid of other herpes virus entry mediators (Sato and Arase, 2008).

Glycoprotein B along with major nucleocapsid protein are some of the most antigenic EHV-1 proteins (Ahmed et al., 1993). An antigenic determinant recognized by an anti-gB monoclonal antibody is present in the N-terminus of the surface domain (Guo, 1990). Strong virus-neutralizing activity of antibodies to the extracellular portion of gB, amino acids 1 to 844, expressed in insect cells using a recombinant baculovirus, showed that post-translational modification of the EHV-1 gp14 is important for the expression of epitopes necessary for the induction of neutralizing antibodies (Osterrieder et al., 1994).

A number of studies of EHV-1 gB have addressed immune responses to and vaccine potential of recombinant gB. Plasmid DNA recombinant intramuscular vaccines expressing the gB, gC and gD glycoproteins of EHV-1 significantly reduced virus excretion but failed to protect against cell-associated viremia following respiratory challenge of ponies with EHV-1 virulent Ab4 strain (Minke et al., 2006). In another study, vaccination of mice with baculovirus-expressed gB prevented clinical signs of infection, induces rapid clearance of virus from the lungs, reduced pulmonary lesions, and increased T cell peribronchiolar and perivascular aggregations in mice challenged with EHV-1 (Packiarajah et al., 1998). Baculovirus-expressed EHV-1 glycoprotein B vaccinated mice also showed significantly higher rate of litter survival, increased body weight of young, and no virus presence in the fetal tissues when challenged during pregnancy (Kukreja et al., 1998b). Mice infected with EHV-1 (RacL11) gB-negative virus, did not develop disease, even when viruses were grown on complementing cell lines, and gB-negative

virus immunization, protected animals from developing disease inducing both virus-neutralizing antibodies and EHV-1-specific splenic T-cell response following challenge with wild-type EHV-1 (Neubauer et al., 1997a).

### **Glycoprotein C**

Glycoprotein C, gp13, is a gamma-1 gene product, encoded by of ORF 16, a 2.8-kb mRNA (Matsumura et al., 1993). The protein has the characteristic features of a membrane-spanning protein: an N-terminal signal sequence, a hydrophobic membrane anchor region, a charged C-terminal cytoplasmic tail, and an exterior domain with nine potential N-glycosylation sites (Allen and Coogle, 1988).

The glycoprotein C of EHV-1 and other alphaherpesviruses functions as a major virus attachment protein through binding to glycosaminoglycans (GAG), heparan sulfate (HS), a heparin-like moiety on the cell surface (Herold et al., 1991; Kari and Gehrz, 1992; Li et al., 1995; Mettenleiter et al., 1990; Okazaki et al., 1991). The main function of gC binding to GAGs seems to be to aid concentration of the virus on cell surfaces, enabling the more stable interaction of gD with an entry receptor. It has also been shown that while PRV gC binds to heparin only in conjunction with gB (Mettenleiter et al., 1990), EHV-1 gC binds to heparin separately from gB, similarly to that in HSV-1 (Herold et al., 1994; Sugahara et al., 1997). Hydrophilic regions of the gC may be responsible for binding to heparin (Sugahara et al., 1997). Interestingly, that the gC of strains adapted to non-equine cells have been shown to have higher affinity for heparin due to amino acid substitutions of the hydrophilic regions gC, amino acid residues 92 to 175, resulting in the glycoprotein becoming more cationic(Sugahara et al., 1997). This and other cell attachment and entry

adaptations of EHV-1 may explain the expansively broad cell tropism of EHV-1 compared to other alphaherpesviruses.

Another important function of gC is its ability to bind and inactivate the C3b component of complement facilitating immune evasion (Eisenberg et al., 1987; Friedman and Nashold, 1984; Huemer et al., 1995; Huemer et al., 1993; Lubinski et al., 1999; Lubinski et al., 1998). Since complement is one of the most critical defence mechanisms of the innate immunity against cerebral infection by viruses, the function EHV-1 gC is important in myeloencephalopathy presentation of EHV-1 disease.

Glycoprotein C is an important virulence factor of alphaherpesviruses *in vivo*. Infection of mice with a EHV-1 mutant virus that lacked gC ORF does not cause EHV-1-related disease, while wild-type virus infected mice exhibit massive body weight losses, high virus titers in the lungs, and viremia (Osterrieder, 1999). Amino acids 152 to 275 of EHV1 gC specifies one of EHV-1 type-specific epitope (Crabb and Studdert, 1995). Immunization of mice with EHV-1 gC shows accelerated clearance of EHV-1 by virus specific antibodies, high levels of virus neutralising antibodies, and by cell mediated immune responses from the respiratory tissues following intranasal challenge (Packiarajah et al., 1998; Tewari et al., 1995). IgG2b is the predominant antibody isotype produced in BALB/c (H-2K(d)) mice against gC derived from EHV-1-infected cells, while equal amounts of IgG2a/2b are found in the serum of C3H mice (H-2K(k)), indicative of a T-helper(1) response (Alber et al., 2000).

### **Glycoprotein D**

The envelope glycoprotein D (gp17/18, gp60?), a 392 amino acids EHV-1 protein is encoded by a unique short (Us) segment of the EHV-1 genome with 26% and 20% of its

residues matching PRV gp50 and HSV-1 gD, respectively (Audonnet et al., 1990; Colle and O'Callaghan, 1995; Elton et al., 1992; Flowers et al., 1991; Flowers and O'Callaghan, 1992; Love et al., 1992; Whalley et al., 1991; Whittaker et al., 1992),(Elton et al., 1992; Whalley et al., 1991). A 3.8-kb mRNA encoding gD is synthesized as a late (beta-gamma) transcript. It initiates 91 and 34 nucleotides downstream of the CCAAT and TATA elements, respectively (Flowers and O'Callaghan, 1992). Glycoprotein D exhibits features typical of a transmembrane protein: a hydrophobic N-terminal signal sequence followed by a cleavage site (Arg35 and Ala36), four potential N-linked glycosylation sites, and a hydrophobic membrane-spanning domain near the carboxyl terminus followed by a charged membrane anchor sequence (Flowers et al., 1991; Wellington et al., 1996a).

Glycoprotein D proteins are first detected at 6 hr after infection with maximal synthesis of gD between 5 and 8 hr post-infection, resulting in 43.206-kDa polypeptide which then undergoes processing (Flowers et al., 1995a). Polypeptides of 55 and 58 kDa are detectable in EHV-1-infected cells 2.5 hr apart. A 55-kDa protein is a high-mannose N-linked oligosaccharides precursor to the 58-kDa species, a mature polypeptide possessing complex type oligosaccharides and observed in the membrane fraction of EHV-1 virions(Flowers et al., 1995a; Flowers and O'Callaghan, 1992).

Importantly, gD has been shown to act as the viral ligand for entry receptors expressed on cell surface (Spear et al., 2000). As a result, gD is essential for replication *in vitro* gene, absolutely required for virus entry and virus-induced cell-to-cell fusion to occur (Csellner et al., 2000). As discussed earlier, HSV-1 gD has the ability to bind HVEM, nectin-1, nectin- 2, and other cell surface receptors to mediate virus entry. In addition, cells that express gD are resistant to herpes infection in a dose-dependent manner due to a

saturation of the corresponding entry receptors (Campadelli-Fiume et al., 1988b; Johnson et al., 1990). No gD receptor for EHV-1 have been isolated to date and studies show that EHV-1 may utilize a unique unknown to date cellular receptor or employ an alternate entry strategy in the absence of the gD receptor. Noteworthy, is that several dozens of mammalian species encode already identified and cloned herpes virus entry mediators on their cell surface, just as most mammals possess the specie-specific herpes virus. It is likely that EHV-1 cell surface receptor of gD can be identified in the near future.

X-ray structures of HSV-1 gD alone and in complex with HVEM have been determined, revealing that a portion of gD assumes an Ig-like fold with unconventional disulfide-bonding patterns (Carfi et al., 2001) and a hairpin loop in the complex with HVEM (Spear and Longnecker, 2003). N-terminal end of gD has a critical role in its functional interactions with all but nectin-1 entry/fusion receptors (Yoon et al., 2003; Zago and Spear, 2003).

Glycoprotein D plays an important role in eliciting the protective immune response against EHV-1 infection, and numerous studies using various expression systems that included *Escherichia coli*, baculovirus and plasmid DNA evaluated its potential as a subunit vaccine (Flowers et al., 1995b; Ruitenberg et al., 2001; Wellington et al., 1996b). Regardless of the expression system and therefore, despite the lack of glycosylation, inoculation of gD protein alone or in combination with gD DNA evoked neutralizing antibody responses and protected vaccinated mice against a challenge with EHV-1, reducing clinical signs of infection such as lung pathology, fetal loss and fetal damage, showing accelerated clearance of virus from the lungs, and inducing accumulation of mononuclear cells, predominantly lymphocytes (T cells) (Packiarajah et al., 1998; Walker

et al., 2000; Weerasinghe et al., ; Zhang et al., 1998). The protective effects of primarily CD4<sup>+</sup> T cells, were confirmed by adoptive transfer from spleen of baculovirus gD-immunized donors to recipients that were challenged with live EHV-1. (Tewari et al., 1994). EHV-1 gD DNA induces IgG<sub>a</sub> antibody production consistent with T-helper 1 (Th1) type of immune response which is maintained after the protein boost, despite the gD protein alone to directing a Th2 response (Love et al., 1993; Ruitenbergh et al., 2000; Ruitenbergh et al., 1999; Tewari et al., 1994). Antibody isotype can also be species specific, where immunization elicited predominantly an IgG1 response in BALB/c mice (indicative of a T-helper(2) response) and an IgG2a/2b response (indicative of a T-helper(1) response) in C3H mice (Alber et al., 2000).

Phenotypically complemented gD-negative EHV-1 also induces protective immune responses following intranasal or intramuscular inoculation of mice (Csellner et al., 2000). Glycosylation was also not required to elicit EHV-1 gD-specific antibodies including virus-neutralizing antibody in horses (Weerasinghe et al., 2006). In foals and adult horses, recombinant baculovirus-expressed EHV-1 gD induces virus-neutralizing antibody, including colostrally-derived, and significantly decreases viral shedding, however does not protect foals born from vaccinated mares (Foote et al., 2005; Freeman and Ellis, 1984). Isotype analysis revealed elevated gD-specific equine IgG<sub>a</sub> and IgG<sub>b</sub> relative to IgG<sub>c</sub>, IgG(T) and IgA (Foote et al., 2005).

Entry of EHV-1 has been suggested to also occur via endocytic/phagocytic pathway, as an alternative way to infect important cell populations. Interaction between integrins and RSD motif of EHV-1 gD were shown to be important in such entry of the virus into CHO-K1, the chinese hamster ovary, cells (Van de Walle et al., 2008).

## **Glycoprotein H and Glycoprotein L**

The 2517 bp UL22 gene encodes the 838 aa glycoprotein H (gH). gH is a type I membrane glycoprotein containing an 18 aa signal peptide, a long 785 aa ectodomain, a single 21 aa transmembrane hydrophobic domain close to the C-terminus, and a 14 aa C-terminal cytoplasmic tail. The 675 bp UL1 gene encodes the 224 aa glycoprotein L. gL contains a 25 aa signal peptide; however, unlike other herpesvirus glycoproteins, gL does not contain a transmembrane domain. It appears that gL is not an integral membrane protein; rather, its membrane association and incorporation into virus particles is dependent on its heterodimer interaction with gH (Dubin and Jiang, 1995). When gH is expressed in transfected cells in the absence of gL, the resulting gH polypeptide is neither folded or processed correctly. The malformed gH remains in the endoplasmic reticulum and undergoes self-aggregation (Foa-Tomasi et al., 1991; Forrester et al., 1991; Roberts et al., 1991). Similarly, cells infected with a gL-null virus do not produce virions containing gH. When cells are infected with a gH-null virus, gL is neither correctly processed nor incorporated into the plasma membrane or viral envelope (Hutchinson et al., 1992; Roop et al., 1993). Due to the lack of a transmembrane region, the gL polypeptide is secreted into the medium (Dubin and Jiang, 1995). When both UL22 and UL1 genes are cotransfected into cells, the antigenic conformation of the gH/gL heterodimer is identical to that of virus infected cells, indicating that no other viral proteins are necessary for heterodimer formation. The mutual interaction of both glycoproteins is mediated by the N-terminal region of the first 69 aa of gL after cleavage of the 25 aa signal peptide (Roop et al., 1993), while gH interacts through a central region of its extracellular domain. A properly formed gH/gL heterodimer is required for both attachment virus entry and virus-induced



cell-to-cell fusion. Although viruses lacking gH/gL are unable to enter cells, they are able to attach to the cell surface. Therefore, the role of gH/gL in virus entry is during the virus envelope plasma membrane fusion event and is not required for virion or receptor binding.

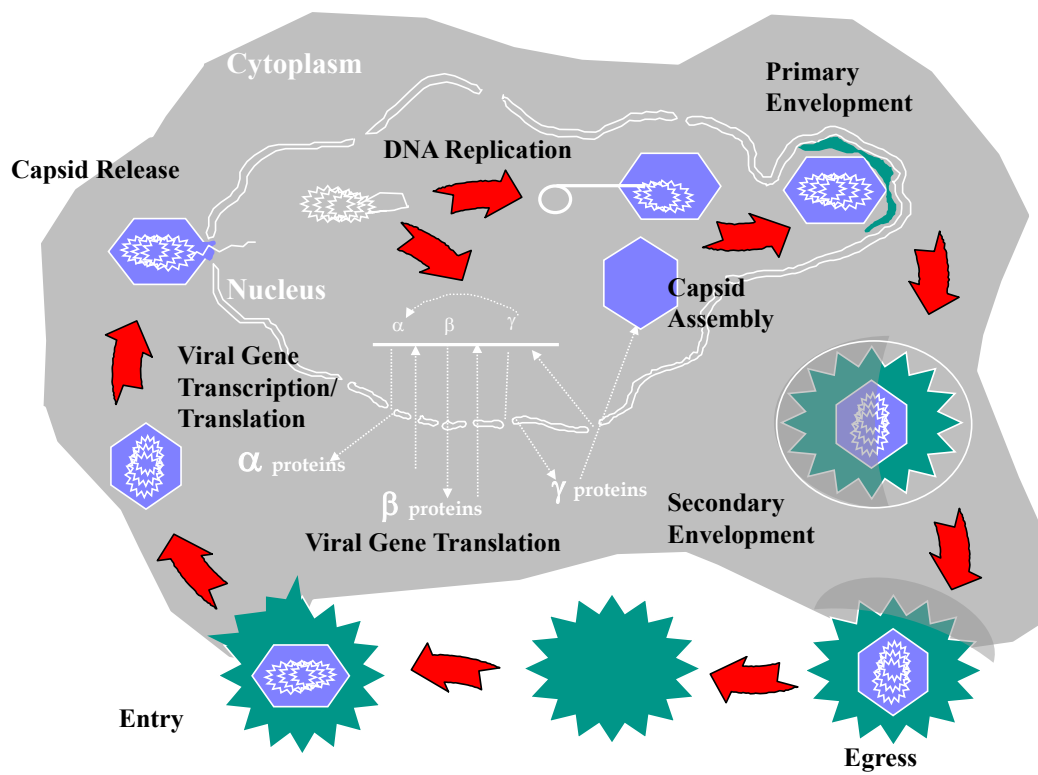
## **The Herpes Virus Life Cycle**

### **Virus Attachment and Entry**

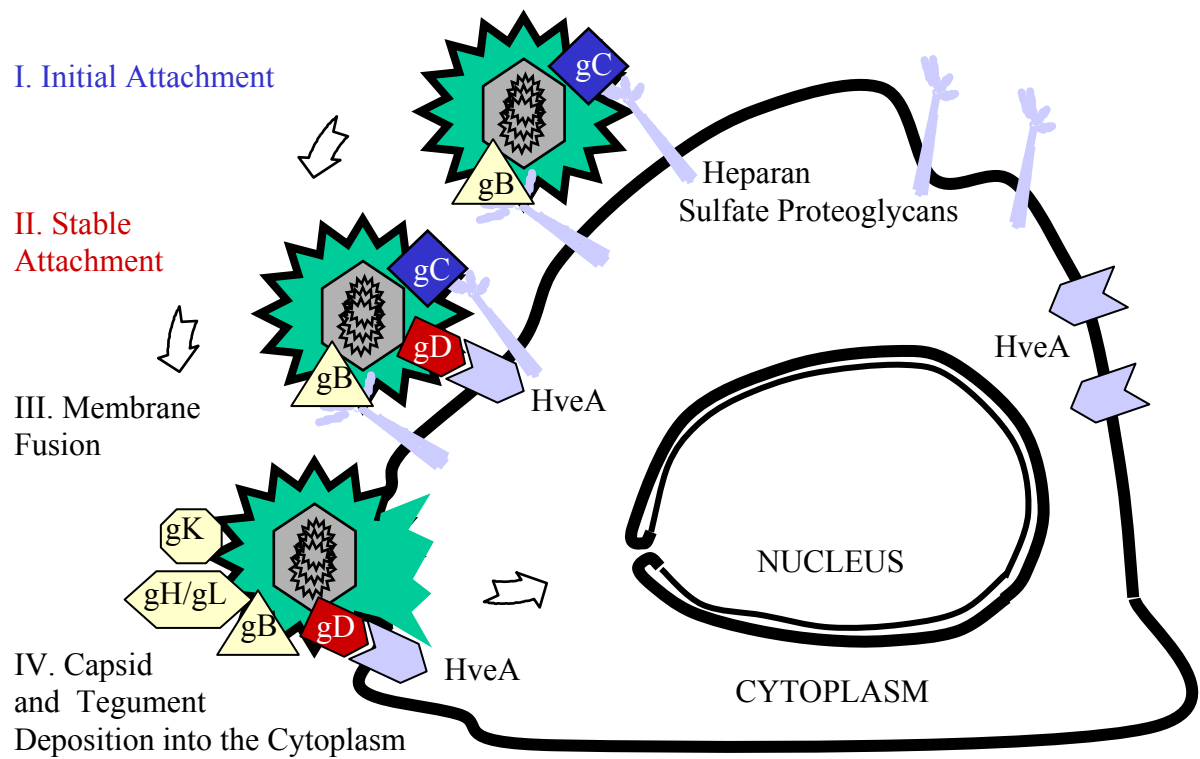
#### **Binding Receptors**

Recent developments in molecular studies of HSV-1 entry, maturation, and spread have contributed to deeper understanding of EHV-1 life cycle and pathogenesis (Figure I-6). Initial contact of the virus to the cell surface membrane is through receptor binding (Figure I-7), where positively charged residues of glycoprotein C and to a lesser extent glycoprotein B, attach to the glycosaminoglycan proteoglycans on cell surface, primarily to the heparan sulfate moieties of ubiquitous in mammals heparin sulfate proteoglycans (Osterrieder, 1999; Shieh et al., 1992; Sugahara et al., 1997; WuDunn and Spear, 1989). The receptor interaction at this point is reversible and the changes in the virion envelope required for fusion and entry do not occur. Presence of heparin sulfate is not essential for virus entry, while gC is also dispensable for either virus entry or replication; their interaction however, confers approximately 10-fold increase in the efficiency of virus attachment (Banfield et al., 1995; Heine et al., 1974).

After initial attachment via gB and gC, alphaherpesvirus gD binds additional cell surface receptors, and thus, confers susceptibility of a certain cell type to the virus infection. Interaction of gD with specific cellular receptors results in its conformational changes and subsequent interaction with gH/gL and gB initiating the fusion of viral and cellular membranes (Csellner et al., 2000).



**Figure I-6. EHV-1 life cycle.**

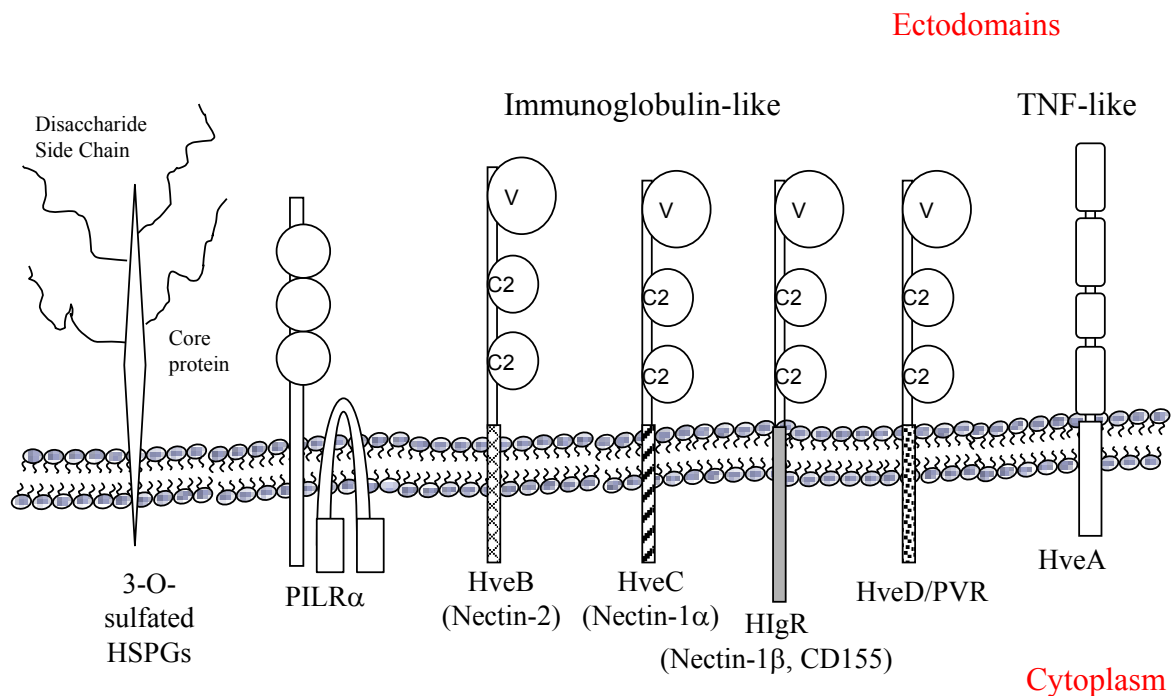


**Figure I-7. Events of virus entry into the cell.**

The importance of alphaherpesvirus gD-receptor binding for virus entry and spread is exemplified by the fact that soluble forms of these receptors (HveA, HveC, and HIgR variants) inhibit virus entry into susceptible cells via their interaction with gD. (Cocchi et al., 1998a; Krummenacher et al., 1998; Rux et al., 1998). Only alphaherpesviruses (except VZV) encode members of the gD family.

Betaherpesvirus and gammaherpesviruses rely on other viral proteins to serve as ligands for cellular receptors. Also, as will be discussed below, endocytic uptake of HSV virions from the cell surface is rapid and independent of any known gD receptor (Cassiani-Ingoni et al., 2005), EHV-1 can potentially employ the same strategy during entry of non-equine cells lacking herpes virus entry mediators. No specific gD receptor have been identified for EHV-1 at this time (Frampton et al., 2005). The increase of EHV-1 gB-negative titers by polyethylene glycol (PEG) treatment, is considerably lower compared to gB-negative pseudorabies virus, suggesting that EHV-1 gB might not be as stringently required for virus penetration as are its homologs in other Alphaherpesvirinae (Neubauer et al., 1997b).

Several human and animal cellular receptors have been identified for various alphaherpesviruses, by their ability to mediate virus entry into normally herpesvirus resistant Chinese hamster ovary (CHO) cells (Figure I-8). These receptors include HveA, a member of the TNF receptor family (Montgomery et al., 1996), as well as several nectins, which are members of the immunoglobulin superfamily (Cocchi et al., 1998a; Cocchi et al., 1998b; Geraghty et al., 1998; Warner et al., 1998). Various homologs of some of these receptors have also been isolated from other cell-types (Foster et al., 1999; Shukla et al., 2000). HSV-1 gD and by analogy all gD homologues specified by alphaherpesviruses bind



**Figure I-8. The three classes of cell surface receptors for HSV entry.** The tumor necrosis factor (TNF) receptor family includes HveA. The immunoglobulin superfamily consists of nectins and PILR-alpha. The third family includes 3-O-sulfated heparin sulfate. Only viral attachment can occur in the absence of an HSV entry receptor.

these receptors and facilitate virus entry into susceptible cells. The importance of gD binding to receptors for virus entry and spread is exemplified by the fact that soluble forms of HveA, nectin-(HveC, and HIgR variants) have been shown to bind to HSV-1 gD (Cocchi et al., 1998a; Cocchi et al., 1998b; Krummenacher et al., 1999) and inhibit virus entry into susceptible cells. HveA and nectin-1 (HveC) are associated with lipid rafts during herpes simplex virus entry (Cassiani-Ingoni et al., 2005).

Immunoglobulin-like type 2 receptor (PILR) alpha is a cellular receptor associates with gB and required for HSV-1 infection (Sato et al., 2008). HSV-1 infection of human primary cells expressing both HVEM and PILRalpha was blocked by either anti-

PILRalpha or anti-HVEM antibody, suggesting that both gB and gD receptors are required for HSV-1 infection.

The corresponding receptors in the equine cells have not been yet identified. However, unlike EHV-4, EHV-1 has a remarkably broad cell tropism and is known to infect and replicate efficiently in dozens of cell lines empowered by unknown mechanism in the otherwise common to alphaherpesviruses entry process. One study, noted the flexibility of EHV-1 to adapt to a non-equine cell line by incorporation of mutations in glycoprotein C that strengthened its affinity to heparan sulfate moieties (Sugahara et al., 1997). gC and gB of EHV-1 strains bound to heparin regardless of their passage history, whereas heparin markedly interfered with infection only by the strains adapted to non-equine cells. Therefore, gC may function as a major viral attachment protein in EHV-1 entry into non-equine cell cultures. Another study proposed that EHV-1 utilizes a unique gD-binding receptor for entry into the cells, different from known mediators of herpes simplex virus, based on studies that EHV-1 was able to enter efficiently into CHO cells lacking HveA as well as HveB and HveC (Frampton et al., 2005). Additional consideration is that current EHV-1 strains used have been passaged solely in non-equine cell lines. The receptors of alphaherpesvirus entry described below have been studied extensively and identified for dozens of mammalian cells, therefore, a brief review of the current knowledge is included for completeness.

### **Tumor Necrosis Factor Receptor Family**

Herpesvirus entry mediator A (HveA), also known as HVEM, TNFRSF14, ATAR, LIGHTR, TR2, and CD40-like protein precursor, is a member of the tumor necrosis factor

receptor (TNFR) family and is present and expressed at high levels in lymphocytes. It has been studied by various disciplines of biology under various designations.

HveA is also found on some epithelial cells, carcinomas, lymphoid dendritic cells, other leukocytes, and fibroblasts (Cassiani-Ingoni et al., 2005). Human HveA is expressed in various tissues including liver, lung, kidney, spleen, and peripheral leukocytes, and it is the principal mediator for HSV-1 entry into human lymphoid cells, but not a primary mediator in other cell types (Montgomery et al., 1996). The cytoplasmic region of this receptor was found to bind to several TNFR-associated factor (TRAF) family members, which may mediate the signal transduction pathways that activate the immune response. Although a TNF receptor, HveA binds ligands with no structural similarity to TNF.

The natural ligands for HveA include LIGHT, lymphotoxin-alpha, and B and T lymphocyte attenuator (BTLA) (Mauri et al., 1998; Sedy et al., 2005). LIGHT can function as a second signal for T-cell activation, and the interactions between LIGHT and HveA are the focus of investigations into the regulation of immune responses (Jung et al., 2003). HveA binding to BTLA, an Ig family member, which inhibits T cell proliferation is probably an important pathway regulating lymphocyte activation and/or homeostasis in the immune response (Sedy et al., 2005). The interaction between HveA and gD has been suggested to initiate the signaling pathway leading to nuclear factor (NF)-kappaB activation (Teresa Sciortino et al., 2007).

### **Immunoglobulin Superfamily**

The second family of herpes simplex virus entry receptors is a part of the immunoglobulin superfamily and includes several isoforms present in various mammalian cells, each diverse due to alternative mRNA splicing. The prototype molecule of the family

is the poliovirus receptor, PVR/CD155. Nectin1, also known as poliovirus related receptor type 1 (PRR1), is assigned to CD111 as a member of a new family of receptors (Campadelli-Fiume et al., 2000).

The family also includes nectin2/PRR2 and nectin3/PRR3. All members are structurally related and consist of three immunoglobulin (Ig) domains, one variable (V)-type domain and two constant (C)-type domains. Nectins are highly conserved among mammalian species in respect to structure, function, and the ability to mediate herpesvirus entry (Milne et al., 2001; Shukla et al., 2000). The nectins are expressed in a variety of cell types including epithelial cells, fibroblasts, keratinocytes, and neural and hematopoietic cells (Cocchi et al., ; Geraghty et al., 1998; Takai et al., 2003). As adhesion molecules, nectins localize to cell-to-cell junctions of endothelial and epithelial cells, in which the carboxyl-terminal domains bind to L-fodrin, a PDZ-binding protein that anchors the receptors to the cytoskeleton and adherens junctions (Takahashi et al., 1999). Nectin-1 $\alpha$ , also known as herpesvirus entry mediator C (HveC) and the mRNA splice variant isoform, nectin-1 $\beta$ , Herpesvirus Immunoglobulin-like receptor (HIgR), contain common ectodomain and are capable of mediating entry of human and animal alphaherpesviruses, including HSV-1 and -2, pseudorabiesvirus (PRV-1), and Bovine herpesvirus (BHV-1) (Geraghty et al., 1998). HveC and HIgR are expressed in human cells of epithelial and neuronal origin and are therefore the primary candidates for co-receptors that mediate entry into epithelial cells at the initial site of infection and into neuronal cells for the establishment of latency (Cocchi et al., 1998a; Geraghty et al., 1998). Nectin-2 $\alpha$ , Herpes Virus Entry Mediator B (HveB), and nectin-2 $\delta$  are also mRNA splice variants, and they mediate the entry of HSV-2, PRV, and certain viable mutant forms of HSV-1 but not wild-



type HSV-1 (Lopez et al., 2000; Warner et al., 1998). Other nectin splice variants have been identified (Cocchi et al., 2004b; Takai and Nakanishi, 2003).

### **3-O-sulfated Heparan Sulfate**

Heparan sulfate proteoglycans can function as entry mediators for HSV-1 as modification of heparan sulfate by D-glucosaminyl 3-O-sulfotransferase isoforms-3 and/or -5, creates 3-O-sulfated heparan sulfate (3-OS HS) generates a gD-binding site. 3-O-sulfated heparan sulfates are broadly distributed on human cells and tissue and mediate HSV-1 but not HSV-2 entry (Shukla et al., 1999).

By analogy with other alphaherpesviruses, EHV-1 was suspected to attach to cells through a nonessential interaction of gC with heparan sulfate proteoglycan and through an essential interaction of gD with one of three cellular receptors: nectin-1, herpesvirus entry mediator (HVEM), or a specifically modified heparan sulfate (Spear, 2004). Binding of gD and HveA receptor causes the former to undergo a conformational change in which a C-terminal segment of the ectodomain polypeptide chain is released from a strong intramolecular contact (Carfi et al., 2001; Krummenacher et al., 2005) and may interact with gB or the gH/gL complex to trigger molecular rearrangements and, ultimately, fusion (Figure I-9). The precise functions of gB and gH/gL in entry process are unknown. Both are required for entry and both receive the signal from gD, to which they respond, by undergoing a conformational change. gD itself is thought not to participate in the fusion process (Cocchi et al., 2004a; Jones and Geraghty, 2004). Neither gB nor gH/gL has an obvious fusion peptide, but an indication that gB might be a fusion effector comes from the notable syncytial phenotype caused by certain mutations within the cytoplasmic domain of gB (Bzik et al., 1984a; Foster et al., 2001a; Gage et al., 1993; Heldwein et al., 2006).

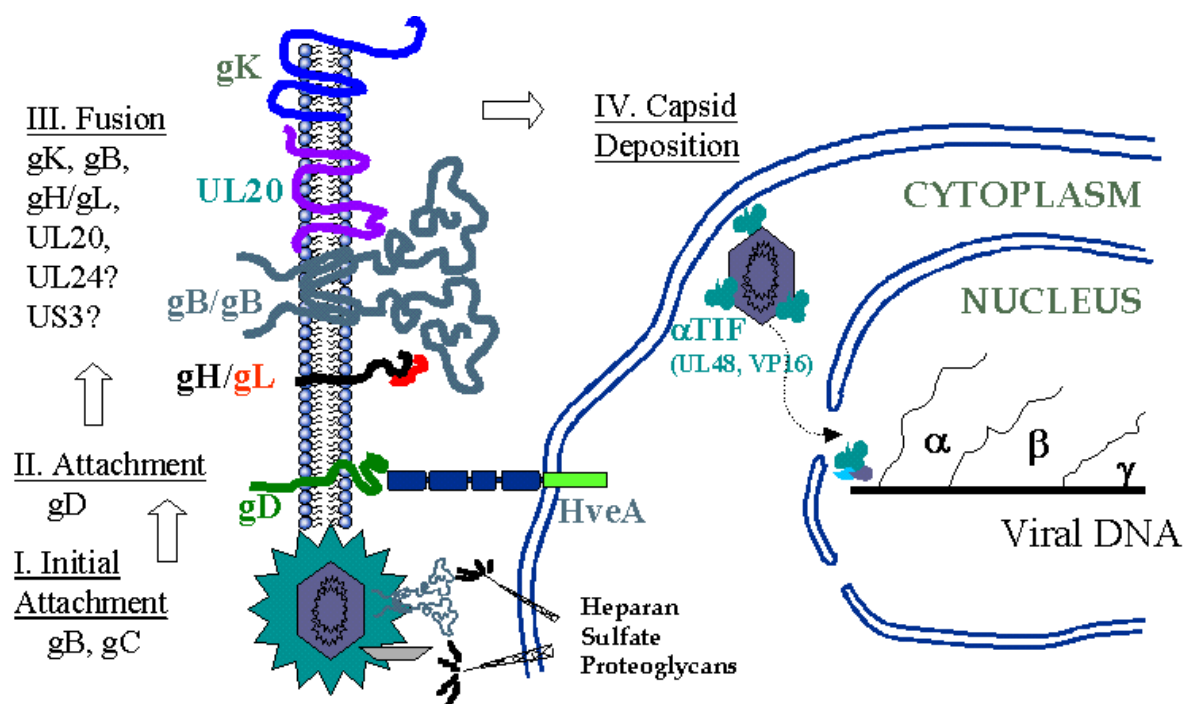


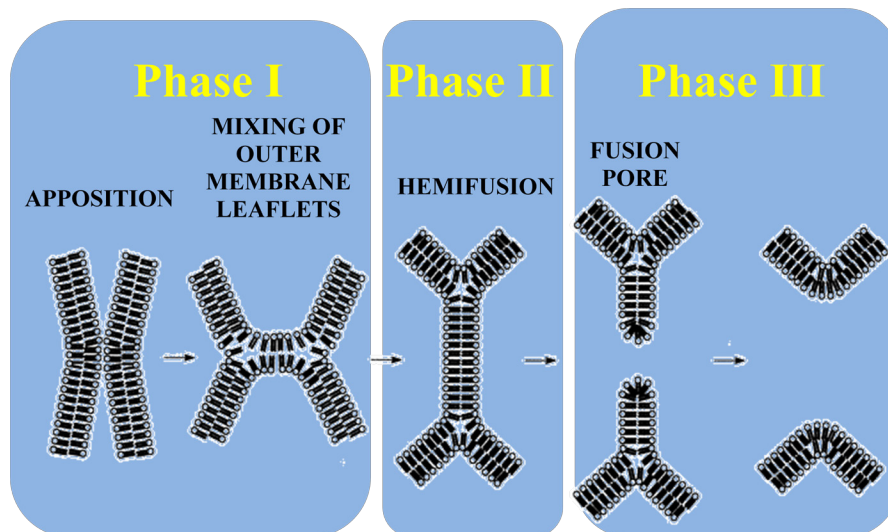
Figure I-9. Sequential protein-protein interactions during EHV-1 entry into the cell.

## **Virus-to-Cell Membrane Fusion**

After binding of gD to a HSV-1 virus entry receptor, the last step in virus entry is fusion of the virion envelope with the plasma membrane of the target cell (Morgan et al., 1968). Potentially, receptor binding triggers a conformational change in gD that translates to a change in gB and gH/gL, resulting in activation of the HSV-1 membrane fusion machinery (Figure I-9). Alphaherpesviruses can utilize two distinct entry pathways, depending on the type of cell encountered, entering some cells through pH-independent fusion with plasma membrane and other cells through an endocytic pathway, where low-pH environment of the endosome triggers fusion of the virion envelope with cellular membranes. Studies of HSV-infected cells at early times post infection using electron microscopy (EM) have detected virions fusing with the plasma membrane as well as virions inside membrane-bound vesicles (Campadelli-Fiume et al., 1988a; Fuller et al., 1989; Fuller and Spear, 1987; Sodeik et al., 1997). The details of the fusion mechanism are unknown but have been under investigation for quite some time (Campadelli-Fiume et al., 2000; Spear et al., 2000). Entry via endocytosis is a recent postulate, based on the study in which inhibitors of endocytosis (energy depletion or hypertonic medium) and endosome acidification (weak base ammonium chloride or the ionophore monensin), blocked virus entry into the cells expressing herpesvirus entry receptors (Nicola et al., 2003; Nicola and Straus, 2004). Endocytic uptake of HSV virions from the cell surface is rapid and independent of any known gD receptor (Cassiani-Ingoni et al., 2005).

Herpes simplex virus entry into cells requires four glycoproteins, gB, gD, gH, and gL, regardless of the entry pathways taken. Glycoprotein B is in the form of a trimer, while gH forms a noncovalent complex with gL (Cairns et al., 2005; Farrell et al., 1994;

Heldwein et al., 2006; Hutchinson et al., 1992; Ligas and Johnson, 1988; Peng et al., 1998; Sarmiento and Spear, 1979). Virus-induced membrane fusion is subdivided into three sequential phases (Figure I-10). During Phase I, two membranes are brought into close proximity through viral glycoprotein binding of cellular receptors. In alphaherpesviruses, such as EHV-1, gC interacts with heparan sulfate glycosaminoglycans (HSGAG) (Osterrieder, 1999), gB interacts with HSGAG and paired immunoglobulin-like type 2 receptor (PILR) alpha (Roller et al., 2008), and gD interacts with HveA and other receptors on cell surface (Spear et al., 2000). Glycoprotein D is the only interaction required for Phase I. Binding of gD to one of its receptors triggers conformational change that exposes the normally hidden receptor binding residues of gD. This results in transient interaction between gD and gH/gL, where gH/gL carries out Phase II hemifusion, followed by stable complex between gD and gB, where gB completes Phase III full fusion.



**Figure I-10. Phases of virus-induced membrane fusion.** During Phase I, two membranes are brought into close apposition. Phase II marks the mixing of the outer membrane leaflets leading to formation of a hemifusion intermediate. A fusion pore stably forms and expands in Phase III, thereby completing the fusion process.

Specifically, Phase II involves the initiation of lipid mixing between the two apposed membranes and is completed when the outer membrane leaflets are mixed to form an intermediate called hemifusion. In Phase III the mixing of the inner membrane leaflets continues from hemifusion to the pore formation and expansion and until completion of the fusion process (Atanasiu et al., 2007; Subramanian and Geraghty, 2007). Interestingly, Varicella zoster virus (VZV) fusion formation occurs upon expression of the gH/gL complex alone. In contrast, Pseudorabies virus (PRV) requires expression of gH, gL and gB, while the Herpes simplex virus (HSV) types 1 and 2 require the quartet of gH, gL, gB and gD. EHV-1 core fusion complex is not defined, but suspected to parallel the HSV-1 model and include gH, gL, gB and gD.

### **Virion Transport to the Nucleus**

After fusion of the virion envelope with the plasma or endosome vesicle membrane of the infected cell, the capsid and the associated tegument complex are deposited into the cytoplasm. Some tegument proteins disassociate from the complex and remain in the cytoplasm. The remaining capsid-tegument complex is then transported along the microtubule network utilizing the cell's dynein motor to be docked at the nuclear pore complex (NPC), potentially disrupting the microtubule interactions in the process (Sodeik et al., 1997; Ward et al., 1998). VP1/2 gene is required for the release of the DNA from nuclear pore associated capsids and its deposition into the nucleus (Batterson and Roizman, 1983; Copeland et al., 2008; Knipe et al., 1979; Ojala et al., 2000). Change in NPC conformation allows translocation of the genome as a densely packaged, rod-like structure, rapidly and efficiently ejected through dilated NPCs towards the nucleus into the nucleoplasm, where it is transcribed and replicated to propagate the infection. After release

of the genome, empty capsids dissociate from the NPC (Batterson and Roizman, 1983; Lycke et al., 1988; Sodeik et al., 1997).

## **Biosynthesis**

### **Coordinate Gene Expression**

During the course of infection, more than 80 genes are expressed in highly regulated cascade fashion. As with other alphaherpesviruses such as herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV), bovine herpesvirus 1 (BHV-1), and pseudorabies virus (PRV), expression of EHV-1 genes is temporally regulated and coordinately expressed in an immediate-early (IE or  $\alpha$ ), early (E or  $\beta$ ), and late (L or  $\gamma$ ) fashion. The regulation of this cascade of gene expression is governed by the action of at least four characterized EHV-1 regulatory proteins: the sole IE protein (IEP), the EICP22 protein (formerly known as IR4), the EICP27 protein (formerly known as UL3), and the  $\alpha$ -gene transactivating factor ( $\alpha$ -TIF).  $\alpha$ -TIF protein, a  $\gamma$  gene product present in the tegument, activates initial transcription of alpha genes, which contain the “TAATGArATT” promoter response element, which binds cellular Oct-1 bound to viral DNA. Meanwhile, VP16 is released from its interaction with VHS and the tegument complex and binds to a cellular protein, the host cell factor (HCF) or C-1 (Katan et al., 1990; Kristie and Sharp, 1990). HCF carries VP16 into the nucleus and the VP16-HCF complex binds to Oct-1. The viral gene expression is then autoregulated, expression of  $\beta$  genes activated, then  $\gamma$  gene expression is activated by  $\alpha$  and  $\beta$  genes, viral genome replication is initiated as  $\gamma$  genes turn off  $\alpha$  and  $\beta$  genes late in infection (Roizman and Knipe, 2001). RNA polymerase II transcription of viral DNA takes place in the nucleus (Alwine et al., 1974; Costanzo et al., 1977).

Viral alpha genes are expressed at peak levels at 2 to 4 hours post infection. Out of six  $\alpha$  genes: ICP0, ICP4, ICP22, ICP27, ICP47, and Us1.5, five stimulate viral  $\beta$  gene expression in at least some cell types. In particular, ICP4 is required for all post- $\alpha$  gene expression (Clements et al., 1977; Dixon and Schaffer, 1980), and its effect is exerted at the transcriptional level (Godowski and Knipe, 1986). ICP4 or possibly it's also responsible for downregulation of itself and ICP0, gene products of ORF P and ORF O (Gelman and Silverstein, 1987; Petzoldt et al., 1987). The absence of ICP4 at low grade infection leads to a 100 fold decrease in virus yield (Sinclair et al., 1994; Stow and Davison, 1986).

The  $\beta$  genes products, involved in viral DNA replication and nucleotide metabolism, are produced at peak levels between 4 and 8 hours post infection, and are subdivided into two groups based on timing of expression.  $\beta_1$  genes such as single-stranded DNA binding protein, ICP8, and the large subunit of ribonucleotide reductase, ICP6, are expressed shortly after the synthesis of the alpha proteins. The  $\beta_2$  genes such as viral thymidine kinase encoded by UL23, are expressed with a certain delay after  $\alpha$  gene expression possibly due to presence of ICP27 requirement for expression (Roizman and Knipe, 2001).

The  $\gamma$  (late) genes are produced at peak levels only after viral DNA replication has started, and require ICP4, ICP27 and ICP8 for efficient levels of transcription.  $\gamma_1$  (leaky-late) genes, are expressed throughout infection and their transcription is increased only a few fold after DNA replication has occurred.  $\gamma_2$  genes, which do not accumulate in appreciable amounts until after DNA replication (Wagner et al., 1985). Typical  $\gamma_1$  genes include the major capsid protein ICP5, gB, gD, and ICP34.5, whereas typical  $\gamma_2$  genes

include gC, UL41 (VHS), UL36, UL38, UL20, and gK. Over 30 viral structural proteins have been identified in the alphaherpesvirus particle, eight of which are associated with the capsid (McNab et al., 1998; Steven and Spear, 1997; Thurlow et al., 2005).

### **Viral Inhibition of Host Protein Synthesis**

While making their own proteins, herpesviruses shut off the synthesis of cellular proteins through degradation as well as inhibition of further synthesis and processing of host mRNA. The virion host shutoff protein (VHS) is a structural component of the virion and functions without the need for *de novo* protein synthesis upon infection (Fenwick and Walker, 1978; Nishioka et al., 1978). In complex with the translation factor eIF-4H protein induces endoribonucleolytic cleavage of 5' end of mRNA (Frink et al., 1981) (Elgadi and Smiley, 1999; Karr and Read, 1999; Kwong et al., 1988; Roizman and Knipe, 2001; Zelus et al., 1996). Early in the infection VHS accelerates the degradation viral mRNAs as well as cellular, thus facilitating the transition from  $\alpha$  to  $\beta$  to  $\gamma$  gene expression (Kwong and Frenkel, 1987; Oroskar and Read, 1987). As a  $\gamma_1$  gene product, newly made VHS accumulates late in infection. VHS is unable to degrade the viral mRNAs that predominate at this late stage of infection as it is immediately incorporated with VP16 into a tegument complex to be delivered to the next cell for inhibition of protein synthesis (Lam et al., 1996)

### **Viral Genome Replication**

Parental viral DNA is circularized upon being deposited into the nucleus of the infected cell. After the  $\beta$  genes have been expressed and translated, several proteins assemble on the parental circular viral DNA initiating replication in a “theta” structure, which then transitions to a rolling circle mechanism producing head-to-tail concatemers of



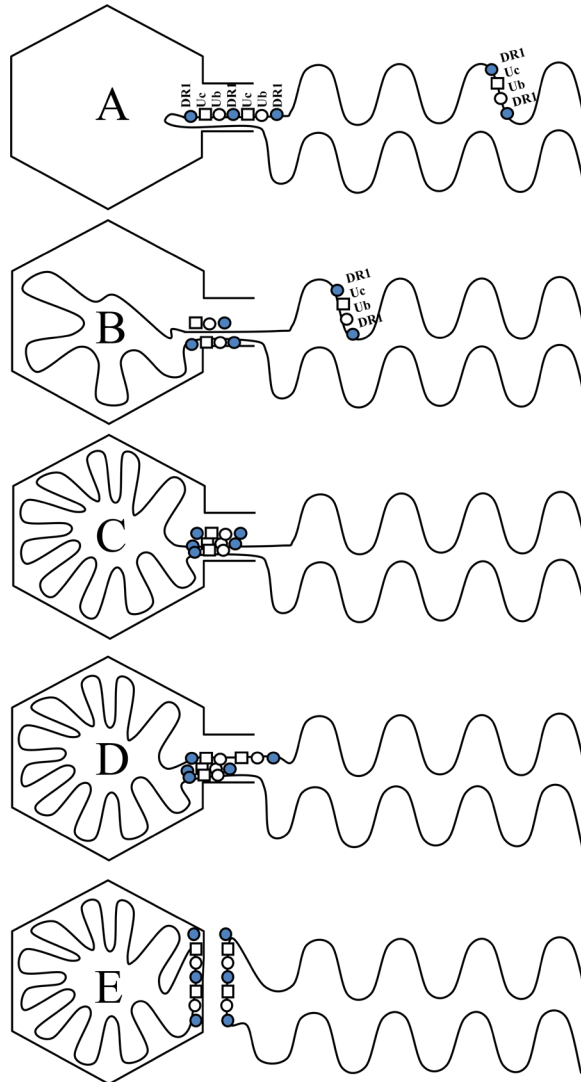
viral DNA (Ishov and Maul, 1996; Jacob et al., 1979; Uprichard and Knipe, 1996). There are seven viral proteins absolutely required and sufficient for viral DNA replication. These are the viral DNA polymerase, UL30 (Purifoy et al., 1977), its accessory protein, UL42 (Conley et al., 1981), an origin-binding protein, UL9, the single stranded DNA binding protein, ICP8, and the helicase-primase complex of UL5, UL8, and UL52 (Challberg, 1986; Wu et al., 1988). Host DNA polymerase  $\alpha$ -primase, DNA ligase, and topoisomerase II are also required. Although only one origin of replication (*Ori*) is needed for replication *in vitro*, viral genomes of alphaherpesviruses has three. Studies *in vivo* have shown that the origins have distinct functional properties. *OriS*, a palindromic sequence of ~45 bp that is located in the “c” repeats flanking the unique short ( $U_S$ ) region of the genome and present in two copies. *OriL*, a palindromic sequence of ~144 bp, is located at the center of the unique long ( $U_L$ ) region of the genome (Deb and Doelberg, 1988; Igarashi et al., 1993; Knopf et al., 1986; Lockshon and Galloway, 1986; Polvino-Bodnar et al., 1987; Roizman and Knipe, 2001). *OriL* contributes significantly to morbidity and mortality of HSV-1 infection in mice and is required for efficient viral replication and reactivation in neurons (Balliet and Schaffer, 2006). Both *oriL* and *oriS* contain palindromic sequences as core elements, share greater than 90% homology, and reside within the promoter/enhancer regions of genes essential for viral replication. UL9 binds to either *oriL* or *oriS* and begins to unwind the viral DNA. UL9 then recruits the single stranded DNA binding protein, ICP8, to the unwound portion of the viral DNA. At this point, UL9 and ICP8 engage the remaining five proteins to the replication forks. The helicase–primase and viral DNA polymerase complexes assemble at each replication fork and initiate theta form replication that switches the rolling circle form of replication through an unknown mechanism. UL9 is

not required for rolling circle replication because it is not origin dependent. The rolling circle replication forms long head-to-tail concatemers of viral DNA, which become cleaved into individual units during packaging of viral DNA into capsids (Roizman and Knipe, 2001).

### **Capsid Assembly and DNA Packaging**

Following initiation of DNA replication and transcription  $\gamma$  genes encoding capsid proteins, capsid assembly proceeds by way of small oligomers of major capsid (VP5) and scaffold proteins added to the edges of the growing capsid and secured in place by triplexes (VP23) (Nicholson et al., 1994; Rixon et al., 1996). Assembly requires the individual subunits to be synthesized in an assembly-inactive form that is subsequently triggered to polymerize, either by recognition of a growing surface or by binding to a scaffolding protein (Figure I-11).

The process is initiated in the cytoplasm and is completed in the nucleus, so most of the proteins are able to localize to the nucleus on their own, while VP5, VP26 (outer tip of hexons), and VP23, can be carried to the site. VP5 uses a triplex protein VP19C or a scaffolding protein pre-VP22a. VP23 uses VP19C, while VP26 uses both VP5 and VP19C or pre-VP22a (Nicholson et al., 1994; Rixon et al., 1996). Once in the nucleus, VP5-pre-VP22a complexes come together as a result of self assembly of pre-VP22a, which is lost as DNA is packaged. The triplex proteins VP19C and VP23 are then added to form a partial capsid. Hexons and pentons made up of VP5. The individual capsomers are linked by triplex structures consisting of VP19C and VP23, the two minor capsid proteins. Each triplex consists of one molecule of VP19C and two molecules of VP23 (Newcomb et al., 1993).



**Figure I-11. Packaging of viral DNA.**

Eventually a single portal complex (comprised of 12 copies of UL6) is incorporated into the growing structure at a unique vertex (Dasgupta and Wilson, 1999; Newcomb et al., 2001) and nascent capsid closes forming the procapsid. The portal complex is an integral part of the capsid structure and harbors the binding sites for the terminase complex (UL15 with UL28 and possibly UL33) (Adelman et al., 2001; Beard et al., 2002; Davison, 2002; White et al., 2003; Yu and Weller, 1998). The portal not only functions as a DNA-translocating machine in conjunction with nonstructural viral factors (terminase/ATP) but also interacts with the minor proteins that seal the portal after DNA packaging and reopen it for genome ejection at onset of infection. Procapsid matures undergoing structural transformation. This process results in the conversion of the spherical procapsid into a more angular form. The internal protein scaffold (VP21 and VP22a) is cleaved by the viral protease and is displaced from inside the capsid as the DNA genome is packaged into the capsid (see Figure I-11). The internal protein VP24 is retained (Sheaffer et al., 2000).

Many aspects of capsid assembly and DNA packaging in the herpesviruses are similar to those in dsDNA bacteriophages such as  $\lambda$ , T4, and P22 (Brown, 2002; Prevelige and King, 1993) (Figure I-11). Cleavage of DNA concatemers by the terminase complex occurs at specific sites and requires the *pac1* and *pac2* packaging signals to generate unit-length genomes (Deiss et al., 1986; Smiley et al., 1990; Varmuza and Smiley, 1985). The process of encapsidation of viral DNA requires several gene products, including UL6, UL15, UL17, UL28?, UL32, UL33, UL36? and UL37? (Brown, 2002; Lamberti and Weller, 1998; Roizman and Knipe, 2001; Taus et al., 1998). UL17 and UL32 have been implicated in targeting of capsids or capsid proteins to the sites of DNA packaging (Lamberti and Weller, 1998; Taus et al., 1998). Of the three capsid-associated DNA

packaging proteins, UL17, UL25, and UL6, only UL17 and UL6 appear to be components of the procapsid (Thurlow et al., 2006). Subsequent to DNA cleavage, UL25 is required and added for packaging process for retention of DNA within the capsid, potentially sealing it (McNab et al., 1998; Stow, 2001). By further extrapolation from HSV-1, portal protein UL6 interacts with the DNA cleavage and packaging proteins (putative terminase subunits) UL15 and UL28 to form part of the terminase enzyme, a protein complex essential for the cleavage of newly synthesized, concatemeric herpesvirus DNA and packaging into preformed capsids (Ladin et al., 1980; Ladin et al., 1982). Another DNA cleavage and packaging protein is encoded by the UL 33 gene associates with capsids (Beard and Baines, 2004). The mature capsid is then able to proceed along the viral egress pathway.

According to separation by density gradient centrifugation, three capsid species have been identified for EHV-1 based on their sedimentation properties, Lights which are abortive and correspond to A-capsids of HSV-1, Intermediate - (B-capsids), and Heavy - minor component (C-capsids) containing packaged DNA. Both A and B capsids represent dead-end products of infection. In the absence of DNA packaging, cleavage of the protein scaffold and structural transformation of the procapsid still occur forming B capsids, devoid of DNA as are lights but containing an additional protein, VP22, (Newcomb et al., 1989; Newcomb et al., 1993). Abortive light (A) capsids contain neither DNA nor the scaffolding proteins (Baker et al., 1990).

### **Egress and Envelopment**

The egress occurs via a two-step process, the “de-envelopment re-envelopment” pathway. Primary envelopment occurs at the inner nuclear membrane, followed by the

fusion of primary envelope with the outer nuclear membrane and subsequent de-envelopment and loss of the envelope, primary tegument, UL31, and UL34 and translocation of the capsid into the cytoplasm of the infected cells (Enquist et al., 1998; Mettenleiter, 2000). (Gershon et al., 1994; Granzow et al., 2001; Harms et al., 2000; Reynolds et al., 2002; van Genderen et al., 1994). The mechanism and the role of major glycoproteins in virion de-envelopment from the perinuclear space is unclear (Cai et al., 1987; Granzow et al., 2001; Jayachandra et al., 1997; Steven and Spear, 1997; Steven et al., 1997). Final tegumentation and envelopment (re-envelopment) occur in cytoplasmic compartments.

Herpesvirus tegument is highly complex. At least 15 proteins have been identified that are part of the HSV-1 tegument. Even more proteins make up the tegument of other herpesviruses such as VZV or CMV (Gibson et al., 1996; Spengler et al., 2001). Tegumentation is an intricate pattern of redundant protein-protein interactions (Mettenleiter, 2002a). The tegument proteins interact with the capsid on one side and the viral envelope proteins on the other side linking the structural components of the virus particle (Mettenleiter, 2002b).

The steps in capsid tegumentation are still largely undefined. Virion morphogenesis still proceeds in the absence of several tegument proteins, including UL13, US3, UL41, UL46, UL47, and UL49 (Frame et al., 1987; Mettenleiter, 2002a; Rafield and Knipe, 1984; Roizman and Knipe, 2001). UL36 and UL37 appear to be the only tegument proteins conserved in all herpesvirus subfamilies, and the absence of the HSV-1 UL36 and UL37 proteins abolishes virus maturation (Desai et al., 2001; Desai et al., 2000; Klupp et al., 2001). The absence of UL48, ( $\alpha$ -TIF), which is responsible for transducing  $\alpha$  gene

promoters and makes up a major part of the virus tegument, interferes with virion assembly, affecting tegumentation and reenvelopment in the cytoplasm (Batterson et al., 1983; Heine et al., 1974; Mossman and Smiley, 1999). UL48 has been shown to interact with other tegument components, UL49 and UL41 (VHS), and may potentially interact with gB, gD, and gH (Read et al., 1993; Smibert et al., 1994; Zhu and Courtney, 1994). UL39 or PRV interacts with gE/I & M. HSV-1 capsid protein VP26 interacts with dynein light chains RP3 and Tctex1 and also plays a role in retrograde cellular transport (Douglas et al., 2004).

Following tegumentation in the cytoplasm, capsids acquire their final envelope by budding into endosomes or cytoplasmic vesicles derived from the trans-Golgi Network (TGN). Following the final envelopment step, nascent virions egress to extracellular spaces, potentially utilizing the cellular transport machinery. The late stages in viral egress are still unclear and may differ depending on the cell type that is infected.

In the absence of capsids, tegument assembly could proceed anchored on UL49 instead of UL36 and UL37, resulting in the formation of light (L) particles, that (McLauchlan and Rixon, 1992; Mettenleiter, 2002b; Rixon et al., 1992). L particles appear to contain the full complement of tegument proteins as well as an authentic envelope containing all appropriate glycoproteins.

## **Latency**

Productive infection of respiratory epithelial cells, endothelial cells and lymphocytes is characterized by active expression of all viral genes in a highly ordered temporal cascade as described above. In contrast, latent infection of sensory neurons is characterized by profoundly restricted viral-gene expression, the failure to synthesize viral

DNA, and the absence of infectious virus. In the trigeminal ganglia, circulating T-lymphocytes and lymphoid tissues draining the respiratory tract of their hosts,

Trigeminal ganglia, circulating T lymphocytes and lymphoid tissues draining the respiratory tract, are the sites for establishment and maintenance of the lifelong state of latency characteristic of all herpesviruses. In latently infected animals, reoccurrence of viremia and shedding of the virus in nasal secretions of horses has been produced by immune suppression through the administration of corticosteroids (Slater et al., 1994). The cycle of a persistent latent infection with intermittent reactivation and shedding is thought to keep EHV circulating within the equine population. Viremia occurring in primary and all recrudescing infections occurs subclinically and poses a threat of inducing abortion, as well as neurological disease.

Many different stressors, such as parturition, transportation, inclement weather, corticosteroid treatment, or mixing of social groups, can result in the reactivation of previously latent EHV-1 infection (Slater et al., 1994). The virus remains latent in the trigeminal nerve ganglia and lymphocytes of infected horses for life.

### **EHV-1 Economic, Epidemiologic, and Clinical Significance**

#### **Economic Significance**

Horse industry is large and diverse involving recreation, showing, racing, sport, breeding, as well as work, and impacts national, state, and local economies. There are about 9.2 million horses in the United States. The recreational segment accounts for \$31.9 billion of the total economic impact, followed by the horse show segment at \$28.7 billion and the racing segment at \$26.1 billion. The horse industry pays \$1.9 billion in taxes to all levels of government. In terms of total effect on the gross domestic product, California



leads the way at \$6.97 billion a year, followed by Texas (\$5.23 billion), Florida (\$5.15 billion), Kentucky (\$3.54 billion), and Louisiana (\$2.45 billion). Texas has the most horses with 978,822, followed by California (698,345), Florida (500,124), Oklahoma (326,134), and Kentucky (320,173). There are 164,000 horses in Louisiana, and 54,200 Louisianans are involved in the industry as horse owners, service providers, employees, and volunteers, while even more participate as spectators (LLP, 2005).

World-wide equine herpesvirus-1 continues to be a major cause of epidemic abortion, perinatal mortality, respiratory disease and occasionally neurological disease in horses (Allen et al., 2000; Bryans, 1989; Whalley, 1998). Herpes virus infections are economically detrimental to the industry. Abortion is the most dramatic outcome of EHV-1 infection, and an epidemic outbreak can be financially disastrous for breeders, with loss of clients and large insurance payments. Equine herpesvirus infection also renders horses more susceptible to other diseases, and in young animals, strangles (*Streptococcus equi*) and rattles (*Rhodococcus equi*) are already of significance to the industry. In addition, considerable losses are encountered due to quarantine of the racing facilities during respiratory herpesvirus outbreaks in adult equine population. Neurologic cases have been on the rise, and demand advanced and costly veterinary care. Nonetheless, with the animal health as the outmost goal, the effort and the expense to combat EHV-1 infection has been considerable throughout past decades.

### **Epidemiology**

Equine herpesvirus-1 (EHV-1) is an alpha herpesvirus which causes upper respiratory tract infections in horses. These infections may be mild or asymptomatic and can result in serious sequelae such as abortion, neonatal syndrome, peracute pulmonary

vasculitis, myeloencephalopathy, ocular disease and secondary bacterial infections. As a primary respiratory pathogen, no contributing factors are necessary for the outbreak of disease. However, environmental and management factors still play a role in the incidence of disease and its prevention (Sellon, 2001). Risk factors contributing to an outbreak include: overcrowding, heavy parasite burden, poor nutritional state, climatic extremes, the presence of concurrent disease and mixing of horse populations (Allen et al., 2002b). In addition, air quality, sanitation, storage and quality of feed and prolonged periods of transport impact the incidence of infection and disease (Sellon, 2001).

EHV-1 is enzootic in most equine populations with horses being exposed early in life to the virus. It is estimated that 80%-90% of horses are infected by EHV-1 or its close relative, EHV-4, by two years of age (Allen et al., 2002a; Gilkerson et al., 1999b), determined that 30% of broodmares and racehorses on a stud farm were positive for EHV-1 antibodies. Once a horse is infected with EHV-1 it is infected for life (Figure I-12). The virus becomes latent in the trigeminal nerve ganglia and in the T lymphocytes of the lymphoid tissue draining the upper respiratory tract (such as the submandibular and retropharyngeal lymph nodes) (Allen et al., 2002b). Under periods of stress the virus can be reactivated. Stressors include parturition, lactation, weaning, inclement weather, prolonged transportation times, surgery, mixing of different groups of animals as well as the use of certain medications such as corticosteroids (Huang Ja et al., 2002). The animal with a reactivated infection may or may not show obvious clinical signs but it can shed virus in its nasal secretions and potentially infect other horses. The ability to become latent is one of the factors contributing to the amplification and maintenance of the virus within equine populations (Huang Ja et al., 2002). A latently infected mare can pass the infection

to her foal that then also can become latently infected. Periodically throughout the horse's life the infection can become reactivated and be passed horizontally to other horses. The cycle of infection, latency, reactivation, viral shedding and infection or re-infection maintains the virus within the population. It should be noted that as the risk of re-infection or reactivation remains with the horse throughout its life, so does the risk of development of any of the sequelae associated with EHV-1 infection. The risk of abortion, neurological disease and severe respiratory disease does not diminish with time (Huang Ja et al., 2002).

The virus is transmitted via close contact with an infected horse shedding virus in its nasal secretions or through aerosolized respiratory secretions. Fomites and the ingestion of contaminated feed are possible sources of infection with virus. An important source of infection for pregnant mares is the fetus, placenta or any birth fluids from an aborted EHV-1 infected fetus (Leblanc, 1999). The incubation period for the respiratory form of EHV-1 infection is 2 to 10 days.

EHV-1 upper respiratory tract infection is primarily a disease of young horses aged from weaning to 2-3 years. It is unusual for foals under the age of 3 months to show clinical signs (unless they were congenitally infected), especially if their mothers were vaccinated. Maternal antibodies are most likely responsible for the protection of these foals (Huang Ja et al., 2002).

The virus is circulated year round within the horse population. As mentioned above, the virus goes into latency and can be reactivated at times of stress, providing a source of infection for other horses. The immunity to the virus is weak and short-lived (3 to 5 months) whether a horse is vaccinated or not, so it is possible for horses to be re-infected (Carman et al., 1997). Respiratory tract infections are a major cause of lost

training days in thoroughbred race horses (Wood et al., 1999). Respiratory tract infections may be caused by a variety of different pathogens, including bacterial and viral agents. Which respiratory pathogen is responsible at any given time in any year varies depending on the particular population of horses involved and the management practices employed, but EHV-1 is one of the more common viral pathogens. There is no data available that gives the annual incidence of infections caused by EHV-1 or the proportion of upper respiratory tract infections attributable to EHV-1 (Huang Ja et al., 2002). In addition to the economic losses secondary to lost training days within the racing industry, EHV-1 is significant because of the potentially serious sequelae that may follow infection with the virus. These sequelae include abortion, neonatal death, myeloencephalopathy, severe respiratory disease, ocular disease and secondary bacterial infections.

The presence of EHV-1 carriers in the horse population makes eradication of EHV-1 difficult. Therefore, management should focus on disease prevention, through vaccination and management strategies, rather than eradication or treatment regimes.

### **Recent Outbreaks**

There is a growing concern in the U.S. horse industry over the increased number of neurologic cases of EHV-1 reported in recent years, as well as the occurrence of several high-profile outbreaks. The Center for Emerging Issues, part of the Department of Agriculture's Animal and Plant Health Inspection Service, recently labeled the neurologic form of EHV-1 as a potentially emerging disease.

Despite the availability and use of currently approved vaccination protocols for EHV-1, in January of 2003, EHV-1 swept through the University of Findlay's English riding facility, infecting 90% of the 138 horses. During the outbreak, both respiratory and

neurological forms of the disease were seen. Forty-two of these horses exhibited severe neurological symptoms and were admitted to the Ohio State Veterinary Hospital where they all subsequently died or were euthanized (Cassiani-Ingoni et al., 2005). Significant outbreaks prior to Ohio have been reported in Southwestern Virginia, March 1998; Johnson County Wyoming, July 2001; Northern Virginia, April 2002; and Canada, October 2002 and January 2003. Most recently, a subsequent outbreak of respiratory and neurological disease has been reported in horses at the Pennsylvania National Racecourse outside of Philadelphia (Grantville, PA). The recrudescence of latent infections may lead to disease despite maintenance of closed populations. Furthermore, with increasing affiliations within the equine industry, the potential devastating losses during EHV-1 outbreaks are no longer confined to individual farms.

Minimum four cases of equine herpes closed the Northville Downs racetrack in February 2005, cancelling the live horse racing for a month. In December prior to that, the racetrack was quarantined after two horses were found to have equine herpes and were euthanized. A third horse was discovered to have the disease in January in a separate barn, which alarmed state officials and the barn was under quarantine until February (Cassiani-Ingoni et al., 2005).

Several cases of equine herpes virus were reported at Truro Raceway N.S. in February 2005, and the local Racing Commission sent out a cautionary advisory to racetracks (Cassiani-Ingoni et al., 2005). In 2006, 11 outbreaks of the neurologic form of EHV-1 were reported, four in Maryland and one each in Colorado, Florida, Georgia, Michigan, New Jersey, Pennsylvania, and Wisconsin (AVMA News Bulletin March 6, 2007). Early 2007 outbreaks included six horses in Virginia, one in Maryland and others.

## **Transmission and Seroprevalence**

Transmission of EHV-1 occurs via the respiratory route after contact (direct or indirect) with infected nasal secretions, aborted fetuses, placenta, or placental fluids (Timoney, 1992). Indirect contamination is also possible through fomites such as human hands, endoscopes, and feed and water buckets (Huang Ja et al., 2002). Within a few hours of infection the virus penetrates the epithelial surfaces of the upper and lower respiratory tracts (Figure I-13). It replicates and disseminates into the stroma, causing collapse of the lamina propria. Infection then spreads to the draining lymph nodes of the respiratory tract where the virus infects lymphocytes, monocytes and dendritic cells. The infection of these immune cells causes immunosuppression and thereby increases the likelihood of secondary bacterial infections. Cell associated viremia results in the dissemination of the virus throughout the body. The virus is endothelialtropic, and the interleukin-2 molecules released during inflammation induce the expression of leukocyte adhesion molecules on endothelial cells. Virus is passed onto vascular endothelial cells by the attached infected leukocytes. Infection of the blood vessels occurs within 24 hours of exposure (Mair, 1999). Endothelial cell infection and ensuing virus replication causes vasculitis and thrombosis. Vasculitis and thrombosis can cause abortion when it occurs in the vessels supplying the fetus and placenta, and it can cause neurological disease when it occurs in the CNS (Wilson, 1997).

An important mechanism by which herpesviruses evade the immune system is through the suppression of class I MHC molecules. Herpesviruses also evade the immune system through latency, which is established in the neurons of the trigeminal nerve ganglia and in the T lymphocytes of the lymphoid tissue draining the upper respiratory tract

(Huang Ja et al., 2002). During latency only one viral gene is expressed, thereby preventing neuron apoptosis. When the host undergoes stress of any nature, proteins made by the host under these conditions activate expression of the herpesvirus. The virus replicates, travels down the axons, and reinfects epithelial cells. It is then shed by the host and infects other hosts.

EHV-1 is enzootic in most equine populations world-wide. The seroprevalence of EHV-1 has been increasing over the past decade, reaching approximately 30% according to a 1999 survey (Gilkerson et al., 1999a). Studies showed that vaccinations do not alter the prevalence of EHV-1 antibody-positive foals, such that the cycle of EHV-1 infection continues in vaccinated mares and their unvaccinated unweaned foals. Foals as young as 11 days old become infected (Foote et al., 2003; Foote et al., 2004). The virus also can be carried and spread in fetal tissues, the placenta, and the uterine fluids from mares that have aborted. Virus spread via nasal secretions among weanlings and adults completes the cycle of EHV-1 transmission to incorporate entire horse population.

### **Prophylaxis**

EHV-1 infections are usually dealt with using management practices that limit spread of the disease, providing symptomatic relief to infected horses, and treating potential secondary bacterial infections with antibiotics. Treatment of neurologic outbreaks with the anti-HSV-1 agent acyclovir (inhibitor of viral DNA replication) did not produce substantial benefits. Of particular importance to the management of EHV infections is the fact that these viruses establish latency or unapparent infection that can recur in times of immune suppression. Upon primary infection of young horses, many will develop rhinopneumonitis, but all will remain subclinically infected throughout their life

periodically shedding the virus to infect new horses. Vaccination against EHV-1 helps to reduce the severity of illness, but it does not prevent the latent infection or the neurological disease caused by EHV 1.

### **Vaccination**

Of particular importance to the management of EHV infections is the fact that these viruses establish latency or unapparent infection that can recur in times of immune suppression. EHV-1 circulates in vaccinated populations of mares and their unweaned unvaccinated foals, continuing the infectious cycle (Foote et al., 2004). Upon primary infection of young horses, many will develop rhinopneumonitis, but all will remain subclinically infected throughout their life, periodically shedding the virus to infect new horses.

As of molecular interest, generally, all herpesviruses have evolved mechanisms to facilitate virus-entry into cells through the use of multiple glycoproteins embedded within their viral envelope. The glycoproteins are efficient primary targets of virus-specific neutralizing antibody, development of which is coincident with resolution of clinical signs and resistance to homologous reinfection for 3 to 4 month. When used in conjunction with appropriate management practices, the current EHV-1 vaccines help to curtail the respiratory disease and abortion but are ineffective to prevent the neurological form of disease (Allen et al., 1999a). Horses become seriously ill despite regular vaccinations in very short intervals. In devastating recent outbreaks, an unusually high number of horses exhibited the neurological form of the disease. This is alarming because this form of EHV-1 infection used to be sporadic and contained to individual animals of an affected herd. Better treatment options are in high demand.



Since 1930s, there is ongoing research to design the vaccination programs for EHV-1, but still numerous limitations are encountered. At its simple, vaccine administration is complicated by practical difficulty to match the duration of immunity and the risk period for the disease.

A modified live EHV-1 vaccine of monkey cell line origin was shown to be associated with neurologic disease in 486 of 60,000 recipients, prompting its withdrawal from the US market in 1977 (Studdert et al., 1981). There are no reports of EHV-1 myeloencephalopathy associated with use of a modified live vaccine currently approved for use in horses in the United States. However, there are several limitations with the current vaccine regimen. Immunity induced by the vaccine only lasts two to three months, and thus, a year round vaccination program must be administered (Cassiani-Ingoni et al., 2005). Additionally, whereas these vaccines have been shown to experimentally reduce disease, the efficacy of vaccination against EHV-1 in the field has yet to be firmly established.

Although both sporadic and epizootic EHV-1 abortions occurred in immunized mares, the numbers of fetal and neonatal foal losses decreased significantly ( $\chi^2 = 15.75$ ;  $p$  less than 0.001) from 11.8% (343 of 2897 pregnancies) during the seasons 1969-1973 to 8.9% (334 of 3763 pregnancies) in the years 1975-1982 during which EHV-1 vaccinations had been carried out (Frymus et al., 1986).

All horses at risk of contracting an EHV-1 infection should be vaccinated. Vaccinating young horses against EHV-1 does not prevent respiratory infection; however, vaccination does reduce the severity of clinical signs and the level and duration of viral shedding. Therefore, goals of vaccination against EHV-1 respiratory infection should

include minimizing the intensity of clinical signs and limiting spread of the virus within the population. As immunity arising from EHV-1 vaccination is short lived, booster vaccinations are required. A typical vaccination schedule begins with two vaccinations, 3 weeks apart, just prior to weaning followed by a booster every 3 to 6 months, depending on the risk of exposure. Athletic horses that compete in shows and/or races will require more frequent vaccination. Vaccination should be given 7-10 days prior to an event in case a local vaccine reaction occurs (Huang Ja et al., 2002).

In order to prevent abortions, pregnant mares can be vaccinated with a killed EHV-1 vaccine. Modified live virus vaccines should not be given to pregnant mares. As the neurological form of the disease may be immune mediated, vaccination is unlikely to offer protection against this EHV-1 manifestation (McClure and Lunn, 1999). No vaccines to date have demonstrated definite protection against the neurological form of EHV-1 infection (Huang Ja et al., 2002), (Goodman et al., 2006)

### **Management**

Overcrowding, heavy parasite burdens, poor feed and air quality, transportation, inclement weather, and mixing of horse populations have been associated with a higher incidence of infection and disease with EHV-1. Management regimes aimed at preventing EHV-1 infections should include isolating incoming horses from the resident population for a minimum of three weeks and minimizing stressors. Regular vaccination and deworming programs can contribute to reducing the risk of infection. Maintaining the general health of horses can be achieved by providing good quality nutrition, a clean environment and protection from the elements (Huang Ja et al., 2002; Sellon, 2001).

Mares that have aborted should be isolated, contaminated stalls and paddocks should be cleaned and disinfected, bedding burned, staff should use protective wear to avoid animal to animal transfer, and facility quarantined for 30 days after the last abortion (Leblanc, 1999; Mair, 1999). Booster vaccination (inactivated) during an outbreak may limit the spread of infection and protect some horses (Mair, 1999).

Prevention is achieved by segregating horses based on age (young horses away from the mares) and keeping mares in small groups isolated from temporary or new populations. Any newly acquired animals should be placed in quarantine for 3 weeks before being introduced to the resident population (Mair, 1999). Minimizing the stress on mares (stressors, such as parturition, transportation, inclement weather or mixing of social groups), especially later in gestation, may be of benefit in preventing reactivation of a latent infection. Vaccinating pregnant mares with an inactivated vaccine at 5, 7 and 9 months gestation has helped reduce the incidence of EHV-1 related abortion. The mares in addition should receive a yearly booster. If other horses are kept on the property, the pregnant mares preferably should be handled first (Mair, 1999).

In any presentation of EHV-1 infection isolation of infected horses and a 3 week quarantine of the farm are paramount. Facilities must be either left empty for three weeks or disinfected appropriately. The most effective strategy to curtail the infection is to keep horses minimally stressed and physically segregated, the method poorly applicable to the modern horse industry with densely populated intermingling high stress environments of race tracks, boarding stables, and show events. It is important that large equestrian events have requirements for entry of the horse into a facility and a good plan for temporary quarantine of new arrivals as well as rapid infection and disease control response.

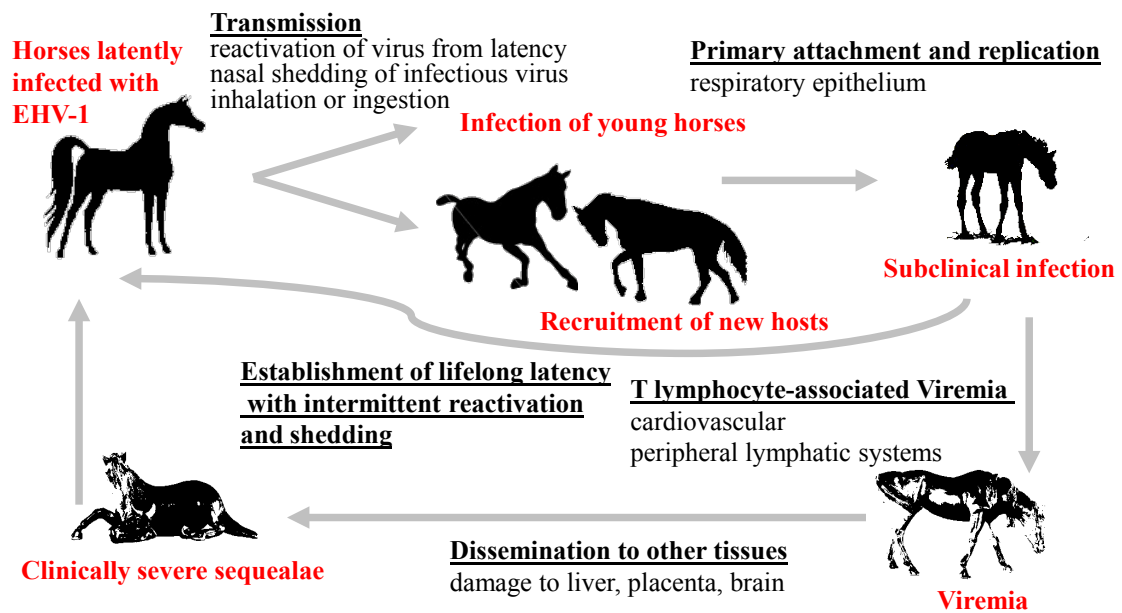


Figure I-12. Pathogenesis of EHV-1 infection.

## **EHV-1 Pathogenesis and Clinical Presentation**

### **Respiratory**

Of the two most common viruses, EHV-4 is by far more commonly isolated from cases of herpesvirus respiratory disease (Foote et al., 2004; van Maanen, 2002), while respiratory infection by EHV-1 has the greater potential for clinically severe sequelae including abortion, neonatal foal death, neurological disorders, peracute pulmonary vasculitis, and ocular disease. Disease severity depends on previous exposure or infection, health status, concurrent infections and the virulence of the particular EHV-1 strain (Huang Ja et al., 2002). Among the eight equine herpesviruses identified to date, EHV-1 is one of the most pathogenic herpesviruses of horses.

Equine respiratory disease associated with EHV-1 infection of the upper airway mucosal epithelium is a condition routinely seen primarily in young horses, weanlings and up to 2 year old horses in training or performing (Timoney, 1992). Clinical signs include depression, poor appetite, nasal discharge and cough. Clinical upper respiratory tract disease (URTD) caused by EHV-1 is acute and infection spreads rapidly within a population via aerosolized respiratory secretions or virus-contaminated fomites. The majority of respiratory infections run a subclinical course. Older horses generally show mild or subclinical signs, while younger horses are usually protected by passive immunity (Mair, 1999). Diagnosis cannot be made on the basis of presenting clinical signs alone and requires laboratory confirmation.

Natural infection with EHV-1 occurs by inhalation or ingestion, after which the virus attaches to, and rapidly replicates in, cells of the nasopharyngeal epithelium and associated lymphoreticular tissues, causing necrosis, exudation, and infiltration of

phagocytic cells (Figure I-13). The incubation period is from 2 to 10 days (Timoney, 1992). Bronchial and pulmonary tissues also become infected, particularly in foals, predisposing them to secondary bacterial pneumonia. The respiratory form is referred to as rhinopneumonitis and is characterized by rhinopharyngitis and tracheobronchitis.

Clinical signs are the most severe and viral shedding greatest during the first few days of infection. Bilateral nasal discharge is most common clinical finding. Initially the discharge is serous, in 2-3 days of infection, the discharge becomes mucopurulent with inflammatory cells and desquamated respiratory cells, and commonly progresses to purulent with an advent of a secondary bacterial infection (Huang Ja et al., 2002). Affected horses may be febrile (39° - 42°C) and the viremia may coincide with a second fever spike (Timoney, 1992). For 9–14 days starting from 4–6 days post EHV-1 infection, an extensive cell-associated viremia is detectable (Gibson et al., 1992).

Migration of virus-infected phagocytes into the circulation results in a T lymphocyte associated viremia. Viremia is associated with, T-cell lymphopenia and appearance of blastic cells (McCulloch et al., 1993) and may occur in the presence of virus-neutralizing antibodies (Doll and Bryans, 1963; Mumford et al., 1987). T-lymphocytes are the most susceptible of the peripheral blood mononuclear cells (PBMC) and carry EHV-1 to distal organs (Scott et al., 1983). Both neutropenia and lymphopenia are often present. Other clinical signs include mandibular and/or retropharyngeal lymphadenopathy, conjunctivitis with mild ocular discharge, depression, anorexia, coughing, and respiratory distress (Huang Ja et al., 2002; Timoney, 1992). Juvenile horses may develop vesicular and erosive lesions in the mucous membranes of the upper respiratory tract (van Maanen, 2002). By contrast, infections with EHV-4 are restricted to respiratory tract epithelium and

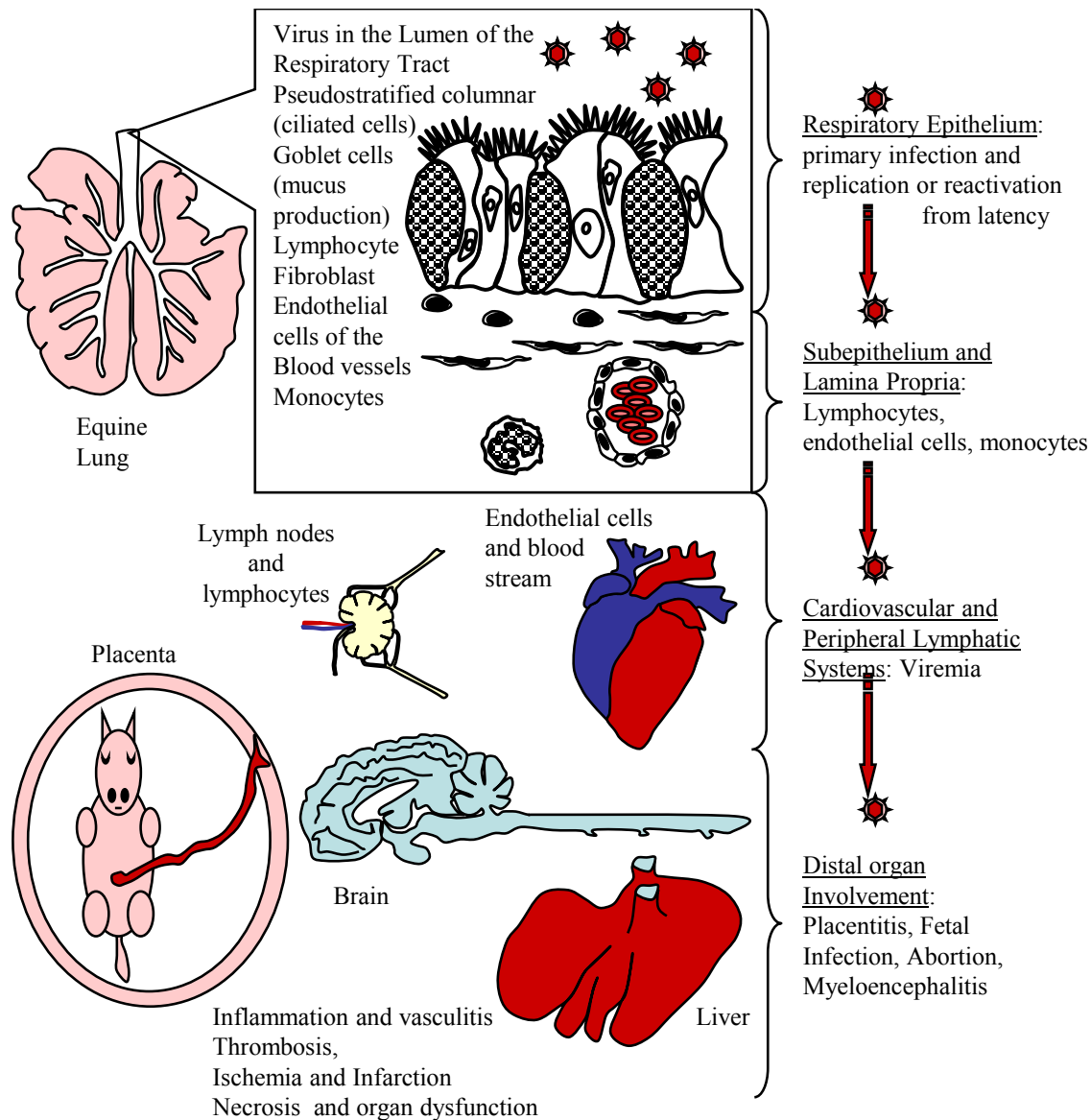
associated lymphatic glands. Leukocyte associated viremia is not found with EHV-4 infections (Timoney, 1992). Complete recovery from uncomplicated infection generally occurs within several weeks, (Mair, 1999).

EHV-1 viremia occurring in primary and all recrudescing infections occurs subclinically, but poses a threat of inducing abortion, as well as neurological disease. The ability of EHV-1 to spread from cell-to-cell without an extracellular phase, via virus mediated cell-to-cell fusion, enables it to avoid inactivation by circulating neutralizing antibody and permits dissemination to other tissues. Trigeminal ganglia, circulating T lymphocytes and lymphoid tissues draining the respiratory tract, are the sites for establishment and maintenance of the lifelong state of latency characteristic of all herpesviruses. Immunity following recovery lasts only 3-6 month and reinfection and asymptomatic viral shedding may occur at these times (Timoney, 1992).

The cycle of a persistent latent infection with intermittent reactivation and shedding keeps EHV-1 circulating within the equine population.

### **Peracute Pulmonary Vasculitis**

This is a more recently described, sporadic condition affecting young adult horses, where EHV-1 induces nonneurological fatal disease, characterized by prominent vasculotropism. In this syndrome, horses exhibit high fever, depression, loss of appetite, and respiratory distress with no neurological signs. Clinical deterioration is rapid and mortality rates from respiratory failure are high (Huang Ja et al., 2002; van Maanen, 2002). The main target of the virus in this condition is the pulmonary endothelium, and post-mortem examination shows severe pulmonary vasculitis with acute edema, leukocytosis, and perivascular hemorrhage (Blunden et al., 1998; Hamir et al., 1994).



**Figure I-13. Virus spread among tissues and body systems.** In response to infection, re-infection or reactivation from latency, the respiratory epithelium is invaded by the virus, which gains access to the subepithelium and lamina propria. Here the virus is able to infect the monocytes, endothelial cells of the blood vessels as well as lymphocytes in route for lymphatic vessels. Once the lymph nodes are infected, infected lymphocytes leave the node resulting in a cell-associated viremia and spread of the virus to various sites around the body via the blood and lymph vessels. Endothelial cells at distal sites are then infected resulting in inflammation and vasculitis. Thrombosis, ischemia, and infarction lead to further tissue necrosis and organ dysfunction.



## **Abortion and Neonatal Disease**

Equine herpesvirus type-1 is the most common cause of infectious abortion in horses (Leblanc, 1999), accounting for diagnosed 10% of abortions in thoroughbreds (van Maanen et al., 2000). The prevalence of EHV-1 abortion varies and it may appear as sporadic cases or as abortion “storms” on stud farms, with few to large number of mares affected. The risk for abortion is increased if the mares are kept in crowded conditions and if they are exposed to new horses (Leblanc, 1999). An infected, re-infected or reactivated mare can then provide a source of infection for non-immune mares resulting in abortion storms and cases of neonatal syndrome and death (Murphy et al., 1999).

The period between infection and abortion varies from 9 days to 4 months but most mares abort within 21 days (Powell, 1991). Ninety-five percent of abortions usually occur late in gestation, typically between 7 to 11 months (Powell, 1991) in a mare showing no clinical signs. The mare is potentially infectious to other horses for 4 weeks. While the future reproductive ability of a mare is not compromised (Huang Ja et al., 2002), it is recommended not to re-breed the mare for 30 days. The risk of abortion remains in future pregnancies due to reactivation of a latent infection or re-infection at that time. The aborted fetus, placenta and birth fluids are a potential source of infection for other mares and should be removed and disposed (Powell, 1991).

To result in abortion, EHV-1 must infect in sequence three separate types of cell and three separate organ systems. In response to infection, re-infection or reactivation from latency, as described previously, the respiratory epithelium of the affected mare is invaded by the virus, which gains access to the subepithelium and lamina propria. Here the virus is able to infect the monocytes, endothelial cells of the blood vessels as well as lymphocytes

reaching lymphatic vessels. Once the lymph nodes are infected, infected lymphocytes leave the node resulting in a cell-associated viremia and spread of the virus to various sites around the body via the blood and lymph vessels. Endothelial cell infection triggers inflammatory cascade which combined with direct cell damage may lead to thrombosis, ischemia, and infarction.

Consequently, microcotyledons of the placenta undergo necrosis and premature separation of the placenta may be triggered. If the thrombosis and infarction are severe, the fetus dies of hypoxia and is expelled. He may not be positive for viral antigen. If the thrombosis is localized and is not severe, the virus may reach the fetus and affect various organ systems. The foal then dies because of viremia, interstitial pneumonia, and focal liver necrosis (Leblanc, 1999) and is expelled. It is also possible for thrombosis and infarction to be minimal and cleared, allowing for the birth of a normal foal (Allen et al., 1999b).

Foals that are infected by EHV-1 in the mare and are born alive, are either sick at birth or become ill within the first few days and up to 2 weeks from birth. Clinical signs may include weakness, depression, difficulty nursing, pyrexia, lymphopenia, and respiratory distress secondary to interstitial pneumonia. The foals may also have evidence of gastrointestinal disease (watery diarrhea) and neurological signs such as visual and vestibular (Dixon et al., 1978). Secondary bacterial infections with *Salmonella* species and *Escherichia coli* are common. EHV-1 has a predilection for vascular epithelium of the nasal mucosa, lung, adrenal, thyroid, and CNS. The disease progression is rapid and mortality due to interstitial pneumonia that results in hypoxia and respiratory failure is at 100%.

## **Myeloencephalopathy**

The neurological form of EHV-1 is an uncommon but serious manifestation of infection with the virus. Neurologic outbreaks occur concurrently with respiratory illness or abortion, or they may occur alone (Hahn et al., 1999; Timoney, 1992). The exact reasons why some EHV-1 infections are associated with a high incidence of neurological disease, whereas others are not, or why horses show different clinical manifestations of infection during an outbreak are still under investigation (Wilson, 1997).

There is no breed or sex selectivity to EHV-1 myeloencephalopathy, though pregnant mares in early to mid gestation may be more susceptible. Route of infection and the immune status of the horse seem to be contributing factors. While foals may develop the disease; it is much more common in older horses. The majority of EHV-1 infections that lead to neurologic signs represent reinfection rather than a primary infection (Wilson, 1997). Therefore, immunological reaction to the EHV-1 infection leading to vasculitis and thrombosis was thought to be partially responsible for the observed neurological damage.

Supporting this, one study showed that vaccinated horses were significantly more likely to develop a neurological manifestation of infection than were non-vaccinated animals (Wilson, 1997). In contrast another study showed that vasculitis was not present in vessels in which endothelial cells did not support viral replication, suggesting that certain EHV-1 strains may be particularly neurotropic. Although unproven, it was proposed that the 1-p and 1-b substrains may preferentially produce the neurological form (Wilson, 1997). The propensity of certain EHV-1 isolates to induce myeloencephalopathy was also considered to be a reflection of marked endotheliotropism rather than specific neurotropism (Hasebe et al., 2002). Thus, virus recognition and infection of specific cell

types, neuronal, endothelial, or lymphocytic, through various cellular receptors may be the ultimate basis for the specific clinical manifestations associated with EHV-1 infection outbreaks.

The acute onset of clinical signs of diffuse multifocal hemorrhagic myeloencephalopathy usually peaks within 48 hours and appears to be the result of vasculitis and thrombosis of arterioles in the brain and especially the spinal cord (Hasebe et al., 2002; Wilson, 1997). The primary lesion is a vasculitis (arteritis), with neural parenchyma affected by secondary hemorrhagic and ischemic infarction. The vasculitis and thrombosis of arterioles in the brain and especially the spinal cord lead to functional impairment of blood flow and metabolic exchange. Sometimes hypoxic degeneration and malacia with hemorrhage into adjacent neural tissues of the white and to a lesser extent, the grey matter occurs (Wilson, 1997).

Clinical signs are variable and depend on the location and severity of the lesions (Wilson, 1997). A fever occurs (up to 41.1 °C), with or without a cough and serous nasal discharge. The horse may begin to show signs of clumsiness or stiffness, progressing to paresis and ataxia of the trunk and limbs with dog sitting and lateral or complete recumbency (Timoney, 1992). Paralysis or paraplegia of the hind limbs leading to recumbency, if it occurs, is usually within the first 24 hours. Paralysis may be so severe that the horse cannot lift its head, though appetite and mentation remain normal. Conscious proprioceptive deficits can be observed (Wilson, 1997) as well as loss of sensation and sensory reflexes in the perineal area, inguinal areas and hind limbs (Timoney, 1992). Hindlimb edema is a frequent sign, and may be accompanied by scrotal edema (Wilson, 1997). There is often bladder distention that leads to urinary incontinence and vulvar or

penile flaccidity with cystitis as a common complication. Tail elevation, decreased or loss of tail tone is inconsistent finding. Depression, nystagmus, eye deviation, and other signs of brainstem involvement may occur (Hahn et al., 1999). Clinical signs are most commonly bilaterally symmetrical, though may be unilateral (Wilson, 1997).

Morbidity rates range from less than 1% to almost 90% of exposed horses, while mortality rates range from 0.5% to 40% (Wilson, 1997). Time to recovery depends on severity of first signs and ranges from several days to 18 months (Hahn et al., 1999), clinically varying from complete recovery, through recovery with retention of neurological deficits, to death (Timoney, 1992).. Horses that are only mildly affected may recover in as little as hours to days. Recumbent horses may die in coma or convulsion, and many are euthanised due to secondary complications, however recumbent horses should not be preemptively euthanised, as many can make a full recovery (Wilson, 1997). Recurrence of neurological signs in recovered horses has not been documented (Wilson, 1997).

### **Ocular Disease**

A potentially serious complication following equine herpesvirus -1 infection, particularly in foals, is severe ocular disease. Upon recovery from mild, transient EHV-1 infection foals may develop visual impairment secondarily to chorioretinitis (Slater et al., 1992). Uveitis and chorioretinitis have also been reported in foals following an outbreak of EHV-1 myeloencephalopathy (McCartan et al., 1995), with nursing foals of mares with EHM being at highest risk of developing this manifestation of EHV-1. Virus is capable of producing ocular and neural damage, even without obvious neurological impairment (Slater et al., 1992). Currently, it is unclear whether ocular disease represents a separate complication in itself or whether it is another manifestation of EHM.

## **Laboratory Diagnosis**

### **Respiratory**

Respiratory EHV-1 infection can be diagnosed by virus isolation from nasopharyngeal swabs or PCR. PCR is a more sensitive method. Antigen capture ELISA can be used, but it is the least sensitive approach. Samples should be taken in the early febrile period of the disease (van Maanen, 2002). Differentiation from equine influenza, equine viral arteritis, EHV-4, and other respiratory infections can only be made with acute and convalescent serum samples in addition to virus isolation (Timoney, 1992). Virus can be isolated in a variety of cell cultures, including those derived from the horse, monkey (Vero), and rabbit (RK-13). By contrast EHV-4 cannot be propagated in cell lines of non-equine origin, providing additional method of virus differentiation.

### **Abortion**

The diagnosis of EHV-1 abortion is made through history, the gross and histological findings on necropsy, virus isolation and evidence of viral antigen in fetal tissues (Timoney, 1992). An aborted fetus secondary to EHV-1 infection will usually not be autolyzed. Clinical specimens for viral abortion diagnosis include placenta, fetal lung, liver, thymus, adrenal glands and spleen sent for virus isolation, immunofluorescence, PCR and histological examination. One set of tissues is submitted on ice and a second set in formalin. The chance of diagnosing a viral abortion is cut in half if the placenta specimen is not provided. Gross lesions at necropsy include severe pulmonary edema, interstitial pneumonia, myocardial and adrenal gland petechiation, multifocal hepatic necrosis and thymic necrosis. On histological examination, focal hepatic necrosis, pulmonary inflammation and lymphoid necrosis in the lymph nodes, spleen, thymus and Peyer's

patches are characteristic. The finding of eosinophilic intranuclear inclusions in fetal tissues by histopathologic examination of the liver, thymus and bronchial epithelium is supportive (Leblanc, 1999). Confirmation is most easily and rapidly obtained by the demonstration of viral antigens in infected cells by immunofluorescence examination of cryostat sections of affected tissue. Serology from aborted mares is not very useful because of the delay between infection and abortion (Powell, 1991).

### **Neonatal Disease**

As for abortion cases, the diagnosis of neonatal disease secondary to EHV-1 perinatal infection is often based on history, clinical signs, necropsy findings as well as immunofluorescence and virus isolation from tissue at necropsy. If a foal has not had colostrum, it may have significant virus neutralizing antibodies demonstrable by serology. (Leblanc, 1999). As compared to septic and premature foals, EHV-1 infected foals commonly have higher mortality, white blood cell counts less than  $3 \times 10^9/L$ , and icterus. Despite common profound hepatic necrosis in the herpes positive foals, liver enzymes may not be elevated (Perkins et al., 1999). On gross examination at necropsy, foals will have interstitial pneumonia with heavy edematous lungs and potentially, areas of atelectasis and evidence of secondary bacterial infection. Splenic and thymic hypoplasia, adrenocortical hyperplasia, hemorrhage and necrosis as well as ventricular subepicardial and subendocardial petechiae may be evident (Bryans et al., 1997; Hartley and Dixon, 1979). On histological examination, lung tissue from affected foals shows congestion, edema and focal necrotizing bronchiolitis. Hyaline membrane formation may be present. Intranuclear inclusion bodies may be found in bronchiolar epithelial cells and hepatocytes, but generally may not be as clearly evident in organs of older foals (Hartley and Dixon, 1979;

Leblanc, 1999; Savage, 1999). The liver may have foci of hepatocellular necrosis (Savage, 1999), while massive thymic parenchymal necrosis may be evident (Hartley and Dixon, 1979). EHV-1 in liver, spleen, and lung can often be detected by immunohistology and electron microscopy (Jonsson et al., 1989).

### **Myeloencephalopathy**

Diagnosis of EHV-1 associated central nervous system disease is more difficult. Presumptive diagnosis of EHV-1 myeloencephalopathy can be made from the history. Sudden onset and early stabilization of neurological signs are characteristic of EHV-1. When there is a recent history of fever, abortion, or viral respiratory disease in the horse or its herd mates, EHV-1 should be suspected (Wilson, 1997). Clinical specimens include nasal swabs, whole blood, cerebrospinal fluid, acute and convalescent sera, brain, and spinal cord from horses with central nervous system disease. Infection may be assumed if a significant increase in specific antibody can be demonstrated between acute and convalescent sera, but most horses with EHV-1 myeloencephalopathy do not show a four-fold rise in serum neutralizing titer and in some, there is even a decline (Wilson, 1997). Antibodies to EHV-1 in the CSF are a variable finding (Wilson, 1997). Elevated protein and CSF to serum albumin ratio reflect vasculitis and leakage into CSF (Wilson, 1997). Xanthochromia and an elevated protein level in CSF are supportive evidence for EHV-1 (Hahn et al., 1999). The virus may be isolated from nasal swabs and buffy coat of the blood from acutely affected horses, but the virus shedding has often ceased at the time of onset of neurological signs. The virus is difficult to isolate from central nervous system tissue post mortem (Timoney, 1992; Wilson, 1997). At necropsy, a brownish patchy discoloration may be seen grossly in the brain and spinal cord. Histologically, vasculitis



with ischemic and hemorrhagic infarction as well as perivascular edema and necrosis of parenchyma are observed (Timoney, 1992).

### **Ocular Disease**

On histological examination of ocular disease in foals, a mononuclear cell infiltrate is present in the cerebral cortex, cerebellar choroid plexus and cervical dorsal root ganglion. The optic nerve is demyelinated. Bilateral degeneration of the neurosensory retina, retinal pigment epithelium and choroidal layers is evident in the eyes.

## **EHV-1 Treatment**

### **Current Recommendations**

EHV-1 infections are usually dealt with using management practices that limit spread of the disease, providing symptomatic relief to infected horses, and treating potential secondary bacterial infections with antibiotics. Current recommendations of treatment of EHV-1 infections in horses include the use of steroids, non-steroidal anti-inflammatory agents, and nucleoside analogs as well as some other compounds used to treat EHV-1-associated neurological symptoms like paresis, paralysis, ataxia, distention of the urinary bladder, stiffness of pelvis or ocular damage, but no specific treatment is available.

### **Supportive Care**

EHV-1 infected horses should be kept quiet and have adequate supportive care. Stall rest in a well ventilated, dust-free environment is essential for the EHV-1 infected horse showing respiratory signs. Antibiotics may be given prophylactically to prevent secondary bacterial infections (van Maanen, 2002). More specific antibiotics are prescribed to target any secondary infections that develop (Wilson, 1997). Supportive treatment for

neonatal disease including fluid therapy, antibiotics, steroids and assisted ventilation are minimally effective (Dixon et al., 1978).

Treatment of equine myeloencephalopathy is limited to supportive therapy and management of patient comfort and secondary complications. Horses should be encouraged to remain standing, and slings should be used to support moderately affected horses that cannot stand on their own. Recumbent horses should be rolled every 2 to 4 hours to reduce the risk of myonecrosis and decubital ulcers. Intravenous fluids are indicated in dehydrated patients. To maintain gastrointestinal system, laxatives or bran mashes should be given, and manual evacuation of the bowel may be necessary to prevent impaction and to maintain patient comfort. Some horses may need to be fed and watered via a stomach tube or intravenously. Cystitis is a frequent complication, especially in recumbent horses. Careful catheterization may be indicated, as well as treatment with antibiotics. Urine scald can be prevented and managed with washing of the perineum and application of repellent ointment, as well as wrapping the tail to keep it out of the way (Wilson, 1997).

### **Corticosteroids**

The treatment with corticosteroids is recommended because vasculitis, hemorrhage, and edema are prominent early lesions of EHV-1 myeloencephalopathy and may have an immune basis. However no objective data to document their efficacy is available, and their use has to be weighed against the detrimental effects on immune function thus exacerbating virus shedding as well as the possibility of inducing laminitis.

A short course glucocorticoid treatment with prednisolone acetate (1 to 2 mg/kg q24hr) or dexamethasone (0.05 to 0.25 mg/kg q12hr for 2-3 days) is used to treat

inflammatory conditions by blocking the release of arachidonic acid (Cassiani-Ingoni et al., 2005). Experiments with HSV 1 in the past showed that the viral yield increased when cells were treated with dexamethasone (Cassiani-Ingoni et al., 2005). The beneficial anti-inflammatory effects of corticosteroids can be dramatic, but substantial complications may ensue including immunosuppression, prolonged virus shedding, delayed healing, and corticosteroid-associated laminitis.

### **Non-steroidal Anti-inflammatory Agents**

Non-steroidal anti-inflammatory drugs (NSAIDs), such as phenylbutazone or flunixin meglumine, are typically given to febrile horses to reduce the fever, thereby encouraging horses to eat and drink (Allen et al., 2002b). Flunixin meglumine, a cyclooxygenase inhibitor, is a potent analgesic, antipyretic, and anti-inflammatory agent. It can be used to treat EHV-1-induced vasculitis in the CNS at 1.1 mg/kg (Cassiani-Ingoni et al., 2005). Dimethyl sulfoxide can be used to relieve typical IC, where symptoms including pelvic pain, perineal pain, nocturnal urinary urgency, urinary frequency and constant sensation of the urge to void.

### **Nucleoside Analogs in Treatment of EHV-1 infection**

Dosing protocols for known antiviral drugs used to treat EHV infections are currently based on extrapolation from well established dosage regimens used for treatment of human infections with HSV-1 and VZV and have not proved to be effective (Huang Ja et al., 2002). Data describing the pharmacokinetics, bioavailability, and safety of acyclovir in horses are lacking.

Acyclovir, a synthetic purine nucleoside analog with viral DNA replication inhibitory activity against several human herpesviruses, has been shown to exert an

inhibitory effect on EHV-1 *in vitro* (Smith et al., 1983). Nucleoside analogues such as acyclovir and penciclovir have been used off label with variable effects in the treatment of EHV-1 myeloencephalopathy. Several cases showing significant improvement when treated with acyclovir were reported, but it is not known whether acyclovir actually influenced the outcome in these cases (Friday et al., 2000; Murray et al., 1998). Treatment of neurologic outbreaks with the acyclovir did not produce substantial benefits (Cassiani-Ingoni et al., 2005).

EHV-1 and -3, HSV-1 and -2, and cercopithecine herpesvirus virus were all sensitive *in vitro* to 9,((2-hydroxy-1-(hydroxymethyl)ethoxyl) methoxyl) guanine (BIOLF-62), another nucleoside analogue, at concentrations of less than 0.55 micrograms/ml (Cassiani-Ingoni et al., 2005).

### **Antivirals**

The common classes of antiviral agents currently in overall use are nucleoside and pyrophosphate analogues, direct non-nucleotide DNA polymerase or reverse transcriptase inhibitors, and endogenous cytokines, while countless other classes are being investigated.

Newest class of antiviral agents was developed to combat HIV-1 infections and includes such compounds as Amantidine and Rimantidine, which inhibit the M2 protein of the virus to decrease hydrogen ion influx through a specific H<sup>+</sup> ion channel, inhibiting viral uncoating during entry and coating process at egress. Currently available fusion inhibitors such as Enfuvirtide and T1249 were also developed against HIV-1. Amprenavir is a sulfonamide compound that was introduced in the market as an HIV protease inhibitor. Despite the success of this drug, it has some shortcomings including gastrointestinal side effects and long term metabolic disturbances (Hanlon et al., 2004; Miller et al., 2004).

## **Current Antiherpetics**

Nucleoside analogues such as acyclovir and ganciclovir have been the mainstay of therapy for alphaherpesviruses, such as HSV, VZV, and CMV infections (see Table I-8). Active compounds currently available for the treatment of herpes virus infections include the nucleoside analogues: acyclovir (Zovirax, GlaxoSmithKline, Middlesex, UK) and its valyl-ester prodrug valacyclovir (valtrex, GlaxoSmithKline, Middlesex, UK), the valyl-ester prodrug of penciclovir - famciclovir (famvir, Novartis, New York, NY), penciclovir, vidarabine, and ganciclovir (cytovene, Hoffmann LaRoche) and its prodrug valganciclovir (valcyte, Hoffmann LaRoche, Nutley, NJ), the acyclic nucleoside phosphate (ANP) derivative cidofovir (vistide, Gilead Sciences, Boulder, CO), the pyrophosphate analogue foscarnet (foscavir, Astra-Zeneca, Boston, MS) as well as the CMV antisense molecule fomivirsen (vitravene, Novartis, New York, NY). More specifically, antiviral agents currently licensed for the treatment of HSV and VZV infections include acyclovir, valacyclovir, famciclovir, penciclovir, foscarnet. For CMV infections: ganciclovir, valganciclovir, cidofovir, foscarnet, and fomivirsen. Except for the CMV antisense molecule, all compounds terminate viral DNA synthesis by inhibiting the viral DNA polymerase (Table I-8).

While some of these antiviral therapies are considered safe and efficacious (acyclovir, penciclovir), some have toxicities associated with them (ganciclovir and foscarnet). Several of the currently available therapies can result in mild to severe side effects making the discovery of less toxic drugs desirable. Prolonged use of these compounds in the clinical setting has led to the emergence of human viral resistance against most of these drugs. Because nucleoside analogues share a similar mechanism of

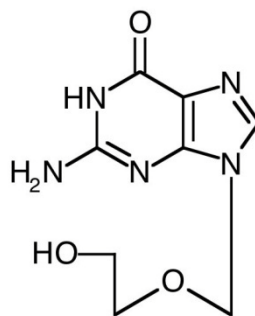
action, treatment options are limited once resistance develops. Efforts over the last decade have focused on the identification and development of improved therapies including less toxic compounds with novel mechanisms of action.

Acyclovir, 2-amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6*H*-purin-6-one, is a white, crystalline powder with the molecular formula  $C_8H_{11}N_5O_3$  and a molecular weight of 225 (Figure I-14). Recently, acyclovir was shown to inhibit the wild-type HIV strain infection at a 50% inhibitory concentration (IC<sub>50</sub>) of ~ 5  $\mu$ M (McMahon et al., 2008).

Current published dosing of acyclovir for treatment of EHV-1 starts with the loading dose of 27 mg/kg of acyclovir every 8 h for 2 days, followed by a maintenance dose of 18 mg/kg every 12 h, will maintain effective serum acyclovir concentrations (L. K. Maxwell, 2008). Small interfering RNA (siRNA) designed against ORF of gB and origin-binding viral helicase resulted in reduction of viral replication in the murine model of respiratory disease (Fulton et al., 2009).

**Table I-8. Antiviral compounds against alphaherpesviruses.**

Compound	Trade Name	Chemistry	Clinical Application
2 <sup>nd</sup> generation	foscarnet	pyrophosphate analogue	HSV, VZV, CMV
	cidofovir	acyclic nucleoside phosphonate derivative	CMV
acyclovir	Zovirax, GlaxoSmithKline		HSV, VZV
valacyclovir	valtrex, GlaxoSmithKline	valyl-ester prodrug	HSV, VZV
famciclovir	famvir, Novartis	valyl-ester prodrug of penciclovir	HSV, VZV
	penciclovir		HSV, VZV
	Valtrex/Zelitrex		HSV, VZV
	Denavir		HSV, VZV
	Abreva		HSV, VZV
	vidarabine		HSV, VZV
ganciclovir	cytovene, Hoffmann LaRoche		CMV
valganciclovir	valcyte, Hoffmann LaRoche	ganciclovir prodrug	CMV
cidofovir	vistide, Gilead Sciences	acyclic nucleoside phosphate (ANP) derivative	
foscarnet	foscavir, Astra-Zeneca	pyrophosphate analogue	
fomivirsen	vitrovene, Novartis	CMV antisense molecule	CMV
	Cytovene, Valcyte		CMV



**Figure I-14. Chemical structure of Acyclovir, 2-amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6*H*-purin-6-one.**

## **CHAPTER II. ANTIVIRAL SCREENING**

### **INTRODUCTION**

#### **Hypothesis and Goals**

The central hypothesis of the proposed investigations is that porphyrin compounds can specifically inhibit membrane fusion phenomena required for virus entry and virus spread. Such inhibition would be achieved via interactions with one or more viral glycoproteins required for membrane fusion. It is expected that the antiviral activity can be enhanced by further modification of the chemical structure of the porphyrin compounds.

Our immediate goal addressed in this chapter, was to identify a few relatively small organic compounds that possess strong antiviral properties against EHV-1 infections. Specifically, two classes of compounds were investigated: porphyrin and platinum compounds. Representatives of these classes of compounds have been already found to possess potent antiviral activities against human immunodeficiency virus (HIV), vaccinia, and coronaviruses (Vzorov et al., 2003). The following chapter describes our approach to define the molecular basis for their actions in cell culture experiments and to further modify their chemical structure to enhance their antiviral potency.

It was envisioned that the proposed investigations will lead to the development of antiviral strategies used as prophylactic treatment for successful combating EHV-1 acute infections of horses.

#### **Significance of EHV-1 Infections and Need for Therapeutic Options**

The development of new antiviral or virucidal compounds for the prophylactic and therapeutic treatment of EHV1 infection is necessary to avoid economic loss to the horse industry. Louisiana ranks fifth nationwide in terms of economic impact on its horse



industry, contributing over \$2.45 billion to the state's economy (Deloitte-Consulting, 2005). As described previously, EHV-1 is enzootic in most equine populations. The seroprevalence of EHV-1 has been increasing over the past decade, reaching approximately 30% according to a recent survey (Gilkerson et al., 1999a). Clinical upper respiratory tract disease (URTD), rhinopneumonitis, caused by EHV-1 infection of the upper airway mucosal epithelium is an acute condition seen primarily in young horses. Infection spreads rapidly within a population via aerosolized respiratory secretions or virus-contaminated fomites. Although, the majority of respiratory infections run a subclinical course, viremia occurring in primary and all recrudescing infections have a great potential for clinically severe sequelae such as late term abortion, neonatal foal death, neurological disorders (paresis/paralysis), myeloencephalopathy, ocular disease, or death by peracute pulmonary vasculitis. Diagnosis cannot be made on the basis of presenting clinical signs alone and requires laboratory confirmation.

Current EHV-1 vaccines help to curtail the respiratory disease and abortion, but are ineffective against the neurological form of infection. Horses become seriously ill despite regular vaccinations in very short intervals. In devastating recent outbreaks, an unusually high number of horses exhibited the neurological form of the disease. This is alarming because this form of EHV-1 infection used to be sporadic and contained to individual animals of an affected herd. Better treatment options are in high demand. Currently, several compounds have been used to address EHV-1-associated neurological symptoms like paresis, paralysis, ataxia, distention of the urinary bladder, stiffness of pelvis or ocular damage, but no specific treatment is available. Dosing protocols for known antiviral drugs used to treat EHV infections are based on extrapolation from well established dosage

regiments used for treatment of human infections with HSV-1 and VZV. Vaccine ineffectiveness to curtail neurologic manifestation of the infection or virus spread through the population, as well as limited therapeutic options, make the development of vaccine and other prophylactic strategies, as well as options for antiviral treatments of affected horses necessary.

## **Literature Review of Porphyrins**

### **Classes of Potential Microbicides**

Porphyrins are a group of naturally occurring and intensely colored compounds, whose name is drawn from the Greek word *porphura*, a word for purple (Anderson, 1999; Milgrom, 1997). Porphyrins were originally under consideration as topical microbicide, specifically for intravaginal application for HIV prophylactic. There are various groups of potential microbicides (see Table II-1): polyanionic molecules, surfactants, various natural products, peptides, heterocycles, and various virucidal agents. The specific microbicides exemplifying each group are those that are best described, since they have been evaluated for their potent anti-HIV activity.

The first generation antiviral/microbicides candidates were surfactants, with most notable of these products, nonoxynol-9 (N-9). It has been shown to be ineffective in preventing HIV, or even harmful (Roddy et al., 1993). The second generation microbicides were polymers. Having more focused spectrum of activity, they interfered with viral attachment to host cells. Several efficacy trials with different polymers, such as PRO 2000, Carraguard, Cellulose Sulfate, and Dextrin-2-Sulfate, have been underway. Several other entry inhibitors are currently in pre-clinical stages of development. The latest generation of microbicides targets attachment, a critical step of the viral life cycle, during which the

virus binds to the receptors on the cell surface. The future of the anti-HIV microbicide development would likely focus on attachment and fusion inhibitors or co-receptor blockers. The main consideration in such microbicide or any antiviral design, is the level of toxicity at therapeutic doses, potential for rapid establishment of resistant isolates, as well as the expense of production using currently available technology.

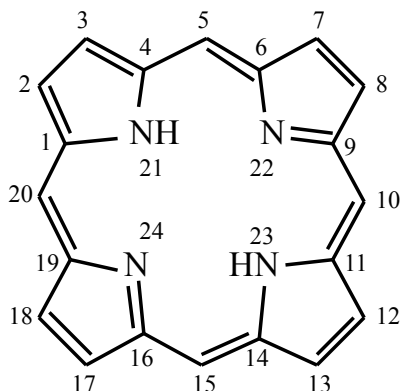
**Table II-1. Examples of potential microbicides evaluated for anti-viral activity.**

<b>Group</b>	<b>Potential Microbicides</b>
Polyanionic molecules	naphthalene sulfonates, sulfated polysaccharides, polycarboxylates and polyoxometalates
Buffers	engineered lactobacillus, hydrogen peroxide/peroxidases, Buffergel
Surfactants	N-9, Benzalkonium chloride, C31G-Savvy, Chlorhexidine zinc gel, detergents
Entry blockers	Carraguard/PG515, PRO2000 gel, Emmelle/Dextrin-2-Sulfate
Fusion inhibitors	CCR5 inhibitors, soluble CD4
Natural products	tannins, plant lectins, betulinic acid derivatives and macrolides
Peptides	natural and synthetic surface active agents, CPFs, T20, T21 and lexitropsins
Proteins	negatively charged albumins and cyanovirin-N
Heterocycles	bicyclams, monensins, porphyrins, diaminoacridones and phenazines
Virucidal agents	azodicarbonamide
Antiretroviral agents	non-nucleoside RT inhibitors, inhibitors of post-fusion replication, nucleoside RT inhibitors (e.g. Tenofovir), NNR-TIs (UC-781), protease inhibitors

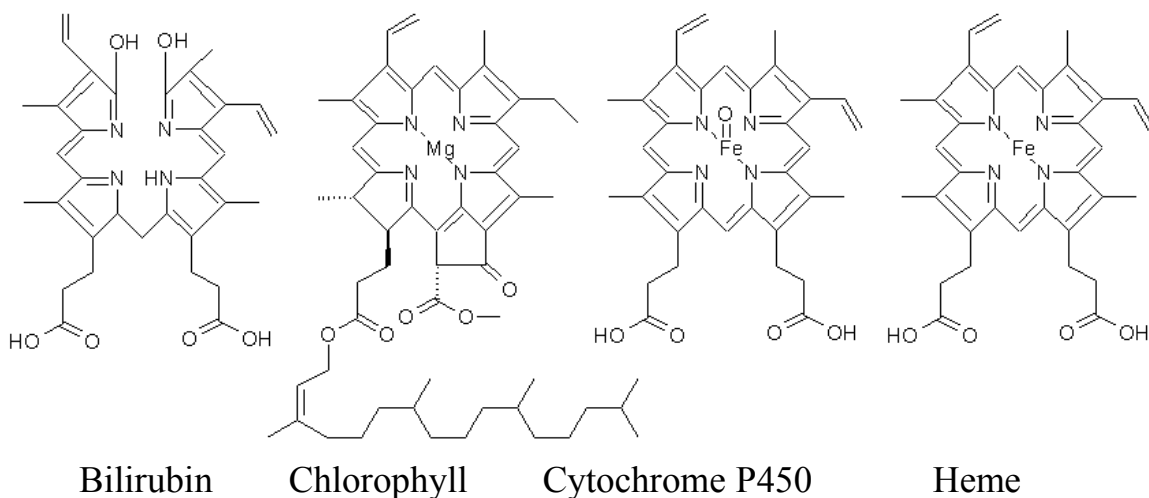
## Structure of Porphyrins

Tetrapyrrole is a term referred to a member of class of compounds whose molecules have four rings of the pyrrole type ( $C_4H_5N$ ), generally linked together on opposite sides by single-atom bridges, usually the four methine ( $=C-$ ) bridges (Figure II-1). The common arrangements of the four rings for which this name is used are macrocyclic, as in the porphyrins and linear, as in the bile pigments (Figure II-2). The famous members of this family include bilirubin, chlorophylls, cytochrome, and heme as part of

hemoglobin. Natural tetrapyrroles are critical for biological processes of energy and electron transfer like photosynthesis or in the respiratory chain. Synthetic tetrapyrroles, like phthalocyanines become more important not only as colors, but also as photoactive compounds in photovoltaic, photomedicine, molecular electronics and other fields.



**Figure II-1. The macrocyclic tetrapyrrolic ring system named porphyrin.**



**Figure II-2. Examples of natural tetrapyrroles.** These critical to life molecules play important biological roles. They are natural pigments containing four pyrrole rings joined by one carbon each into a macrocyclic tetrapyrrole.

## **Current Use of Porphyrins**

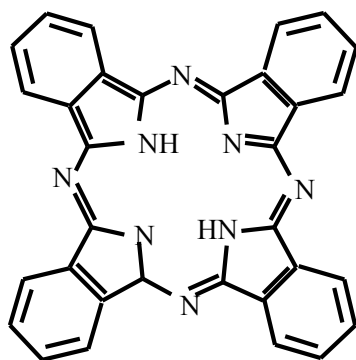
Porphyrins are in use as photodynamically activated agents for cancer, psoriasis, and macular degeneration. Porphyrins in photodynamic therapy or porphyrin based detection treatment (PDT) are used as tumor localized photosensitizer (chelated metal without a central metal or with a diamagnetic central metal), which reacts with molecular oxygen and other substrates to generate highly cytotoxic species that destroy tumor tissue.

Photosensitizer are molecules, which, when excited by light energy, can utilize this energy to induce photochemical reactions to produce lethal toxic agents. In a cellular environment within the immediate area of light illumination, these agents (singlet oxygen ( $^1\text{O}_2$ ), hydroxyl radicals ( $\cdot\text{OH}$ ), and superoxide ( $\text{O}_2^-$ ) ions) can interact with cellular components including unsaturated lipids, amino acid residues, and nucleic acids; and ultimately, result in cell death and tissue destruction (Detty et al., 2004; Ian J. Macdonald, 2001).

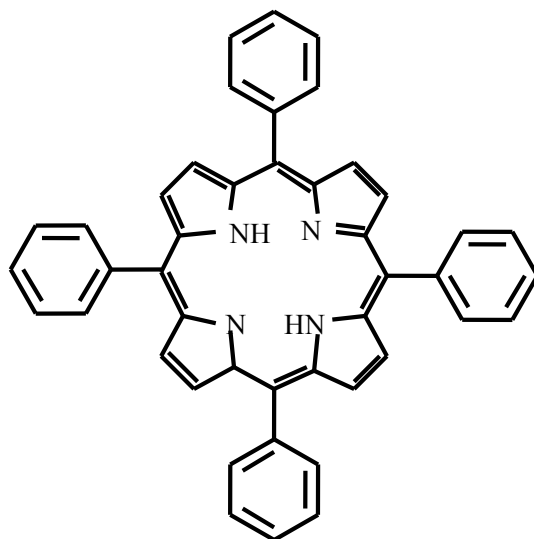
## **Structure-Activity Relationship of Porphyrins**

Synthetic porphyrins are most readily synthesized from pyrrole and benzaldehyde derivatives and constitute two main groups, the metallo-TPPS4 (sulfonated tetraphenyl porphyrin) derivatives (Figure II-3) and phthalocyanines (sulfonated tetra-arylporphyrins) (Figure II-4).

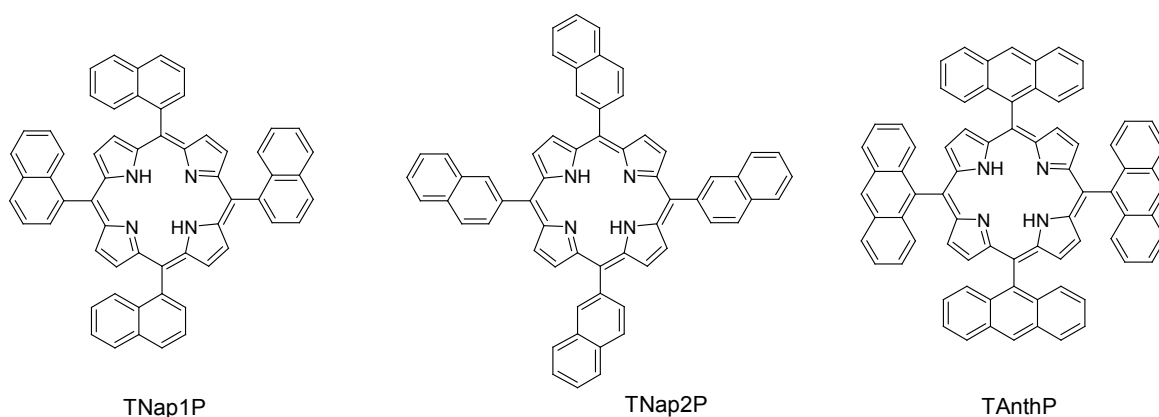
Substantial work performed by Dr. Marzilli of the LSU Department of Chemistry and his collaborators at Emory University, Atlanta, GA has shown that specific porphyrin-based compounds exhibited strong and specific antiviral activities against different viruses such as HIV and vaccinia (Figure II-5). In our laboratory, different groups of antiviral compounds have been tested against Respiratory Bovine Coronavirus (RBCoV) infection.



**Figure II-3. Structures of a synthetic 5,10,15,20-Tetraphenylporphyrin, TPP,  $C_{44}H_{30}N_4$ .**



**Figure II-4. Structures of phthalocyanine, tetrabenzotetraazaporphyrin.**



**Figure II-5. Structures of synthetic porphyrins which were effective against HIV.**

Sulfonated, halogenated tetraaryl porphyrins and sulfonated tetraaryl porphyrins with large or electron-donating substituents like tetranaphthyl porphyrins and tetraanthracenyl porphyrins were the most efficient compounds against HIV (Dixon et al., 2005).

Importantly, some of these compounds have showed exceptional antiviral properties (Vzorov et al., 2003). The molecular basis for these antiviral activities and their action against herpesviruses are not known and is the subject of the proposed investigations.

Virucidal as well as antiviral activities can be found in “natural” and “synthetic” porphyrins. Both classes exist as “free base” form without any central metal or as “metallo-porphyrin” with different metal ions in the centre. A number of studies have investigated the detailed structure–activity relationships of porphyrins (Dixon et al., 2005; Vzorov et al., 2002). Porphyrins were originally under consideration as topical microbicides. They were later shown to inhibit cell fusion induced by the HIV-1 Env protein and to block binding of gp120 to the CD4 receptor. Active over a range of pH values, while having no detectable activity against normal bacterial flora, the compounds have been shown to inhibit transmission of cell-associated HIV and inactivate a broad range of isolates. None of the tested natural compounds were able to inhibit an HIV infection for more than 80% (Vzorov et al., 2002).

Metallated tetrapyrroles are present in most organisms and participate in essential biochemical processes that include photosynthesis, oxygen transport, drug metabolism, transcriptional regulation, nitric oxide synthesis, and oxidative phosphorylation. In nature, metallation of tetrapyrroles is catalyzed by a group of enzymes named chelatases. Heme is an iron porphyrin coordination complex. Metallation in the laboratory can be achieved by heating with  $\text{Fe(II)C}_{12}\text{H}_{20}\text{O}$  in the presence of nitrogen air (Marzilli, 2004 Baton Rouge, LA, May 2004). Addition of naphthalene group ( $\text{C}_{10}\text{H}_8$ ) extends the porphyrin structure. These compounds have two additional benzene rings attached on the periphery of the porphyrin structure (for example, TNap1P and TNap2P in Figure II-5).

Overall, for coronavirus, HIV and vaccinia, the most effective compounds were a combination of porphyrins with sulfonate groups which are associated with antiviral activity and low toxicity (Dixon et al., 2005). With sulfonation using chlorosulfonic acid, the chlorosulfonyl compounds can be converted into derivatives with free sulfonic acid, a sulfonamide or a sulfonate ester (Rocha Gonsalves et al., 1996; Sobral et al., 2002). Sulphonation significantly increases solubility of porphyrin compounds in polar solvents including water, circumventing the need for alternative delivery vehicles (Ali and van Lier, 1999; Detty et al., 2004; Nyman and Hynninen, 2004; Phillips, 1995).

### **Properties of Porphyrins Favorable as Therapeutic Compound**

The different attributes of porphyrins such as independence of pH changes, stability against different redox situations, affinity for serum proteins, favorable pharmacokinetic properties, and no photoactivity renders them attractive candidates as antiviral or virucidal compounds against EHV-1 infections, especially against the neurological form of the viral infection. Most of them are non-toxic, inexpensive, and form stable complexes with a



variety of metal ions (chelate). The time required for complete elimination of porphyrins from biological tissues is reported to be about a week and most porphyrins are known to be non-toxic. These compounds may also be modified to increase their half-life in blood.

### **Porphyrins Mechanism of Antiviral Action**

The mechanisms of action and the stage in the viral life cycle at which the porphyrins inhibit the virus, is not well understood yet. In case of HIV infection, the antiviral effect may be due, at least in part, to inhibition of the reverse transcriptase. Metalloporphyrins as well as natural porphyrin class showed significant inhibition of RT activity. A second way of action may be inhibition of the HIV protease. Furthermore, natural porphyrins seem to inhibit HIV virus entry mediated by the Env glycoprotein interaction with its cellular receptors CD4+, CCR2 (Vzorov et al., 2003).

### **Stages of the EHV-1 Life-cycle as Targets for Porphyrin Therapy**

#### **Cell Tropism and Spread**

Natural infection with EHV-1 occurs by inhalation or ingestion, after which the virus attaches to, and rapidly replicates in, cells of the nasopharyngeal epithelium and associated lymphoreticular tissues, causing necrosis, exudation, and infiltration of phagocytic cells. Bronchial and pulmonary tissues also become infected, particularly in foals, predisposing them to secondary bacterial pneumonia.

Migration of virus-infected phagocytes into the circulation results in a T lymphocyte associated viremia. T-lymphocytes are the most susceptible of the peripheral blood mononuclear cells (PBMC) and carry EHV-1 to distal organs (Scott et al., 1983). Viremia is associated with, T-cell lymphopenia and appearance of blastic cells (McCulloch et al., 1993) and may occur in the presence of virus-neutralizing antibodies (Doll and

Bryans, 1963; Mumford et al., 1987). For 9–14 days, starting from 4–6 days post EHV-1 infection, an extensive cell-associated viremia is detectable (Gibson et al., 1992). The ability of EHV-1 to spread from cell-to-cell without an extracellular phase, via virus mediated cell-to-cell fusion, enables it to avoid inactivation by circulating neutralizing antibody and permits dissemination to other tissues.

### **Virus Entry and Neutralizing Antibodies**

Recent developments in molecular studies of HSV-1 entry, maturation, and spread have contributed to deeper understanding of EHV-1 life cycle and pathogenesis. Herpes simplex virions have evolved mechanisms to deal with multiple membrane barriers during virus-entry and cellular egress through the use of multiple glycoproteins (gB, gC, gD, gE, gI, gJ, gH, gK, gL, gM) and other virus-specified proteins. Initially, virions attach to ubiquitous glycosaminoglycans, e.g., heparan sulfate moieties on cell surfaces via glycoproteins gC and gB. Subsequently, viral glycoprotein gD interacts with specific cellular receptors embedded in plasma membranes initiating the fusion of viral and cellular membranes. These receptors include members of the immunoglobulin superfamily (nectin-1, nectin-2, and CD155) and HveA (or HVEM), a member of the tumor necrosis factor (TNF) superfamily (Campadelli-Fiume et al., 2000; Whitbeck et al., 1997). Viral glycoproteins gB, gH and gL are directly involved in fusion of the viral envelope with plasma membranes (Hagglund et al., 2002). Generally, all herpesviruses have evolved mechanisms to facilitate virus-entry into cells through the use of multiple glycoproteins embedded within their viral envelope. The glycoproteins are efficient primary targets of virus-specific neutralizing antibody, development of which is coincident with resolution of clinical signs and resistance to homologous reinfection for 3 to 4 month. Viral

glycoproteins are also key determinants of membrane-associated events occurring during virion morphogenesis and egress from infected cells, where cell-to-cell transmission of herpesviruses occurs either by release of virions to extracellular spaces or through virus-induced cell-to-cell fusion.

### **Latency**

Trigeminal ganglia, circulating T lymphocytes and lymphoid tissues draining the respiratory tract, are the sites for establishment and maintenance of the lifelong state of latency characteristic of all herpesviruses. The cycle of a persistent latent infection with intermittent reactivation and shedding is thought to keep EHV circulating within the equine population. In latently infected animals, reoccurrence of viremia and shedding of the virus in nasal secretions of horses has been produced by immune suppression through the administration of corticosteroids (Slater et al., 1994).

### **SPECIFIC OBJECTIVES**

This chapter describes the initial experiments conducted with large number of porphyrin, phthalocyanine, and platinum compounds for preliminary determination of their potential antiviral and cytotoxic effects. The tests were designed to screen the available compounds for their inhibition of free virus as well as interference with virus entry and production. A number of leading compounds were then selected based on their *in vitro* efficacy (EC50, 50% effective concentration), toxicity to the cells (CC50, cytotoxic concentration), cell localization, and therapeutic index ( $TI=CC50/EC50$ ). The chemical structures of the selected substances were then then modified by the collaborating laboratory of Dr. Marzilli, and the resulting change in quantity of the EHV-1 antiviral activity and cytotoxicity was determined.

## **EXPERIMENTAL DESIGN**

During lytic replication, EHV-1 destroys the host cells that it infects, observed as rounding of the cells, multinucleated giant cell formation (syncytia), and presence of acidophilic inclusions. The antiviral activity of potential therapeutic agents against EHV-1 can be determined by evaluating the inhibition of this virus-induced cell killing, or cytopathic effect (CPE).

Initially, experiments to determine cytotoxicity of the different compounds to the rabbit kidney cells, RK13, were performed. RK13 cells were chosen, since it is a standard cell line used for EHV-1 propagation, research, and diagnosis. Based on preliminary data from Dr. Marzilli's laboratory and our studies with coronavirus and vaccinia, the baseline concentration of antiviral in cell culture media was chosen at 50µg/ml. For those compounds that were found to be cytotoxic at this concentration, two-fold dilutions were performed to determine optimal concentration that does not exhibit an obvious cytopathic effect on the cells. Second, a collection of up to a 100 antiviral drugs was tested in the EHV-1 cytopathic effect inhibition assay. The set of drugs tested included platinum, tetraphenylporphine, sulfonated and metallo-porphyrin compounds and others. The initial screen was followed by a plaque reduction assay to determine the 50% effective concentration (EC50) of compounds showing positive results.

Following primary and secondary screenings, subsequent chemical modifications of leading compounds was performed by the Dr. Marzilli's group at the LSU Chemistry Department to ascertain whether antiviral properties were improved or diminished, while cytotoxicity avoided.

## **MATERIALS AND METHODS**

### **Cells**

RK13 cells, rabbit kidney epithelial cells, were obtained from American Type Culture Collection (ATCC CCL-37, Rockville, MD). Cells were propagated and maintained in Dulbecco's Modified Eagles Medium (DMEM; Sigma Chemical Co., St. Louis, MI) containing sodium bicarbonate, 15 mM Hepes, and supplemented with 7% heat inactivated fetal bovine serum (FBS). The equine dermal cells, ED (ATCC CCL-57), equine fetal tracheal cells, EFTr (Primary cell line, field strain provided by Dr. Corstvet, LSU SVM, Baton Rouge, LA), and Vero (African Green Monkey Kidney Cells, ATCC CCL-81) were maintained in Dulbecco modified Eagle's minimum essential medium (DMEM) supplemented with 15 mM Hepes, and 7% fetal bovine serum (FBS) and incubated at 37°C, 5% CO<sub>2</sub>.

### **Viruses**

The NY1, NY2, VA, and RacL11Δgp2/EGFP viruses were kindly provided as ethanol precipitated DNA by Dr. Osterrieder of the Cornell University School of Veterinary Medicine, Ithaca, NY. Neurologic field Isolates, NY1 (New York) and NY2, were collected a few years apart, from the animals of the same herd. VA (Virginia) isolate was obtained from the brain tissue of a horse with clinical symptoms of encephalitis. To establish infectious virus stocks, viral DNA was transfected into RK13 cells using SuperFect Transfection Reagent (Cat. No. 301305, Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The virus strain Ab4, parental strain of RacL11Δgp2/EGFP recombinant virus, was isolated in the late 1950s from an aborted foal and exhibits high virulence both in the natural host and in laboratory animals, such as the

Syrian hamster and the mouse. The EHV-1 Ab4 genome has been cloned into bacterial artificial chromosomes (BAC), which facilitated the construction of mutant viruses constitutively expressing the green fluorescence protein (GFP) under the human cytomegalovirus immediate early promoter (HCMV-IE). Specifically, a portion of an F plasmid and an EGFP expression cassette HCMV-IE promoter were substituted for gene 71 encoding glycoprotein gp2 to create a RacL11Δgp2/EGFP recombinant virus. Deletion of the gp2 glycoprotein does not appreciably affect virus entry and virus replication, while a slight reduction (5%) in plaque size is observed (Neubauer et al., 2002).

### **Antiviral Compounds**

About a hundred different inorganic and organic compounds, either commercially available or synthesized by the collaborating laboratory of Dr. Marzilli, LSU Chemistry Department were kindly provided by Dr. Marzilli (Louisiana State University, Baton Rouge, LA) and stored at 3-5°C in containers not permissive to light. Due to patent considerations, some of the tested compounds are referred to as they were named upon synthesis at the laboratory of Dr. Marzilli and not by the corresponding nomenclature of porphyrin chemistry (Moss, 1988). The chemical nomenclature of porphyrin and porphyrin derivatives and the abbreviations used are as follows: *meso*-tetraphenylporphyrine (TPP); 5,10,15,20-tetrakisphenylporphyrin (H2TPP); and 5,10,15,20-(4-chlorosulfonylphenyl) porphyrin (H2TPPS4). An “S” at the end of the abbreviation indicates that the parent porphyrin was sulfonated and the derivatives are mixtures with variable numbers and/or positions of the sulfonates on the ring. Cu(II)-5,10,15,20-tetrakis(4-[p-sulfobenzyl] sulfoamidonylphenyl) porphyrin (Cu(II)TPPS4) is a sulfonated tetraphenylporphyrin with a copper moiety/chelate at the *meso* position, while Fe(III)-5,10,15,20-tetrakis(4-[p-

sulfobenzyl] sulfoamidonylphenyl) porphyrin (Fe(III)TPPS4) is a sulfonated tetraphenylporphyrin with an iron moiety/chelate, and for simplicity, will be referred to as metalloporphyrins in this paper. Distilled water was used as solvent to prepare 2 mg/ml stock solution for each test chemical. Several compounds were provided dissolved in dimethyl sulfoxide (DMSO, (CH<sub>3</sub>)<sub>2</sub>SO) as solvent. DMSO is miscible with water and prior to their use in assays, solutions of porphyrin compounds were diluted in excess of 100-fold in water. Therefore, DMSO was not expected to influence the outcome of the assays via its function to enhance membrane penetration and transport. Preparation of solutions and all experiments were done under red light to prevent chemicals degradation or phototoxicity upon exposure to light. ChemDraw Pro 11.0 (CambridgeSoft Corporation, Cambridge, MS) program was used to present chemical structures of the compounds. All stock solutions of compounds were in the range of pH 8.5 to 9.

### **Initial Screening Assay: Antiviral Effect on Free Virus**

The initial screen of antiviral compounds was a cell-based assay with endpoint detection performed using visual inspection and calculation of ratio of the number of infected cells (EGFP fluorescent cells) counted by FACS to that of untreated control (Figure II-6). RK13 cells were plated in triplicates in 24-well tissue culture plates (Greiner, Frickenhausen, Germany), at a density of 2 x 10<sup>5</sup> cells per well and incubated overnight in a humidified 5% CO<sub>2</sub>-air at 37°C. At 80% confluency, cell monolayers were infected with EHV-1 RacL11Δgp2/EGFP at a multiplicity of infection of 10 and 0.1 PFU/cell in DMEM 25 mM Hepes medium. Prior to infection, virus was incubated with 50 µg/ml concentrations of the compounds for 30 min at 37°C. Virus was allowed to enter the cells during one hour incubation at 37°C. Then, EHV-1 infected cell monolayers were washed

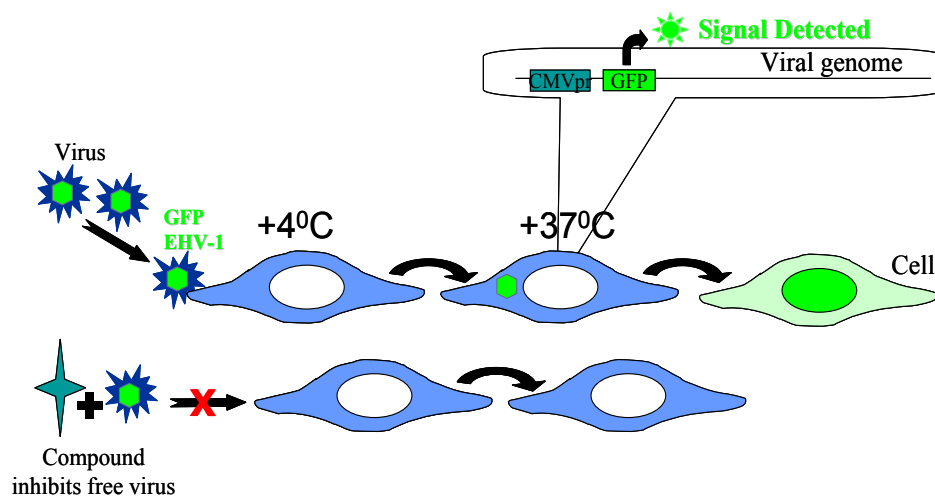
with phosphate-buffered saline (PBS) to remove residual drug and the virus that has not entered, overlaid with fresh 2% FBS DMEM, and incubated at 37°C for 8 h, at which time the cells were harvested, washed and subjected to FACS analysis to determine the percent of infected cells via GFP fluorescence. Acyclovir was used as control antiviral, applied at a concentration 50, 25 and 5 µg/ml. Cells infected with viruses at the same multiplicity of infection (M.O.I.) of 10 and 0.1 PFU/cell and without adding any of the compounds served as a control of a 100% infection. Those compounds that show more than 50% reduction in EGFP fluorescence at the M.O.I. of 10 were selected for further testing.

### **Cytotoxicity Assays and 50% Cytotoxic Concentration**

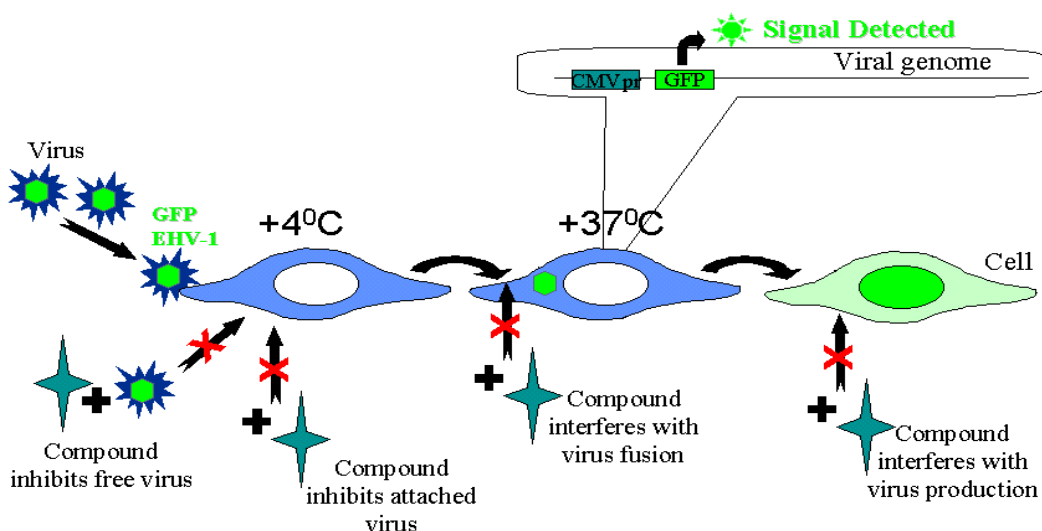
Current assays to monitor the viability of cells include vital dye uptake, tritiated thymidine uptake, and tetrazolium salts (e.g. MTT, WST-1). We used the following assays to determine compound cytotoxicity: (i) Cytopathic effects produced in RK13 cells were determined by daily visual inspection and qualitative assessment of cells exposed to serial dilutions of the candidate microbicides; (ii) The effects of the compounds exerted during two population doublings of RK13, ED, and EFTr cells determined by staining the cells with trypan blue and visually counting the cells that incorporated the dye. Briefly, serial 2 to 10-fold dilutions of the compounds, 0.1 to 100 µg/ml, were added to 20%, 40%, 60%, or 80% confluent monolayer of RK13, ED, EFTr or Vero cells, without viral challenge. Then, plates were left at 37°C and 5% CO<sub>2</sub> for 3 days and examined for toxicity effects by using trypan blue exclusion test. Alternatively, the treated cells were incubated under normal growth condition for 48 hours and then, stained with neutral red containing medium to access cell viability. The cells were subsequently washed, the dye was extracted in each well, and the absorbance was read using a spectrophotometer. The 50% Cytotoxic



Concentrations (CC50) were calculated. Therapeutic Index (TI) values were calculated as the ratio of 50% cytotoxic concentration to %50 Inhibitory Concentration of each compound ( $TI = CC_{50}/IC_{50}$ ). Compounds were scored as active if  $TI > 10$ , moderate if  $1.5 < TI < 10$ , inactive if  $TI < 1.5$ .



**Figure II-6. Diagram of initial antiviral screening assay.**



**Figure II-7. Diagram of the modified screening assay.**

## **Confocal Microscopy**

For laser scanning confocal-microscopy experiments, RK13 cells were cultured in eight-well chamber slides (Lab-Tek II; Nalge Nunc International, Naperville, Illinois). Twenty-four hours after being seeded, the cells were EHV-1 or mock infected and further incubated in the presence or absence of the antiviral compounds. At various times post infection, the cells were fixed with paraformaldehyde and permeabilized with 0.5% Triton X-100 in order to maintain the integrity of cellular structures. For cell surface biotinylation, prior to fixation cells were washed with TBS-Ca/Mg and incubated for 15 minutes at RT in EZ-Link Sulfo-NHS-LC Biotin cell impermeable biotinylation reagent (Pierce Chemical, Thermo Fisher Scientific Inc., Rockford, Illinois), which reacts with primary amines on cell surface proteins. Cells were then washed with TBS and fixed with electron microscopy grade 3% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, Pennsylvania) for 15 minutes, washed twice with PBS-50 mM glycine, and permeabilized with 1.0% Triton X-100. For cell surface labeling, biotinylated cells were reacted with 1:1000 diluted Alexafluor 647 conjugated streptavidin for 20 minutes. For Golgi and ER organelle labeling, cells were incubated with 1:750 dilution of Alexafluor 488 conjugated lectins GSII and concanavalin A, respectively. TGN was identified with a donkey anti-TGN46 primary antibody and an Alexafluor 488 conjugated sheep anti-donkey secondary antibody. Specific immunofluorescence was examined using a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems Inc., Bannockburn, IL) fitted with a CS APO 63x Leica objective (1.4 numerical aperture). Individual optical sections in the z-axis, averaged 6 times, were collected at set zoom in series in the different channels at 1024 x 1024 pixel resolution. Images were compiled and rendered in Adobe Photoshop.

### **Modified Screening Assay: Effect on Virus Entry and Replication**

To analyze whether antiviral compounds affected viral particles prior to and at entry steps of the infection, the virus EHV-1 RacL11Δgp2/EGFP was incubated with the compounds for 30 min at room temperature and then added to the cells (see Figure II-7). Then, after the incubation time of 1.5 hours at 4°C and another hour at 37°C the wells were washed and fresh DMEM 25 mM Hepes medium was added. To determine if the antivirals act at the level of binding, penetration, or post-entry, the antiviral compounds were added to the infected cell monolayers for 10 minutes and then, removed by a wash, either during the 4°C period, at the time that the cells were transferred to 37°C, or immediately after the 1 hr incubation at 37°C respectively. High resolution pictures of the EGFP fluorescent virus infected cells were taken 12 hours post infection and the numbers of infected cells were counted as percent of control, number of infected cells without the antiviral compounds.

### **Cytopathic Effect Inhibition Assay and 50% Inhibitory Concentration**

To determine the efficacy of antiviral compounds, a cell-based assay utilizing cytopathic effect inhibition (CPE) or reduction in the number of viral plaques was set up. The basic assay involved infection of RK13 cells as described above, however neurological isolates (NY1, NY2, and VA) were tested in the presence of various concentrations of test compounds in addition to EHV-1 RacL11 virus. Cells were seeded at a density of  $2 \times 10^5$  cells per well and grown in triplicate wells in 24-well plates overnight. Each drug was added at least eight different concentrations within 0.1 to 30 µg/ml range of concentrations, at the time of the infection and maintained until completion of the assay.

EHV-1 (0.1 to 5 PFU/cell) was exposed to serial dilutions (0.1-30 µg/ml) of the candidate microbicides for 30 min at 37°C. After incubation for 1 h at 37°C, the virus inoculum was aspirated, and a maintenance medium overlaid containing 1% methylcellulose and the appropriate compound concentration added. The plates were incubated at 37°C in 5%CO<sub>2</sub> for 2-3 days and observed daily for CPE. The end point titration was determined as the drug dilution that inhibited 100% of the CPE in triplicate wells. The plates were fixed with 5% formaldehyde and stained with crystal violet. The viral titer in the presence of each concentration of drug was determined by counting the plaques. The ability of the compounds to inhibit EHV-induced cell killing was determined as reduction in plaque number and diameter. Median inhibitory concentration or the IC<sub>50</sub> (or EC<sub>50</sub>, 50% effective concentration) calculated as the concentration required for 50% inhibition. Reported IC<sub>50</sub> values are the average and standard deviation of three separate determinations per experiment, with each experiment repeated three times. The IC<sub>95</sub> values were extrapolated via polynomial of order three.

### **Image Analysis and Quantitation**

For most experiments, high resolution pictures of the fluorescent virus infected cells were taken under fluorescent microscope magnification of x40 at 12 hours post infection and the numbers of infected cells were counted as percent of control. Image analysis was done using the Leica microscopy software (Leica Microsystems Inc., Bannockburn, Illinois). Image masks of fluorophore were generated from the image fluorogram data by defining the specific regions of interest with a bounding box. To determine an percentage of the surviving virus, pixel enumeration and intensity statistics within the Leica software package was applied to a series of individual sections.

## **RESULTS**

### **Initial Screening of Antiviral Compounds for Inhibition of EHV-1 Infection**

Using a recombinant, BAC progeny EHV-1 strain, RacL11- $\Delta$ gp2-GFP, as a model target, we conducted antiviral activity testing of up to 100 different inorganic compounds synthesized by the collaborating laboratory of Dr. Marzilli of Louisiana State University (LSU) Chemistry Department, Baton Rouge, LA. RacL11 encodes a portion of the F plasmid and an EGFP expression cassette under the human cytomegalovirus immediate early promoter (HCMV-IE) that was substituted for EHV-1 non-essential gene 71 encoding glycoprotein gp2 (Neubauer et al., 2002).

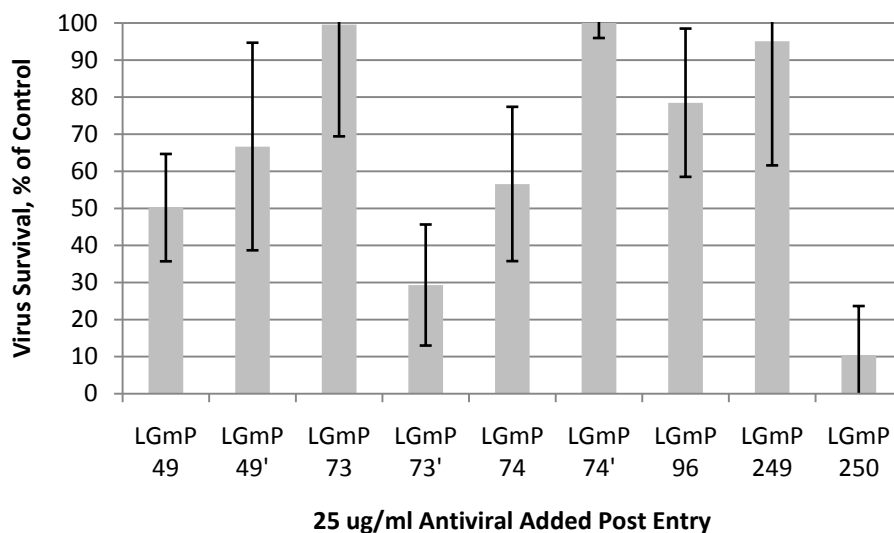
The screening assays were used to estimate the antiviral and cytotoxic effects of the compounds and to identify lead candidate antiviral compounds on the basis of their *in vitro* efficacy and toxicity. Tests were done as cell-based assays with end-point detection performed using visual inspection, Leica image analysis software and FACS detection of percent infected cells showing green fluorescence from EGFP gene encoded by the recombinant EHV-1. The chemical structure of the compounds used in initial screening was undisclosed to the investigators to avoid bias in selecting compounds as well as due to patent and other intellectual property issues. Only compounds selected for further testing were matched to corresponding structural formula, otherwise the compounds were referred to as they were numbered upon synthesis at the laboratory of Dr. Marzilli (Louisiana State University, Baton Rouge, Louisiana).

Initial experiments showed that many of the compounds had potent virucidal activity against the virus, since exposure to virus-stocks for as little as few minutes inactivated more than 99% of the virus (results not shown). These compounds were

selected and further tested for their effect on virus during replication stage of infection.

The results of a representative experiment are shown in Figure II-8. Infected cells were treated with antiviral compounds once the virus has already entered the cell: virus envelope and cellular membrane were fused and the capsid released into the cytoplasm.

Treatment with compound LGmP 250 (sulfonated tetraphenoxypyphenyl porphyrin) and LGmP 73'((3-Cl)4-SO<sub>3</sub> tetraphenyl porphyrin) resulted in about 90 and 70% inhibition of the viral infection respectively, while LGmP 73, 74, and 249 had practically no effect (Figure II-8). Those compounds that when used post entry showed more than 40% reduction in virus infection at the MOI of 10 were selected for further testing and are shown in Table II-2.



**Figure II-8. Blinded initial screening of the antiviral compounds.** Inhibitory effect of photodynamic compounds on post entry events of virus infection resulted from photoinactivation of EHV-1 and decreased cell growth due to cytotoxicity of the photodynamic effects (49, 49', 249, and 250) and chloro groups (73', 74, and 74').

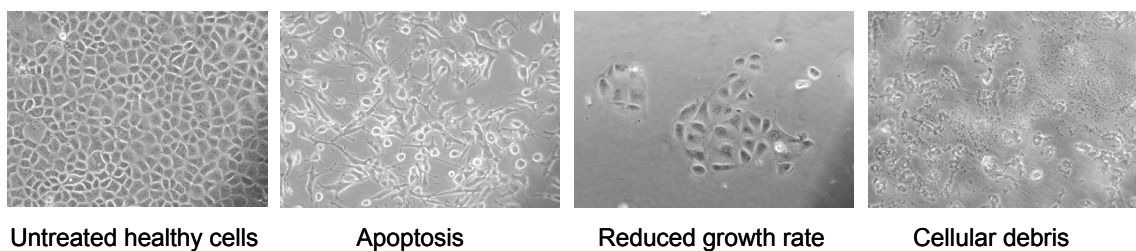
**Table II-2. A selection of the compounds tested.**

Compounds in	Solvent H <sub>2</sub> O or DMSO	Class of Compound	Sulfonate/ Metallo group	Fluorescence/ Photodynamic properties	Cytotoxicity CC50	Inhibition of Free Virus, % 25 µg/ml
LGmP 17	H <sub>2</sub> O			F-		87
LGmP 37	H <sub>2</sub> O	H2TPPS4	M-; S+			
LGmP 38				F-		98
LGmP 39	H <sub>2</sub> O	Cu(II)-tetrasulfonated phenyl porphyrin	Cu; S+	F-	>100	90
LGmP 44	H <sub>2</sub> O	2-fluoro-4-sulfo porphyrin	M-; S+	F+		95
LGmP 45	H <sub>2</sub> O	4 chloro-3-sulfo porphyrin	M-; S+	F+		95
LGmP 46	H <sub>2</sub> O	2,6-difluoro-tetrasulfonated phenyl	M-; S+	F+		100
LGmP 47	H <sub>2</sub> O	(4-F)3-sulfo- tetraphenyl porphyrin		F+		100
LGmP 49	H <sub>2</sub> O		M-	F+		50
LGmP 69	H <sub>2</sub> O	(2-F)3-sulfo-tetraphenyl porphyrin	M-; S+	F+		90
LGmP 71				F-		5
LGmP 73	H <sub>2</sub> O	3-fluoro-4-sulfonato porphyrin	M-; S+	F+		100
LGmP 74	H <sub>2</sub> O	chloro	M-	F+		100
LGmP 96	H <sub>2</sub> O		M-	F+		20
LGmP 106	H <sub>2</sub> O	Phthalocyanine		F-		95
LGmP 128				F-		0
LGmP 129	H <sub>2</sub> O	insoluble		F-		0
LGmP 149	DMSO					0
LGmP 176	DMSO			F-		100
LGmP 177	DMSO					0
LGmP 179				F-		40
LGmP 179b	DMSO	Cis-Platinum, Pt(NH3)2Cl2		F+		82
LGmP 188	H <sub>2</sub> O			F-		0
LGmP 196				F+		98
LGmP 199	H <sub>2</sub> O			F-		0
LGmP 200c	H <sub>2</sub> O			F-		82
LGmP 205a	DMSO	Platinum		F-		0
LGmP 210a	DMSO	Platinum		F-		10
LGmP 212a	DMSO	Porphyrin		F-		0
LGmP 213a	DMSO	Platinum		F-		10
LGmP 226						
LGmP 241	H <sub>2</sub> O	Porphyrin		F-		0
LGmP 246	H <sub>2</sub> O	Fe (III)-tetrasulfonated phenyl porphyrin	M+; S+	F-	>100	100
LGmP 248						70
LGmP 249	H <sub>2</sub> O	Metalloporphyrin	M+	F-		
LGmP 250	H <sub>2</sub> O	tetra-(phenoxyphenyl sulfonated	M-; S+	F+		95
LGmP 252	H <sub>2</sub> O	Cu(II) tetra OCH3SO3 phenylporphyrin	M+; S+	F-	>100	65
LGmP 273		Italian lab		F+	>30	100
LGmP 275	H <sub>2</sub> O	Novel photodynamic porphyrin, H2TPPS8	M-; S+	F+	>100	100
LGmP 276	H <sub>2</sub> O	Novel Cu H2TPPS8	M+; S+	F-	>30	100
LGmP 277	insol	Italian lab	M+		>30	100
LGmP 278		Italian lab	M-		>30	100
LGmP 279	H <sub>2</sub> O	Novel, Naphthalene H2TPPS8	M-	F+	>30	100
LGmP 280	H <sub>2</sub> O	Novel Cu, naphthalene H2TPPS8	M+; S+	F-	>100	100
LGmP 282	H <sub>2</sub> O	Novel Cu, fluoride H2TPPS8	M+; S+	F-	>100	100
LGmP 284	H <sub>2</sub> O	Novel photodynamic naphthalene	M-	F+	>30	100

## **Cytotoxic Effects of Antiviral Compounds**

Cytotoxicity tests were conducted for each individual compound. Different concentrations of the compound were added to rabbit kidney cells, RK13, equine dermal cells, ED, equine fetal tracheal cells, EFTr, and African green monkey kidney cells, Vero, at 20 , 40, 60, or 80% confluency to reach concentrations of 100 µg/ml, 50 µg/ml, 30 µg/ml, 25 µg/ml, 15 µg/ml, 10 µg/ml, 5 µg/ml, and 1 µg/ml in the culture medium. Cells were examined after 12 hr, 24 hr and 36 hr post infection. The observed cytotoxic effects included rounding and shrinking, vacuolization, membrane blebbing/budding, loss of confluency, reduction of growth rate, nuclear damage, and cellular debris. Examples of cytotoxic effects observed are shown in Figure II-9. The results were recorded and used to select compounds without significant cytotoxic effect. Cytotoxicity of compounds was directly related to their fluorescence and therefore photoactivity.

Tetrapyrrole compounds with chloro group, such as LGmP 45 ((4-Cl)3-SO<sub>3</sub> tetraphenyl porphyrin), LGmP 73'((3-Cl)4-SO<sub>3</sub> tetraphenyl porphyrin), LGmP 74, LGmP 74' and others were proven to be cytotoxic even if assayed in the absence of light (results not shown). The chloro group was the only common feature of these compounds and was assumed to have contributed to the cytotoxic effect. Therefore, LGmP 73' was ruled out as a potential antiviral.



**Figure II-9. Cytotoxic effects of antivirals as observed on phase-contrast microscopy.**



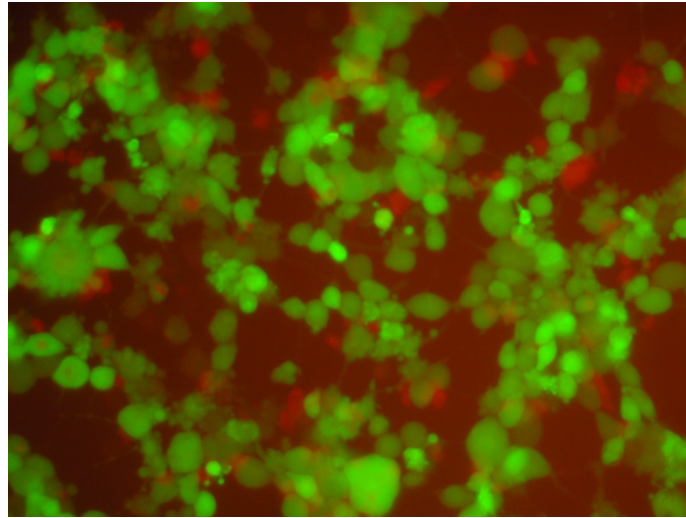
## **Photoactivation**

A flaw of the initial screening approach was that many of the compounds were photodynamic as seen by their red fluorescence when examined under fluorescent microscope (Figure II-10), and therefore, inactivated virus and damaged the cells via light activated production of reactive oxygen species (ROS). As an example, 0.2 µg/ml LGmP 46 inhibited virus by 90% upon exposure to visible and UV spectrum, and only 50% in the dark (Figure II-11). Thus, the exceptionally good antiviral properties all fluorescent compounds were most likely due to photoactivity against EHV-1.

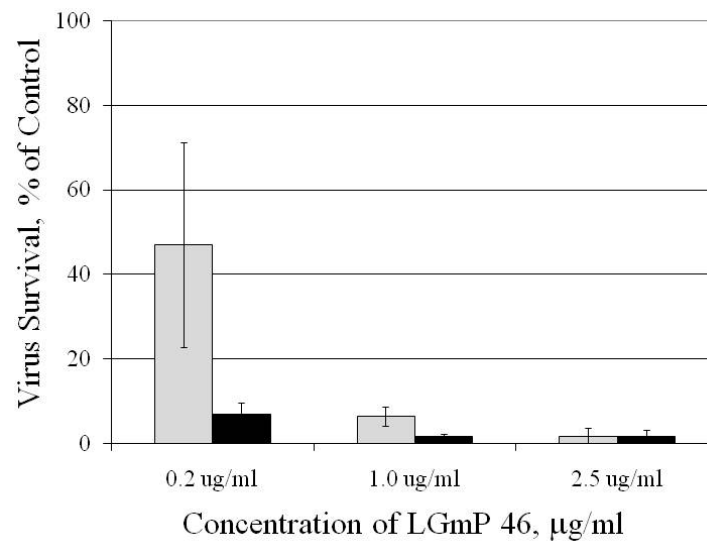
As mentioned previously, the fluorescent compounds were also cytotoxic at concentrations inhibitory to virus infection, resulting in decreased rates of cell growth. Photoinactivation of viruses by diamagnetic porphyrins have been studied previously (Horowitz et al., 1992). The light-activated compounds react with molecular oxygen to produce reactive oxygen species and radicals; in a biological environment these toxic species (singlet oxygen ( $^1\text{O}_2$ ), hydroxyl radicals ( $\cdot\text{OH}$ ), and superoxide ( $\text{O}_2^-$ ) ions) interact with unsaturated lipids; amino acid residues; and nucleic acids causing biochemical disruption to the cell (Detty et al., 2004; Ian J. Macdonald, 2001). If the homeostasis of the cell is altered significantly, then the cell enters apoptosis. Therefore, all subsequent experiments with photodynamic compounds were conducted in dark room conditions to minimize the photoinactivation effect and to evaluate other mechanism of their antiviral function.

## **Cellular Distribution, Cytotoxicity, and Stability of Antiviral Compounds**

We capitalized on the fluorescent property of the porphyrins to evaluate their cellular localization during our preliminary investigations of the cytotoxic effects on RK13



**Figure II-10. Illustration of GFP-expressing virus infected cells in the presence of photoactive porphyrin.** Cells exhibit green fluorescence and syncytia formation characteristic of recombinant GFP-expressing EHV-1 RacL11 infection in the presence of 0.2  $\mu\text{g/ml}$  photodynamic antiviral compound LGmP 37, H2TPPS4 (red fluorescence), examined under fluorescent microscope at x100 magnification.



**Figure II-11. Inhibition of viral infection by photoactive antiviral compound in the presence and absence of visible and UV spectrum.** Prior to infection of RK13 cells, EHV-1 RacL11/EGFP virus was incubated with indicated concentration of LGmP 46 in the absence (gray bars) or presence (black bars) of visible and UV exposure.

cells (Figure II-12, A and B). Compounds were observed to rapidly enter the cytoplasm of both EHV-1 RacL11 and mock infected cells and remain within cytoplasm potentially as either aggregates or as content of endoplasmic vesicles.

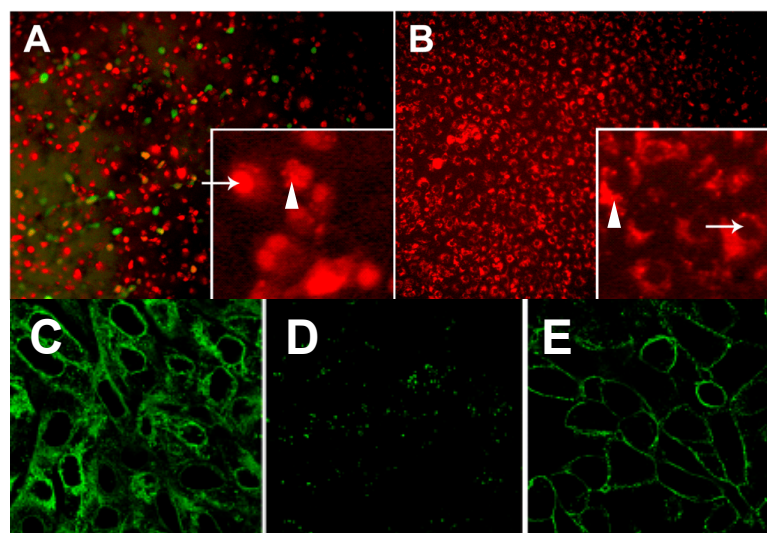
If compounds were added to the cells post infection, they were also seen to concentrate in the nucleus, most likely due to EHV-1 induced modification of cell architecture. If the compounds were preincubated with a virus prior to virus infection, no nuclear staining was observed, as no infection was detected due to complete inhibition of the infection by the compounds.

Confocal microscopy was considered to obtain more detailed information about the internalization and intracellular localization of compounds (Figure II-12, C-E). However, non-photoactive/non-fluorescent compounds could not be evaluated since there is no available marker to localize them to the organelles. The pattern of distribution of photoactive compounds is suspected to be due to the onset of apoptotic changes in the cell.

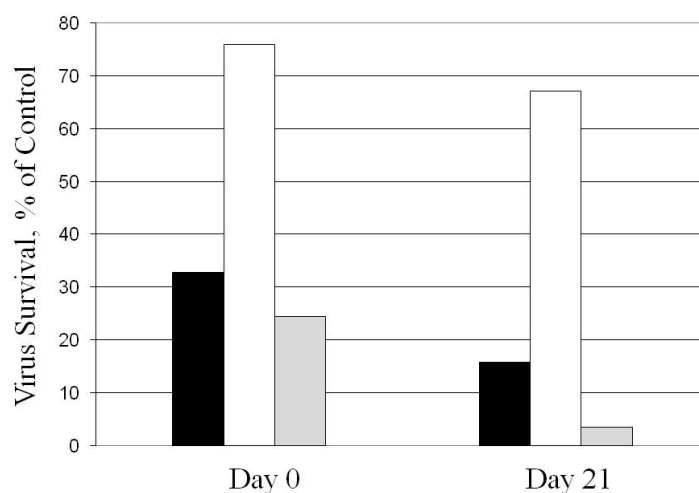
To evaluate stability of antiviral compound upon storage, the activity of several compounds was retested after three weeks of refrigeration. Most compounds showed significant stability or an increase in antiviral activity (Figure II-13), potentially due to evaporation, while other compounds were unstable in storage (results not shown) and were eliminated from further investigations.

### **Modified Screening of Antiviral Compounds Based on Their Activity on EHV-1 Attachment, Fusion and Post-entry Events**

A new set of compounds was synthesized, containing various metals in the *meso* position. Presence of the metal group was expected to decrease or fully abolish photoactivity of these compounds, and therefore, diminish their cytotoxicity. Compounds selected based on initial screening and ruling out cytotoxic compounds and those newly



**Figure II-12. Differential cellular localization of antiviral compounds within cellular compartments.** RK13 cells infected (A) and mock infected (B) with GFP fluorescent RacL11 virus were subjected to antiviral treatment for the duration of infection (12 hours), starting at one hour post infection at a concentration of 20  $\mu\text{g/ml}$  in cell culture media. Porphyrin-based antiviral compound is seen exhibiting red fluorescence under UV exposure. Arrows denote nucleus, arrow head indicates cytoplasmic vacuolization. Porphyrin compound is seen as red fluorescence. Green fluorescence is the GFP expression from the recombinant EHV-1 genome. C, D, E) Laser scanning confocal-microscopy images showing individually stained ER, Golgi and surface cellular compartments of RK13 cells, respectively.



**Figure II-13. Effect of storage on antiviral activity of Fe(III)TPPS4.** Increase in antiviral activity of Fe(III)TPPS4, potentially due to evaporation of the solvent, was seen after refrigerated storage for 3 weeks.

synthesized, were tested for their ability to interfere with EHV-1 infection. Since most of the selected compounds efficiently inactivated the free virus, to better differentiate their antiviral properties and to determine at what stages of viral infection they might exert their antiviral properties, a time course assays were conducted. RK13 cells were precooled to 4°C, and each well inoculated with EHV-1 RacL11Δgp2/ EGFP virus, and the infected cells were incubated for 1 hour at 4°C to allow virus binding. Washing the cells at this time, removed any unattached virus. The shift to 37°C at this step allowed virus penetration into the cells.

To evaluate effect of antiviral compounds on attached virus, following a 1 hour attachment at 4°C, the antiviral compounds were added, cells were incubated for 10 more minutes at 4°C, washed and transferred to 37°C to allow virus entry. To evaluate effect of antiviral compounds on fusion step of virus entry, antiviral was added post 1 hr at 4°C and the cells immediately transferred to 37°C for 10 min, then washed, and transferred to 37°C to continue infectious cycle. To evaluate effect of the antiviral compounds on post entry events, antiviral was added for 10 min at the step after the virus has entered the cells (post 1 hr at 4°C and additional 1hr at 37°C). At 12 hours post infection, numbers of infected cells were counted and are shown in as percent of untreated control, a number of infected cells without the antiviral compounds. The extent of inhibition of infection was determined from the percent of virus that survived.

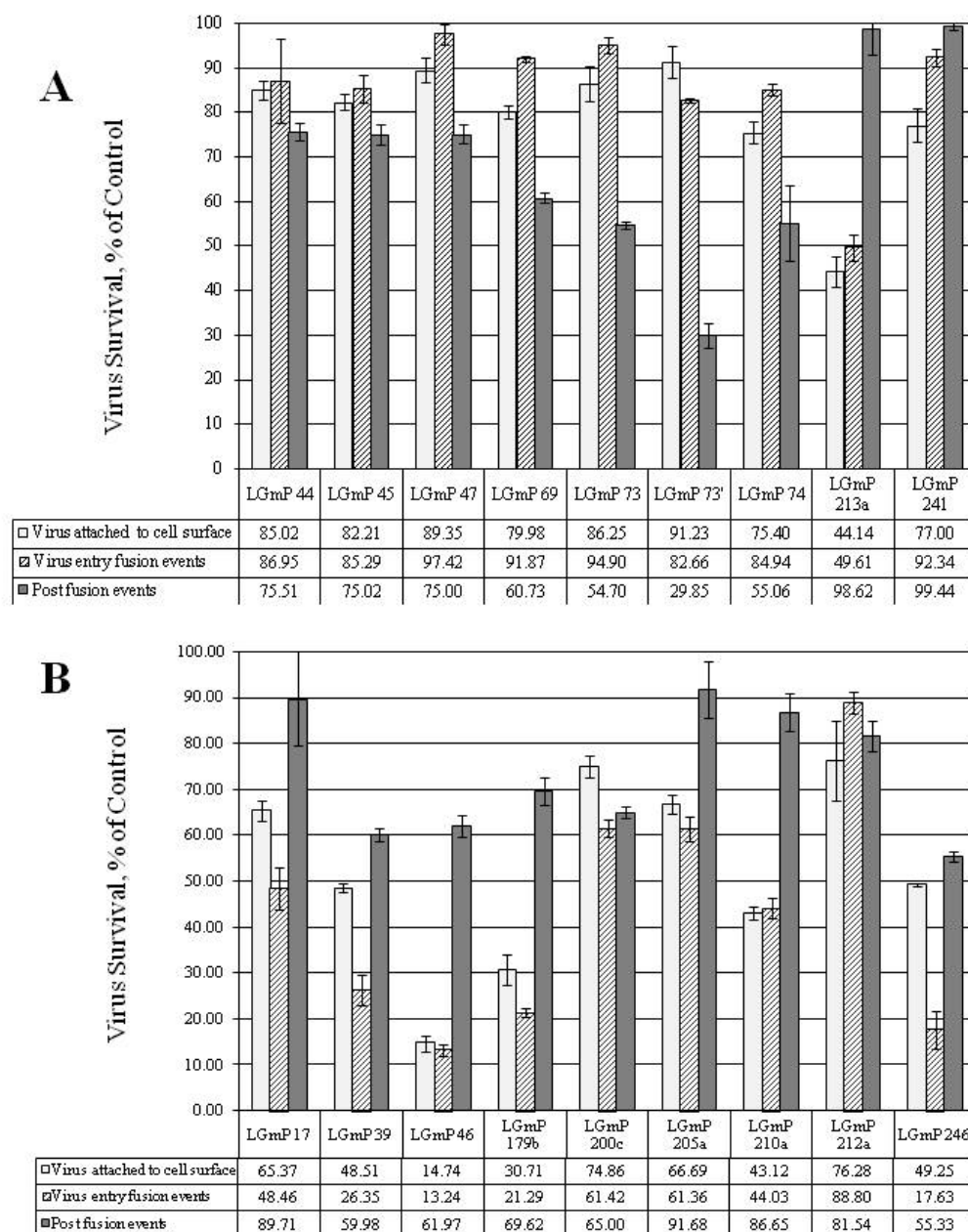
The results of antiviral effect on attachment, fusion and post entry were taken together to select most active compounds for further studies (Figure II-14, A-B). Most compounds had minimal effect on post entry events, inhibiting the virus survival at most by 45%. LGmP 73' inhibited post entry events by 70%, however had minimal effect on

attachment and fusion. Treatment of attached virus or virus undergoing fusion event with LGmP 39, 46, 179b, 210a, 213a, or 246 compounds, reduced virus survival by 50% or more. LGmP 39, 46, 179b and 246 reduced virus survival by 75% or more, when these compounds were added during onset of fusion.

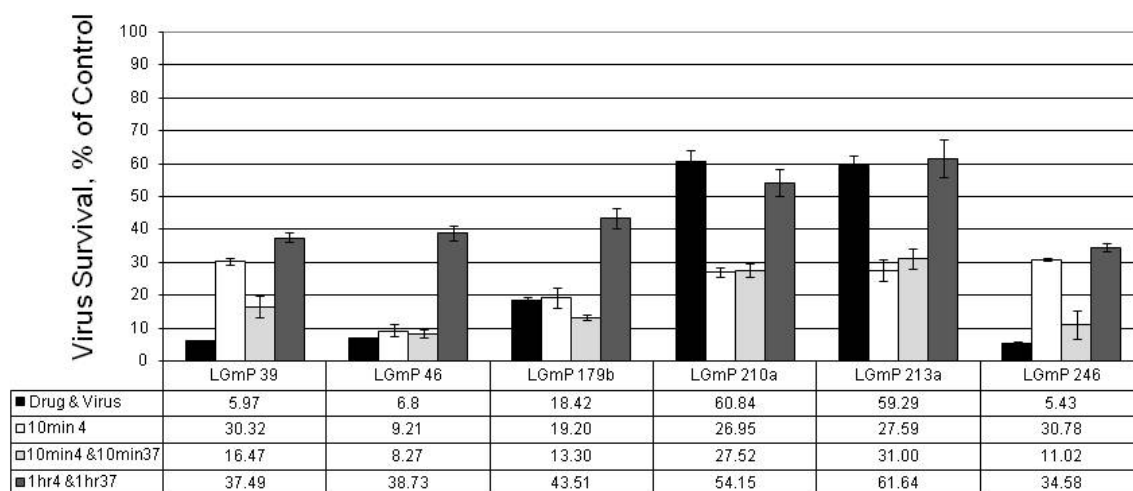
The chemical structures of the selected most active compounds were one non-metallated sulfonated porphyrins LGmP 46 (2,6-difluoro-tetrasulfonated phenyl porphyrin), two metallated sulfonated porphyrins, LGmP 39 (Cu(II)-tetrasulfonated phenyl porphyrin) and LGmP 246 (Iron (III)-tetrasulfonated phenyl porphyrin), as well as three platinum compounds LGmP 179b (Cis-Platinum,  $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$ ), LGmP 210a, and LGmP 213a. Selected compounds were tested at higher concentration of  $10\mu\text{g/ml}$ , for their effect on free virus, attached virus, virus undergoing fusion, and virus that has entered the cell (Figure II-15). Platinum compounds LGmP 210a and LGmP 213a showed only 40% reduction in the free virus survival and post entry. Compounds, LGmP 39 (Cu(II)-tetrasulfonated phenyl porphyrin) and LGmP 246 (Iron (III)-tetrasulfonated phenyl porphyrin), have near identical effect on every stage of virus infection tested. These two compounds have identical chemical structure with the exception of a different metal chelate.

### **Leading Compounds and Synthesis of Derivatives**

The leading compounds chosen for further investigations were LGmP 39, Cu (II) tetrasulfonated phenylporphyrin, and LGmP 246, Fe (III) tetrasulfonated phenylporphyrin (Figure II-16). Since platinum compounds had less effect on free virus, only porphyrin compounds were chosen for further investigation. For the antiviral compounds of interest and at specifically selected concentrations of the compounds, cell viability was determined



**Figure II-14. Modified antiviral screening assay based on the effects of antiviral compound at 5 µg/ml on various steps of virus life cycle during GFP-expressing EHV-1 RacL11 virus infection of RK13 cells.** To determine if the antivirals act at the level of binding, penetration, or post-entry, the antiviral compounds were added for 10 minutes followed by a wash: either during the 4°C attachment period, at the time that the cells were transferred to 37°C to allow fusion, or immediately after the 1 hr incubation at 37°C, respectively. No drug was added to the control wells, however all washes were performed as with other wells.



**Figure II-15. The effect of antiviral compounds at various stages of viral infection.**

RacL11Δgp2/EGFP infected RK 13 cells were exposed to the antivirals at 10 µg/ml at different times post infection: 10 min at 4°C to test the compounds effect on the attachment (white), 10 minutes when the cells were shifted to 37°C to test the compounds effect on the fusion (light gray), after 1 hour at 37°C for 12 hours to test the drugs' effect on post-entry events (dark grey), and for the entire time of infection, including a 20 minutes virus preincubation with compounds (black). Pictures were taken under fluorescence microscopy 12 hours post infection. Cells harvested, washed in PBS and analyzed via FACS. Graphed are the % infected cells when the drug was applied at different times of infection.

using metabolic rate assays, while cell proliferation was ascertained using cellular growth curves (results not shown). LGmP 46 was an excellent antiviral compound, however due to photoactivity was more cytotoxic, reaching 50% cytotoxic concentration (CC50) at 200 µg/ml. The CC50 of LGmP 37 (5,10,15,20-(4-chlorosulfonylphenyl)porphyrin), a parent compound of LGmP 39 and LGmP 246, was only 50 µg/ml, however it was chosen for further studies for comparative purposes. LGmP 37 is a non-metallated tetrasulfonated phenylporphyrin. The 50% cytotoxic concentration of Cu (II) tetrasulfonated phenylporphyrin (LGmP 39) was 450 µg/ml. The 50% cytotoxic concentration of Fe (III) tetrasulfonated phenylporphyrin (LGmP 246) was 600 µg/ml.

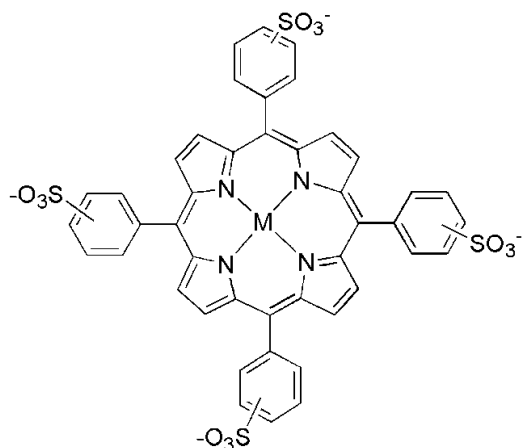


Multiple antiviral assays for selected compounds were conducted to using identical conditions such as cell number, confluency of cell monolayer, incubation temperature, and incubation time were followed throughout the performed experiments. These two selected compounds were used to synthesize new compounds with further modified chemical structure. Presence of the central metal was expected to retain low cytotoxicity properties of compounds due to the absence of photoactivity. Sulfonation has been shown to yield compounds that are more soluble in polar solvents, such as water, thus eliminating the use of DMSO(Phillips, 1995), and to increase antiviral activity against HIV (Dixon et al., 2005) and, as shown here, against EHV-1. Additional sulfonation was expected to yield more active antiviral properties of the compounds.

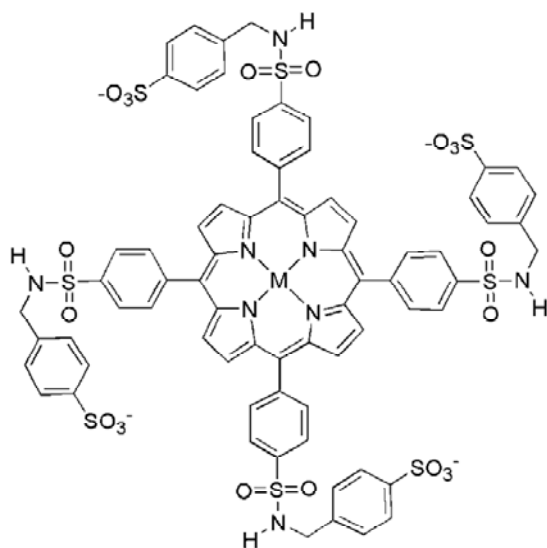
New porphyrin compounds synthesized were LGmP 265, 276, 280, and 282. LGmP 276 has a higher level of sulfonation but otherwise identical to LGmP 39 (Figure II-17). LGmP 275 is a photodynamic non-metallated equivalent of LGmP 276 (Figure II-18). LGmP 280 was derived from LGmP 276 by addition of naphthalene groups, while LGmP 282 was derived from LGmP 276 by addition of fluoride.

### **Evaluation of Antiviral Properties of Novel Compounds**

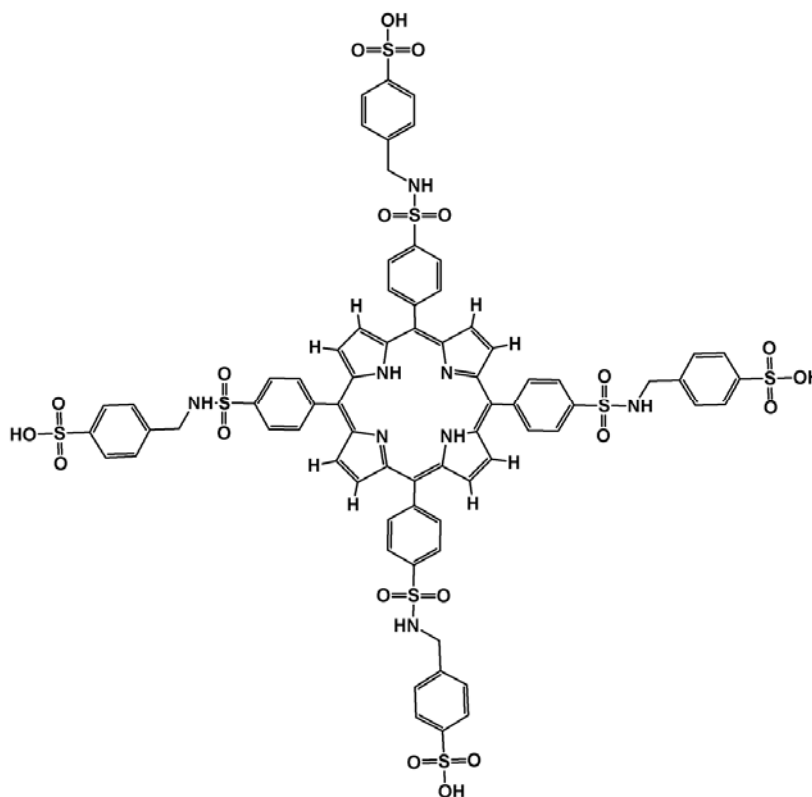
Newly synthesized compounds were tested for their ability to inhibit virus infection. The result of antiviral assays showed little deviation in the antiviral properties of the newly synthesized compounds from that of their parental set (Figure II-19). Decrease of virus survival attributed to the effect of LGmP 275 was actually the result of decrease in cell numbers, due to the cytotoxic effects of this photoactive compound. LGmP 37 was much less effective at corresponding concentrations of antiviral compounds and reached IC50 for free virus at about 15 µg/ml. Presence of central metal, Cu(II) or Fe(III), reduced



**Figure II-16. Chemical structure of leading compounds, metal chelates of 5,10,15,20-(4-chlorosulfonylphenyl)porphyrin.** M represents Cu(II) in LGmP 39 ( $C_{44}H_{28}CuN_4$ , MW 676.28 ) and Fe(III) in LGmP 246 ( $C_{44}H_{28}FeN_4$ ). Position of  $SO_3^-$  at the center of benzene ring indicates that the parent porphyrin compound, LGmP 37, was sulfonated and the derivatives are mixtures with variable positions of the sulfonates on the ring.



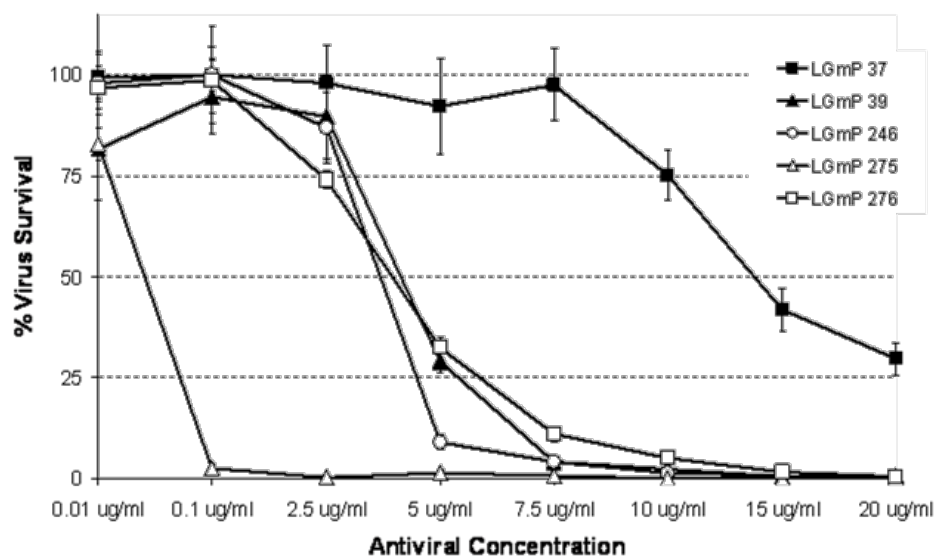
**Figure II-17. Chemical structure of newly synthesized compound, Cu(II) -5,10,15,20-tetrakis(4'-[p-sulfonylbenzyl]sulfonylamidophenyl)porphyrin.** Compound, named LGmP 276, was derived by additional chlorosulfonation of LGmP 39, where M represents Cu(II).



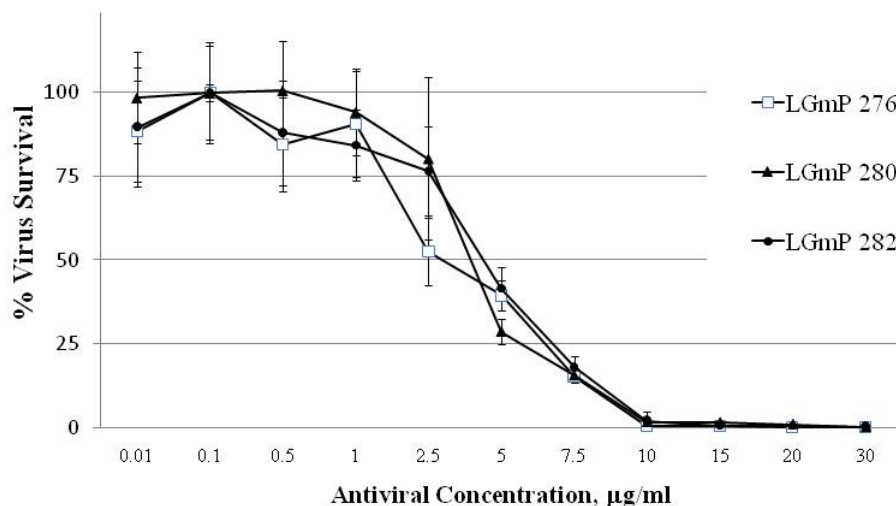
**Figure II-18. Chemical structure of newly synthesized compound, 5,10,15,20-tetrakis(4'-[p-sulfonylbenzyl]sulfonylamidophenyl)porphyrin.** Compound, named LGmP 275, was derived by additional chrolosulfonation of LGmP 37 and is photodynamic. Expanded view of hydrogens is shown.

IC50 to about 3  $\mu\text{g}/\text{ml}$  (LGmP 39 and LGmP 246). Higher levels of sulfonation and additional naphthalene or fluoride moieties, as for compounds LGmP 276, 280, and 282, did not improve IC50 (Figure II-20).

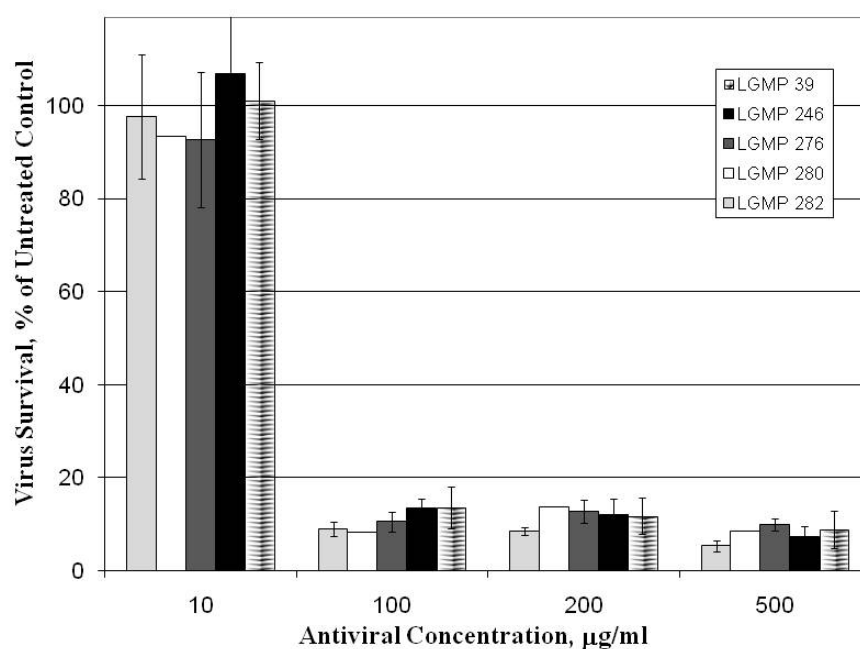
Further study of the newly synthesized compounds revealed that although at concentrations effective to inactivate free virus in solution, virus that has attached to the cell surfaces is not susceptible to the antivirals unless much higher concentrations were used (Figure II-21). At concentrations above 100  $\mu\text{g}/\text{ml}$ , antivirals started to exhibit increasing cytotoxic effects. No significant difference in ability to inactivate attached virus was noted among the compounds studied.



**Figure II-19. Inhibition of EHV-1 RacL11/EGFP virus infection of RK13 cells at various concentrations of antiviral compounds.** The effect of antiviral treatment on free virus was tested at a range of concentration for parental LGmP 39 compound, 5,10,15,20-(4-chlorosulfonylphenyl)porphyrin), its Cu(II), LGmP 39, and Fe(III), LGmP 246, metal chelates, as well as on the higher sulfonation LGMP 37 derivative, LGmP 275, and its Cu(II) chelate, LGmP 276.



**Figure II-20. Inhibition of EHV-1 RacL11/EGFP virus infection of RK13 cells at various concentrations of new antiviral compounds.** LGMP 276 (Cu(II)-5,10,15,20-tetrakis(4'-[p-sulfonylbenzyl]sulfonylamidophenyl)porphyrin) and its naphthalene (LGmP 280) and fluoride (LGmP 282) derivatives.



**Figure II-21. Inhibition of virus attached to cellular membranes at high concentrations of antiviral compounds.** Following 1 hour at 4°C, EHV-1 RacL11/EGFP virus infection of RK13 cells was treated for 10 min at 4°C with indicated concentrations of new antiviral compounds, the cells were then washed and infection allowed to proceed.

## **DISCUSSION**

There is a pressing need for effective antiviral compounds to treat disseminated as well as neurological forms of EHV-1 infections. Porphyrins have been shown to possess strong antiviral properties against HIV and Vaccinia. Preliminary results in our laboratory indicated antiviral activity of porphyrins against EHV-1 and Coronaviruses.

Out of the first set of tested compounds, most showed 99% inhibition of free virus even at concentrations as low as 5  $\mu\text{g/ml}$ . However, most of these compound exhibited high level of cytotoxicity, which was attributed to their photodynamic properties, seen in cell culture as high level of red fluorescence. Subsequently, all assays were performed in a dark room environment. Experimental conditions such as confluency of RK13 cell monolayer used for infections, number of infected cells, incubation temperatures and times were maintained consistent.

For all compounds selected following the screening assays, exposure of the free virus to the antiviral compounds prior to attachment, resulted in zero virus survival at 50  $\mu\text{g/ml}$  or 25  $\mu\text{g/ml}$  concentration of compounds in culture medium. At 5  $\mu\text{g/ml}$  concentration of antiviral compounds in culture medium overlaying the cells at the onset of infection, 20% of the virus survived.

After repeating the experiment, we concluded that most of the antiviral properties of photodynamic compounds seen initially were most likely due to photoinactivation of EHV-1 (free radicals and singlet oxygen). Photoactivity of porphyrins is utilized in palliative photodynamic therapy (PDT) of cancer. For antiviral activity, the important consideration is that the agent is not phototoxic to the cells. In our investigations we were interested in the potential of porphyrins for light-independent inhibition of virus infection

and not a photodynamic mechanism. New compounds were synthesized with metals incorporated into the *meso* position in an attempt to abolish photodynamic properties.

To further study the effect of porphyrins on the cells, we were interested in determining the cellular compartments where porphyrins would localize. We were unable to study localization of porphyrins inside the cellular compartments using confocal microscopy, short of using analytical chemistry analysis of nucleus and cytoplasmic fractions to identify localization. Photoactive porphyrins and metallated non-fluorescent porphyrins have drastically different antiviral and cytotoxic properties, so we cannot assume that their cellular uptake and localization would be identical. Metallated non-fluorescent porphyrins may or may not localize to the cytoplasm as do the photoactive porphyrins.

Interesting to note that without the virus present, the fluorescent compounds localized to the cytoplasm and within vacuoles and did not enter the nucleus, while in the presence of infection porphyrins freely enter the nucleus and occupy the cytoplasm. Thus, potentially, virus infection modifies the nuclear envelope rendering it permeable to the porphyrin compounds.

Irrespective of localization of the fluorescent porphyrins, these compounds were highly cytotoxic with or without the virus infection, and cytoplasmic distribution could be due to initiation of apoptosis by the cell. Metallation of porphyrins abolished the fluorescence, making them significantly less toxic to the cells. The photodynamic compounds that contained chloro group remained cytotoxic, even in the absence of light activation. An initial screening of almost 100 porphyrin and platinum compounds as well as a detailed screening of 18 different compounds revealed that Cu (II) tetrasulfonated

phenylporphyrin and Fe (III) tetrasulfonated phenylporphyrin possessed strong virucidal and antiviral activities against EHV-1, inhibiting 100% of EHV-1 infectivity at a concentration of 10 µg/ml and 15 µg/ml, respectively. The photoactive compound H2TPPS4, a precursor/parental compound to the two metalloporphyrins, was used as control in subsequent experiments. Antiviral compounds showed the most dramatic inhibitory effect on free virus. Virus undergoing fusion was also significantly affected. Much less inhibition of virus infection was seen with antiviral treatment of virus attached to the cellular membranes or virus that has already entered into the cytoplasm.

The excellent antiviral activity and low toxicity of the selected porphyrin compounds led to their selection for further evaluation against additional laboratory and clinical virus strains, which were also inhibited effectively. The selected two compounds were used to design new compounds of modified chemical structure. Additional sulfonation of compounds did not lead to improvement in their antiviral properties against free virus nor virus attached to cell surfaces. Addition of fluoride or naphthalene groups, also did not improve antiviral properties. A more detailed analysis of chemical structures in correlation to their antiviral or cytotoxic properties could not be conducted due to proprietary limitations. Such analysis was left up to the researchers that developed and patented the compounds and is not presented here. Porphyrin compounds were shown to specifically inhibit free virus particles as well as membrane fusion phenomena required for virus entry and virus spread, and the antiviral activity was enhanced by modification of the chemical structure of the porphyrin compounds via primary sulfonation and metallation. However, neither secondary sulfonation, nor the addition of naphthalene or fluoride moieties, improved the effectiveness of compounds to inhibit EHV-1.



# **CHAPTER III. ANTIVIRAL ACTIVITY OF PORPHYRIN COMPOUNDS AGAINST FUSION EVENTS OF EHV-1 INFECTIVITY AND SPREAD**

## **INTRODUCTION**

### **Motivation to Study EHV-1**

*Equine herpesvirus 1* (EHV-1), a member of *Alphaherpesvirinae* family, genus *Varicellovirus*, is ubiquitous to the equine population worldwide, with seroprevalence reaching up to 80% (Carvalho et al., 2000; Taouji et al., 2002). With 9.2 million horses in the United States and horse industry contribution of \$39 billion in direct economic impact (Deloitte-Consulting, 2005), EHV-1 is responsible for economically detrimental diseases: epizootic respiratory diseases in race horses and clinically serious diseases such as abortion among mares at the late stage of gestation, perinatal mortality, as well as occasional outbreaks of disseminated necrotizing myeloencephalitis. Of particular importance to the management of EHV-1 infections, is the fact that these viruses establish latency, regardless of natural or vaccine-induced immunity, and can recur in times of immune suppression. Vaccination against EHV-1 reduces the severity of respiratory illness and occurrence of abortion, but it does not prevent spread to newborn foals, the latent infection or the neurological disease caused by the virus (Foote et al., 2004).

### **Current EHV-1 Treatment**

EHV-1 infections are usually dealt with using management practices that limit spread of the disease, providing supportive symptomatic relief to infected horses, treating potential secondary bacterial infections with antibiotics. Because vasculitis, hemorrhage, and edema are prominent early lesions of EHV-1 myeloencephalopathy (EHM) and may

have an immune basis, controversial treatment with corticosteroids is often recommended (Friday et al., 2000).

### **EHV-1 Antivirals**

Multiple nucleoside analogue anti-viral agents show activity against EHV-1 *in vitro* and in laboratory animals (de la Fuente et al., 1992; Rollinson and White, 1983; Smith et al., 1983). Treatment of neurologic outbreaks with acyclovir (9-[2-hydroxyethoxymethyl]guanine) (Schaeffer, 1982), a viral DNA replication inhibitor active against several human herpesviruses, has been attempted in horses with a few reports of reduced mortality in a study group of up to seven horses (Friday et al., 2000; Murray et al., 1998). There is a lack of statistically significant data on increase of survival. Patent restrictions have only recently been lifted from acyclovir and its use in treatment of EHV-1 is on the rise. Although acyclovir is a remarkably low toxicity drug, in an already compromised equine patient with EHV-1 infection, nephrotoxicity, myelosuppression, gastrointestinal disturbances and fetotoxicity can occur. In addition to toxicity issues, resistance and cross-resistance is on the rise in human herpes patients. Acyclovir resistant strains of HSV-1 are becoming more common and account for growing fraction of new infections worldwide. Data describing the pharmacokinetics, bioavailability, and safety of acyclovir in horses, is limited. Dosing protocols to treat EHV-1 infections are currently based on extrapolation from well established dosage regimens used for treatment of human infections with HSV-1 and VZV.

### **Rationale for Fusion Inhibitors**

There is a need for EHV-1 antivirals that are less toxic than acyclovir and target aspects of the herpes replicative cycle other than DNA replication. Therefore, development

of new class of antiviral agents that prevent entry of herpes into cells is a promising prospect for therapy. Characterizing the viral and host determinants that impact entry inhibition sensitivity will provide information that can be used to guide the clinical application.

## **Hypothesis**

Herpes viruses infect cells by fusion of the viral envelope with the cellular membranes and can spread from cell to cell via fusion of adjacent cells, mediated by viral glycoproteins expressed on infected cell surfaces. While the majority of antiviral compounds in use today interfere with function of viral genome replication enzyme, several compounds have been described to interfere with virus infection at the stage of virus penetration into the cell. The desirable antiviral compounds for *in vivo* use will inhibit viral infectivity through interference with membrane fusion events critical to the virus lifecycle.

The central hypothesis of these investigations is that selected tetraporphyrin-derived compounds can specifically inhibit membrane fusion phenomena required for virus entry and virus spread through specific interactions with one or more viral glycoproteins required for membrane fusion.

## **Objectives**

The aim of the previous part of the study was to conduct screening tests to determine preliminary virucidal and cytotoxic effects of the commercially available and newly synthesized porphyrin compounds. A select group of compounds with virucidal properties was analyzed by definitive tests in order to measure the observed effects and identify potential antiviral substances. Further experiments were conducted to increase the

information base relative to the mechanism of antiviral and cytotoxic effects, specifically, to identify individual stages of virus lifecycle (attachment, virus to cell fusion, infectious virus production, and cell-to-cell spread), which are affected by selected antiviral compounds and to determine what specific viral and cellular components are affected by the compounds.

Antiviral compounds were tested for their ability to interfere with EHV-1 infection of rabbit kidney and equine cell culture during the entry and post entry events of the viral life cycle in order to determine if antivirals act at the level of binding, penetration, or post-entry. Specifically, selected porphyrin compounds were tested to determine whether they selectively inhibit glycoproteins-mediated membrane fusion events of virus entry and virus spread.

### **Porphyryns: Overview, Antiviral Properties and Synthesis**

A number of attributes of porphyrins such as stability over the range of pH and ionic strengths, favorable pharmacokinetic properties, low toxicity and cost, render them attractive pharmaceutical candidates. Previous studies indicated that some porphyrins inhibit the interaction between the human immunodeficiency virus (HIV-1, HIV-2) envelope protein, gp120 and its receptors, by directly interacting with gp120 (Song et al., 1997; Vzorov et al., 2002; Vzorov et al., 2003).

Synthetic porphyrins, namely the sulfonated derivatives of the tetraphenylporphyrin, have also been shown to be active against HSV-1, HSV-2, and pox virus (Vzorov et al., 2002; Vzorov et al., 2003). Sulfonamide (-SO<sub>2</sub>NH-) group occurs in numerous biologically active compounds, including antimicrobial drugs, saluretics, carbonic anhydrase inhibitors, insulin-releasing sulfonamides, antithyroid agents and a

number of other agents with biological activities (Chen-Collins et al., 2003; Remko and von der Lieth, 2004).

Iron containing porphyrins called hemes (Figure III-1, A) are the best known natural porphyrins found in hemoglobins, myoglobins, peroxidase, catalase, bilirubin, and cytochromes. Micromolar concentrations of synthetic metalloporphyrins have been shown to have *in vitro* activity against human immunodeficiency (HIV) and vaccinia viruses (Neurath et al., 1992). Both, natural and synthetic porphyrins exist without any central metal, in a “free base” form (Figure III-1, D), or as metalloporphyrins with various metal ions in the center (Figure III-1, B, C). While “free base” porphyrin derivatives are in daily clinical use as photodynamic agents for cancer, it is critical for virucidal porphyrins to be non-phototoxic and thus, non-cytotoxic. Introduction of a central metal compound abolishes photodynamic properties, potentially reducing cytotoxicity. Virucidal as well as antiviral activities can be attributed to either class of compounds.

A screen of different porphyrins and other compounds presented in the previous chapter revealed that sulfonated metalloporphyrins, Cu (II) tetrasulfonated phenylporphyrin and Fe (III) tetrasulfonated phenylporphyrin, possessed strong virucidal and antiviral activities against as well as low cytotoxicity.

### **Fusion Process**

Herpes simplex virus entry into cells requires four glycoproteins, gB, gD, gH, and gL. Glycoprotein B forms a trimer (Heldwein et al., 2006) and gH establishes a noncovalent complex with gL (Cairns et al., 2005; Hutchinson et al., 1992; Peng et al., 1998). Virus-induced membrane fusion is subdivided into three sequential phases. During Phase I, two membranes are brought into close proximity through viral glycoprotein

binding of cellular receptors. In alphaherpesviruses, such as EHV-1, gC interacts with heparan sulfate glycosaminoglycans (HSGAG) (Osterrieder, 1999), gB interacts with HSGAG and paired immunoglobulin-like type 2 receptor (PILR) alpha (Roller et al., 2008), and gD interacts with HveA and other receptors on cell surface (Spear et al., 2000). Glycoprotein D is the only interaction required for Phase I. Binding of gD to one of its receptors triggers conformational change that exposes the normally hidden receptor binding residues of gD. This results in transient interaction between gD and gH/gL, where gH/gL carries out Phase II hemifusion, followed by stable complex between gD and gB, where gB completes Phase III full fusion. Specifically, Phase II involves the initiation of lipid mixing between the two apposed membranes and is completed when the outer membrane leaflets are mixed to form an intermediate called hemifusion. Phase III begins when the inner membrane leaflets are mixed and continues the pore formation and expansion until completion of the fusion process (Atanasiu et al., 2007; Subramanian and Geraghty, 2007). Interestingly, Varicella Zoster Virus (VZV) fusion formation occurs upon expression of the gH/gL complex alone. In contrast, Pseudorabies Virus (PRV) requires expression of gH, gL and gB, while the Herpes Simplex Virus (HSV) types 1 and 2 require the quartet of gH, gL, gB and gD. EHV-1 core fusion complex is not defined, but suspected to parallel the HSV-1 model and include gH, gL, gB and gD.

## **MATERIALS AND METHODS**

### **Cells**

Rabbit kidney epithelial cells, RK13, were obtained from the American Type Culture Collection (ATCC CCL-37, Rockville, MD). Cells were maintained in Dulbecco modified Eagle's minimum essential medium (DMEM) supplemented with 15 mM Hepes,

and 7% fetal bovine serum (FBS) and incubated at 37°C, 5% CO<sub>2</sub>. Cell lines ED (Equine Dermal, ATCC CCL-57), EFTr (Equine Fetal Trachea Cells, primary cell line, field strain provided by Dr. Corstvet, LSU), Vero (African Green Monkey Kidney Cells, ATCC CCL-81), and COS7 (African Green Monkey Kidney Cells, ATCC CRL-1651) were propagated in DMEM with 15 mM Hepes and 7% FBS.

### **Viruses and Determination of Virus Titer**

EHV-1 RacL11- $\Delta$ gp2-GFP, NY-1, NY-2 and VA virus strains were kindly provided by Dr. Osterrieder (Cornell University School of Veterinary Medicine, Ithaca, NY). RacL11 encodes the F plasmid and an EGFP expression cassette under the human cytomegalovirus immediate early promoter (HCMV-IE) that was substituted for EHV-1 non-essential gene 71 encoding glycoprotein gp2. Deletion of the gp2 glycoprotein does not appreciably affect virus entry and virus replication, while a slight reduction (5%) in plaque size is observed (Neubauer et al., 2002). The parental wild-type HSV-1 strain used in this study, HSV-1 (KOS), was originally obtained from Dr. Priscilla A. Schaffer (Harvard Medical School, Boston, MS). HSV-1 KOS-EGFP was provided by Dr. Prashant Desai (The John Hopkins University, Baltimore, MD). Virus stocks of EHV-1 and HSV-1 were grown on RK-13 and Vero cells respectively. To determine the number of infectious virus particles, the virus stocks were subjected to 3 cycles of freezing and thawing to release virus from the cells or were sonicated. To infect the RK13 cells in triplicate wells, the virus suspension was diluted tenfold across wells of the 24-well plate, starting at 20  $\mu$ L of virus stock and 180  $\mu$ L of media. After 1 h of adsorption at 37°C in DMEM supplemented with 25 mM Hepes, methylcellulose overlay medium (DMEM containing 1.5% methylcellulose and 2% FBS) was added to the infected cell monolayers. The plates

were incubated at 37°C for several days and then fixed with methanol. The cell monolayers were stained with 0.1% crystal violet, plaques were counted for each dilution, and the number of virus particles in the original virus stock estimated.

### **Porphyrin Antiviral Compounds**

According to the chemical nomenclature of porphyrin and porphyrin derivatives, compounds are designated as follows: *meso*-tetraphenylporphyrine (TPP); 5,10,15,20-tetrakisphenylporphyrin (H2TPP); and 5,10,15,20-(4-chlorosulfonylphenyl)porphyrin (H2TPPS4). An “S” at the end of the abbreviation indicates that the parent porphyrin was sulfonated and the derivatives are mixtures with variable numbers and/or positions of the sulfonates on the ring. Cu(II)-5,10,15,20-tetrakis(4-[p-sulfobenzyl]sulfoamidonylphenyl)porphyrin (Cu(II)TPPS4) is a sulfonated tetraphenylporphyrin with a copper moiety/chelate at the *meso* position, while Fe(III)-5,10,15,20-tetrakis(4-[p-sulfobenzyl]sulfoamidonylphenyl) porphyrin (Fe(III)TPPS4) is a sulfonated tetraphenylporphyrin with an iron moiety/chelate, and for simplicity, will be referred to in this paper as metalloporphyrins (Liébecq, 1992). Compounds were either commercially available (Kadish et al., 2003) or synthesized from compounds obtained from Sigma-Aldrich Corp. (St.Louis, MO) by the collaborating laboratory of Dr. Luidgi Marzilli (Chemistry Department, Louisiana State University, Baton Rouge, LA). Distilled water was used as solvent for each test chemical. Preparation of solutions and all experiments were done under red light to prevent chemicals degradation or phototoxicity upon exposure to light. All compounds were stored at 3-5°C in containers not permissive to light. ChemDraw Pro 11.0 (CambridgeSoft Corporation, Cambridge, MA) program was used to present chemical structures of the compounds.



## **Determination of 50% Inhibitory Concentration of Antiviral Compounds**

EHV-1 strain RacL11 at  $2 \times 10^5$  PFU was incubated with indicated concentrations from 0 to 20  $\mu\text{g/ml}$  of porphyrin in media for 20 min at  $37^\circ\text{C}$ , then was used to infect RK13 cells for 1 hour at  $4^\circ\text{C}$ , following which the media was replaced with fresh containing the same concentrations of antiviral compounds, and the infection was allowed to proceed at  $37^\circ\text{C}$  for 2 days. Virus titers were determined by endpoint titration of virus stocks on RK13 cells (Cassiani-Ingoni et al., 2005). The effect of porphyrins at various concentrations was determined as the ratio of virus surviving the treatment with metalloporphyrins to the untreated control.

## **Neutral Red Uptake Cytotoxicity Assay**

The procedure used to determine cell viability via spectrophotometric assay was essentially as described by Ellen Berenfreund and James Borrero of Rockefeller University (Borenfreund and Puerner, 1985). Tissue culture grade Neutral Red (NR) dye was purchased from SIGMA (Cat. No. 289) in a liquid form at 3.3 mg/ml. Fresh stock solution and Neutral Red Medium was prepared within 30 minutes of each experiment. Cells were seeded in 96-well tissue culture plates. Antiviral compounds were serially diluted to eight different concentrations in the range from 2,500 mg/ml to 1 mg/ml, and added to overlay the 50% confluent cell monolayers. The treated cells were incubated under normal growth condition for 48 hours and then, stained with neutral red containing medium to access cell viability. The cells were subsequently washed, the dye was extracted in each well, and the absorbance was read using a spectrophotometer. The 50% cytotoxic concentration (CC50) was calculated.

### **Antiviral Effect on Kinetics of Infectious Virus Production**

Analysis of one-step growth kinetics of total infectious virus was as described previously (Mettenleiter, 1989 ). Briefly, approximately  $8 \times 10^5$  cells of the RK13 cell monolayers were infected with RacL11- $\Delta$ g2-GFP at MOI of 5 or MOI of 0.1. Virus adsorbed to cell surfaces at 4°C for 1 h. Thereafter, prewarmed media was added, and virus was allowed to penetrate the cells for 2 h at 37°C. Any remaining extracellular virus was inactivated by low-pH treatment (0.1 M glycine, pH 3.0). Cells were rinsed and overlaid with DMEM supplemented with 2% FBS with or without Cu(II) TPPS4 or Fe(III)TPPS4 antivirals. Cells and supernatants were harvested immediately thereafter (4 h) or after 6-, 8-, 10-, 12-, 20- or 32-hours post infection and frozen. The samples were then subjected to 3 cycles of freezing and thawing to release virus from the cells and virus titers were determined by endpoint titration of virus stocks on RK13 cells.

### **Antiviral Effect on Kinetics of Virus Entry**

All antiviral compounds were tested for their ability to interfere with EHV-1 infection during virus entry and infectious virus production. The RacL11- $\Delta$ g2-GFP virus was preincubated with or without each compound for 20 minutes at room temperature, and then, the treated virus stock was used to infect cells for 1 hour at 4°C to allow virus binding. Subsequently, infected cells were incubated for 2 hours at 37°C to allow virus penetration, washed with PBS, and overlaid with fresh DMEM with 25 mM Hepes. Alternatively, infected cells were exposed for 10 minutes to the antiviral compounds added at various times post-infection, as follows: 1) immediately following the 1 hr attachment at 4°C; 2) at the time that the cells were transferred from 4°C to 37°C; and 3) after the 1 hr incubation at 37°C to determine the relative antiviral efficacy of each compound at the

binding, penetration, or post-entry lifecycle steps, respectively. The relative inhibition of viral infection was determined at 12 hour post infection, based on the percent reduction in the number of cells emitting fluorescence and thus infected, as determined either via FACS or direct cell counts. Alternatively, cells were inspected under fluorescent microscope at 48 hours post infection.

### **Evaluation of Antiviral Effect on Virus Spread**

The antiviral effect on cell to cell spread and virus-induced cell fusion was determined via comparison of plaque size and the extend of multinucleation of EHV-1 RAcL11 infected RK13 (rabbit kidney) cell monolayers treated with Cu(II)TPPS4 or Fe (III) TPPS4 or neither (Muggeridge, 2000; Turner et al., 1998). Virus was adsorbed to cell monolayers for 1 hour at 4°C. Thereafter, prewarmed media was added, and virus was allowed to penetrate the cells for 2 h at 37°C. Cells were then rinsed and overlaid with methylcellulose overlay medium (DMEM containing 1.5% methylcellulose and 2% FBS) containing the indicated amount of antiviral. At 72 hpi, the number of infected cells per viral plaque as well as the number of nuclei per polykaryocytes and frequency of their occurrence within the plaque were counted microscopically using Leica software in two independent assays. Polykaryocytes were defined as cells containing more than one nucleus. Average values and standard deviation were then calculated.

### **Construction of Plasmids Expressing EHV-1 Glycoproteins**

The glycoprotein genes amplified from the EHV-1 genomes were cloned in the pcDNA<sup>™</sup>3.1 vector (Cat. No. V795-20, Invitrogen Corporation, Carlsbad, CA), under the control of the Cytomegalovirus (CMV) early promoter, as detailed elsewhere. This vector is suitable for constitutive expression in mammalian cells.

### **Assay for Virus-Free Cell-to-Cell Fusion**

Rabbit kidney (RK13) cells were seeded at approximately  $8 \times 10^5$  cells per well in six-well culture dishes. Transfections were performed with SuperFect Transfection Reagent (Cat. No. 301305, Qiagen Inc., Valencia, CA) with 2  $\mu\text{g}$  of each glycoprotein-encoding plasmid. The amount of DNA was equalized by adding appropriate amounts of pcDNA<sup>™</sup>3.1 /V5-His-TOPO<sup>®</sup> vector (Cat. No. K4800-40, Invitrogen Corporation, Carlsbad, CA). DNA was mixed in 150  $\mu\text{l}$  of MEM without serum, and 10  $\mu\text{l}$  of SuperFect Transfection Reagent was added and mixed. After 10 min of incubation at room temperature, 830  $\mu\text{l}$  of medium containing 10% fetal calf serum was added, and the transfection mixture was dispersed in duplicate wells onto the cell monolayer. Cells were incubated at 37°C for 4 hours when transfection mixture was replaced by medium with 10% fetal calf serum. Cells were further incubated at 37°C for 9 to 24 hours as indicated, then fixed with 80% ethanol, incubated with a monoclonal antibody (MAb) directed against V5, and scored for syncytium formation. Nuclei in 100 polykaryocytes per assay were counted microscopically using Leica software in two independent assays. Polykaryocytes were defined as cells containing more than one nucleus. Average values and standard deviation were then calculated (Muggeridge, 2000),(Turner et al., 1998).

### **Polyethylene Glycol Reversal Experiments**

EHV-1 RAcL11 at an MOI of 1 was diluted in PBS with or without 100  $\mu\text{g}/\text{ml}$  of heparin sodium salt or 5  $\mu\text{g}/\text{ml}$  of porphyrin. RK13, CHO-K1, EFTr cells were inoculated with virus suspension. Virus was adsorbed to cell monolayers for 1 h at 4°C and shifted to 37°C for an additional hour. Viral inoculum was then removed and cells washed with PBS, citrate buffer (135 mM NaCl, 10 mM KCl, 40 mM citric acid [pH 3.0]), or

overlaid for 30-60 s with PEG50 (50% PEG 6000-8000 in MEM or PBS wt/vol) wt/wt. PEG50 was removed by consecutive addition of PBS to make a 1:2 and 1:4 dilution of PEG50 in a well. Cells not exposed to PEG were treated similarly in PBS without PEG. Wells were carefully washed three times in PBS or DMEM supplemented with 5% fetal calf serum, overlaid with methylcellulose medium or DMEM containing 8% FBS and incubated further for 2 days at 37°C. The cell monolayers were stained with 0.1% crystal violet and plaques were counted. To determine virus yields after PEG treatment, cells were incubated in DMEM and harvested at 10 h postinfection.

### **Statistics**

The results of the experiments were graphed and statistically analyzed using SigmaPlot (SYSTAT Software Inc., San Jose, CA) and other applicable software.

## **RESULTS**

### **Chemical Structure**

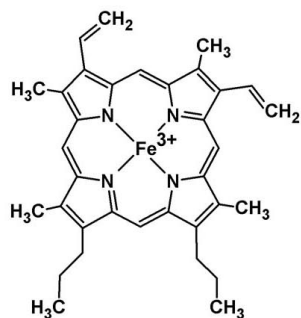
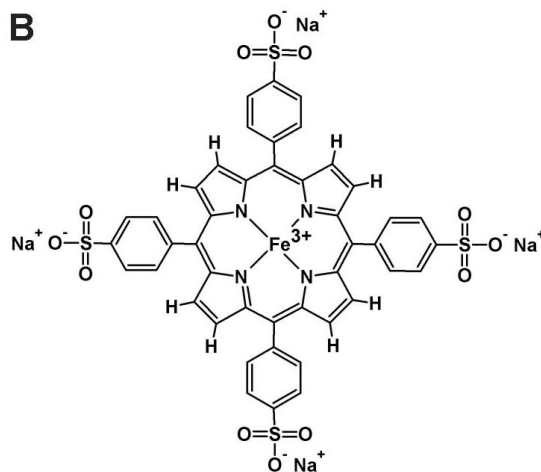
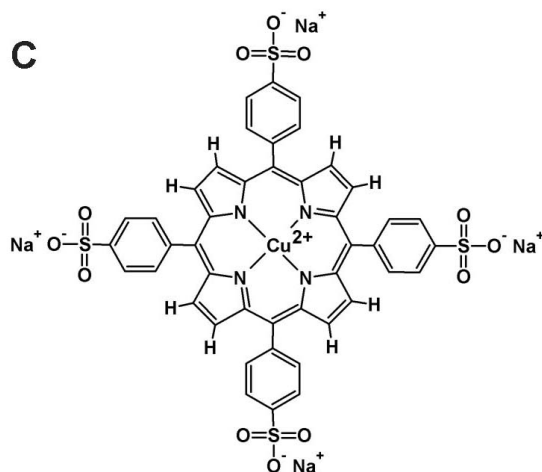
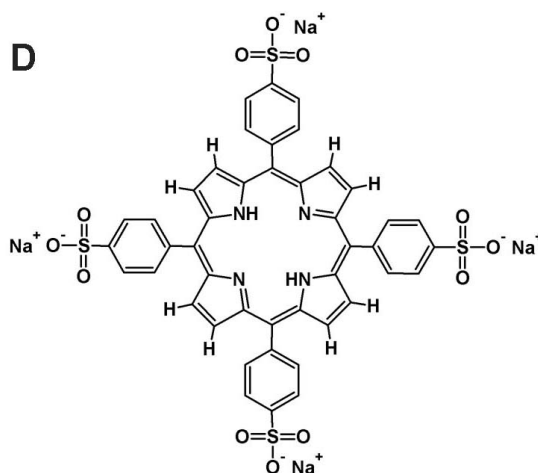
Porphyrins are readily synthesized from pyrrole and aromatic aldehyde derivatives and can be modified into sulfonamide and metal complex derivatives by using the process of chlorosulfonation (Gonsalves et al., 1996). The basic compound is TPP, meso-tetraphenylporphyrine (Figure III-1, D). A 5,10,15,20 (4'-sulfonphenyl) porphyrin or H2TPPS4 was obtained by chlorosulfonation of 5,10,15,20-tetrakisphenylporphyrin (H2TPP) to 5,10,15,20-(4-chlorosulfonylphenyl) porphyrin, which was then hydrolyzed to its water soluble by-product H2TPPS4 (L. Marzilli, LSU, Baton Rouge, LA, May 2004). In most cases, the product of the synthesis is a mixture of compounds, tetraphenyl porphyrin derivatives, containing variable numbers of sulfonates and/or positions of the sulfonates on the ring. Compounds are negatively charged and water soluble. EHV-1 inhibitors, Cu(II)

tetrasulfonated phenylporphyrin and Fe(III) tetrasulfonated phenylporphyrin were synthesized via metallation of water-soluble porphyrin (Figure III-2), H<sub>2</sub>TPPS<sub>4</sub> with Cu(II) or Fe(II) oxide (Figure III-1, B and C), dichloride and tetrachloride. Fe(III) or Cu(II) chelates were inserted into H<sub>2</sub>TPPS<sub>4</sub> by metallation process. H<sub>2</sub>TPPS<sub>4</sub> heated with Fe(II)Cl<sub>2</sub>·4H<sub>2</sub>O in the presence of nitrogen for 30 min, then stirred in air, cooled and evaporated. Then dissolved in water and excess of iron removed (L. Marzilli, LSU, Baton Rouge, LA, May 2004). The molecular mass of Cu(II) tetrasulfonated phenylporphyrin is 1134 g/mol and that of Fe(III) tetrasulfonated phenylporphyrin is 1126 g/mol, considering the chemical structures of C<sub>48</sub>H<sub>24</sub>O<sub>12</sub> N<sub>4</sub>Na<sub>4</sub>S<sub>4</sub>Cu<sub>1</sub> and C<sub>48</sub>H<sub>24</sub>O<sub>12</sub> N<sub>4</sub>Na<sub>4</sub>S<sub>4</sub>Fe<sub>1</sub> formula respectively.

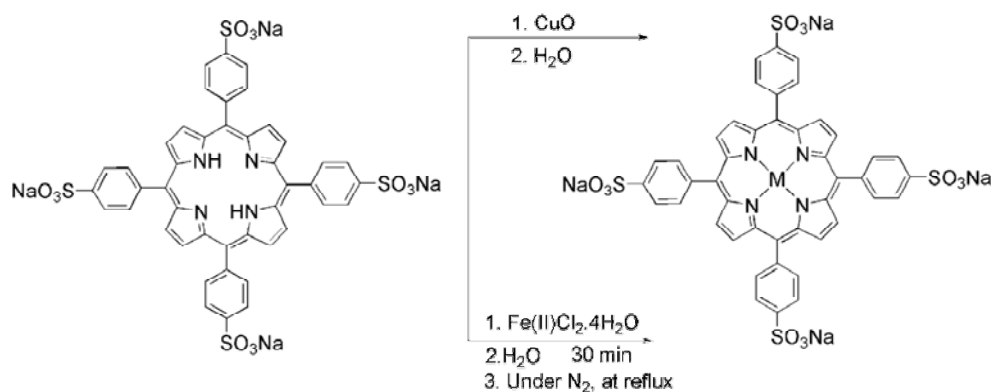
### **Concentration of Antiviral Required to Inhibit Virus Infection**

Rabbit kidney (RK-13) cells were the cells of choice to conduct the experiments, since these cells are primarily used to study the equine herpesviruses and are the recommended cell type in laboratory diagnosis of field infections by virus neutralization assays. The virus was subjected to the porphyrins in solution prior to infection and throughout the infectious life-cycle, at 0 to 20 µg/ml concentration of porphyrin in media (Figure III-3). The effect was cumulative including that contribution from antiviral effect on free virus as well as attachment, fusion and post entry events of the virus life cycle.

Once virus had attached to the cell surfaces, the cells were thoroughly rinsed to remove virus that may have remained unattached. The media was then replaced with fresh media containing the respective concentrations of the antiviral compounds and the infection was allowed to proceed. The effect of porphyrins at various concentrations was determined as the ratio of the virus surviving the treatment with porphyrins to the untreated

**A****B****C****D**

**Figure III-1. Chemical structures of common porphyrin, heme[Fe(II) protoporphyrin-IX complex] (A), and tetrasulfonated phenyl metaloporphyrins used in this study, Fe (III)TPPS4 (B), Cu(II)TPPS4 (C), and H2TPPS4 (D).** Porphyrins have aromatic groups at the meso position. H2TPPS4 has hydrogen substituents on the pyrrole rings, while the central nitrogens have NH. Cu(II) tetrasulfonated phenylporphyrin (Cu(II)TPPS4) and Fe(III) tetrasulfonated phenylporphyrin (Fe(III)TPPS4) were synthesized via metallation of water-soluble porphyrin, H2TPPS4, with Cu(II) or Fe(II) oxide.

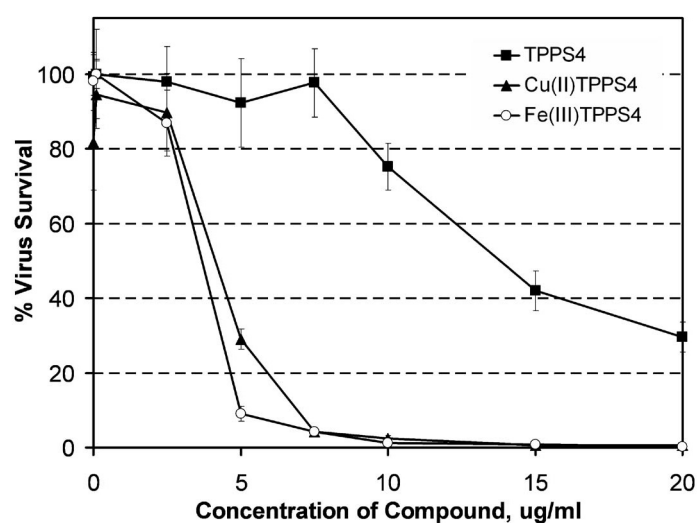


**Figure III-2. Metallation of H<sub>2</sub>TPPS<sub>4</sub> Porphyrin.** The chemical reaction of addition of metal ( M = Cu or Fe) to the meso position of the water-soluble tetraporphyrin.

control infection. Metalloporphyrins, Cu(II)TPPS<sub>4</sub> and Fe(III)TPPS<sub>4</sub>, inactivated all virus particles at the concentrations as low as 10 µg/ml.

At metalloporphyrin concentrations of around 2.5 µg/ml or less, virus survival was reduced only by 15 % or less. At concentration of 2.5 - 5 µg/ml the virus survival had dramatically decreased. Both metalloporphyrins followed a similar porphyrin concentration to virus survival curve, although they contain different metals at their meso position. The parental compound, TPPS<sub>4</sub> had minimal effect on the virus at concentrations up to 7.5 µg/ml, while virus survival dropped linearly from 100 % to 30%, when the concentration was raised to 20 µg/ml. Addition of the metal at the meso position has significantly increased antiviral function of sulfonated tetraporphyrin compound on virus infection, irrespective of the metal compound added. Based on the result of these experiments, most of the subsequent studies were conducted at minimum effective dose of 20 µg/ml. At 20 µg/ml the molar concentration of each compound is around 18 µM. By comparison at 20 µg/ml of acyclovir the molar concentration of acyclovir is 80 µM, while 2 µg/ml is 8 µM.





**Figure III-3. Inhibitory concentration of antiviral compounds on virus infection.**  $2 \times 10^5$  PFU of EHV-1 strain RacL11 was incubated with indicated concentrations of compounds for 20 min at  $37^\circ\text{C}$ , then used to infect RK13 cells for 1 hour at RT, following which the media was replaced with fresh containing the same compound concentrations, and the infection was allowed to proceed at  $37^\circ\text{C}$  for 2 days, when plaques were counted.

## **Cytotoxic Effects of the Antiviral Compounds**

To establish that the compounds have antiviral activity at the concentrations that can be achieved without inducing toxic effects to the cells, cytotoxic effects of the compounds as well as cell viability and proliferation were evaluated by using Neutral red uptake assay. The results were then confirmed by trypan blue dye exclusion cell viability assay and propidium iodide apoptosis assay. Neutral red uptake assay is based on the ability of viable cells to incorporate and bind the supravital dye, neutral red. Neutral red is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes/endosomes. Microtitration plate cultures were exposed to range of drug concentrations during the log phase of growth and viability was determined. Alterations of the cell surface or the lysosomal membrane due to the toxic effects of porphyrins would lead to lysosomal fragility, decreasing uptake and binding of neutral red dye. Spectrophotometry was used to quantitate the amount of neutral red uptake.

The assay was used to determine cytotoxic concentration of the compound that reduced cell viability to 50% and 100%, CC50 and CC100, respectively. Inhibitory concentration 50, IC50, is the concentration of a compound required to inhibit the effect of the virus *in vitro* by 50% of the control value. The relative effectiveness of the compound to inhibit viral infection compared to inducing cell death is defined as the therapeutic index (USDHHS, 2005). Therapeutic index, TI, is CC50/IC50 or the ratio of the amount of the compound that causes toxic effects to the amounts needed for therapy. A narrow therapeutic range and thus smaller TI is less desirable, since even a small increase in the amount of drug may lead to toxicity.

Metalloporphyrins reached cytotoxic dose lethal to all the cells at 1.25 mg/ml and median cytotoxic concentration of 0.6 mg/ml and 0.45 mg/ml for Fe(III)TPPS4 and Cu(II)TPPS4 respectively. Inhibitory concentrations of the compounds were obtained from three sets of independent experiments such as shown in Figure III-3. Metalloporphyrins reached 50% inhibitory concentration at around 3-3.5 µg/ml, while their parental strain reached IC50 at the 4-fold higher concentration. Therapeutic indexes (TI) were computed as the ratio between CC50 and IC50 (Table III-1). Iron metalloporphyrin had a higher two-fold higher TI than its copper counterpart. The parental strain had extremely narrow therapeutic range, as expected due to its phototoxic properties in the absence of the central metal. Compounds with higher TI are better candidates as antiviral medication, due to safety considerations. By comparison, the EC50 of acyclovir against HSV-1 virus infection of MDBK cells was reported as 2.88 µg/ml and CC50 of 9.12x10<sup>4</sup>, resulting in a TI of 3.16x10<sup>4</sup> and one of the safest medications available (Bean, 1992; Clercq, 1995; Wiltink and Janknegt, 1991).

**Table III-1. Quantitation of cytotoxic concentrations and therapeutic indexes of antiviral compounds. Cytotoxicity is expressed as concentration-dependent reduction of the uptake of the Neutral Red dye evaluating both cell integrity and growth inhibition.**

Chemical denomination	CC100 µg/ml	CC50 µg/ml	IC50 µg/ml	TI
Fe (III) tetrasulfonated phenylporphyrin	~1250	~600	3.0	200
Cu (II) tetrasulfonated phenylporphyrin	~1250	~450	3.5	118
Tetraphenoxyphenyl sulfonate porphyrin	~625	~50	13	4

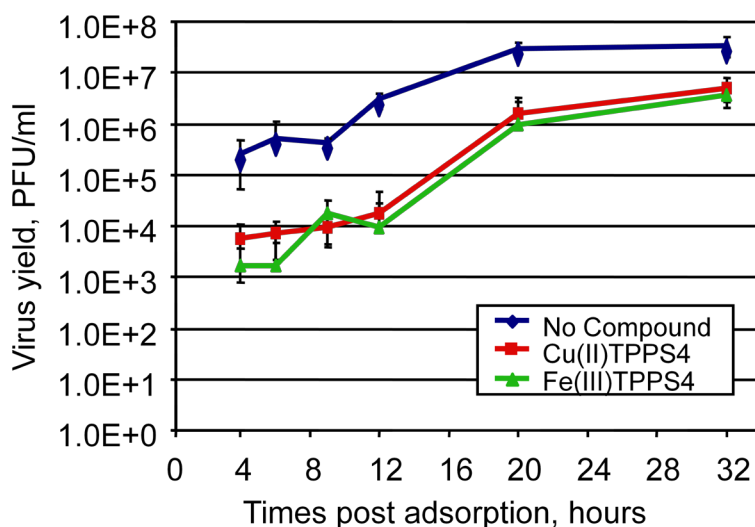
### **Antiviral Effects of Porphyrins: Reduction of Infectious Virus Production**

RK13 cell monolayers were infected with RacL11-Δgp2-GFP at MOI of 5 (Figure III-4) or MOI of 0.1 (Figure III-5). Three hours into infection, once the virus has attached

and entered the cell initiating replication, porphyrin compounds were added to the media overlaying the cell. One hour later and at specified intervals thereafter, supernatants of infected cells and the infected cells were collected and total infectious virus yield determined by plaque assay.

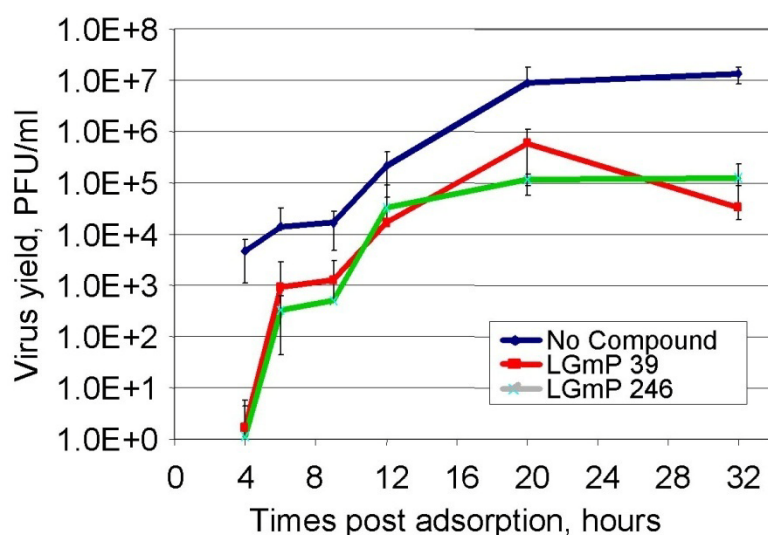
The exponential phase of the virus growth from 4 to 20 hours post adsorption, was graphed on a logarithmic versus linear scale, and therefore, follows a linear pattern. The relative growth rate (RGR) in the presence of either compounds or in their absence remained the same, as indicated by similarity of slope (dy/dx) of all three functions. Decreasing growth rate after 20 hours post adsorption is evident by the leveling of the slope over time.

The kinetics of virus growth is depicted as virus titers determined as the sum of the extracellular free virus in the supernatants and the intracellular infectious virus. The

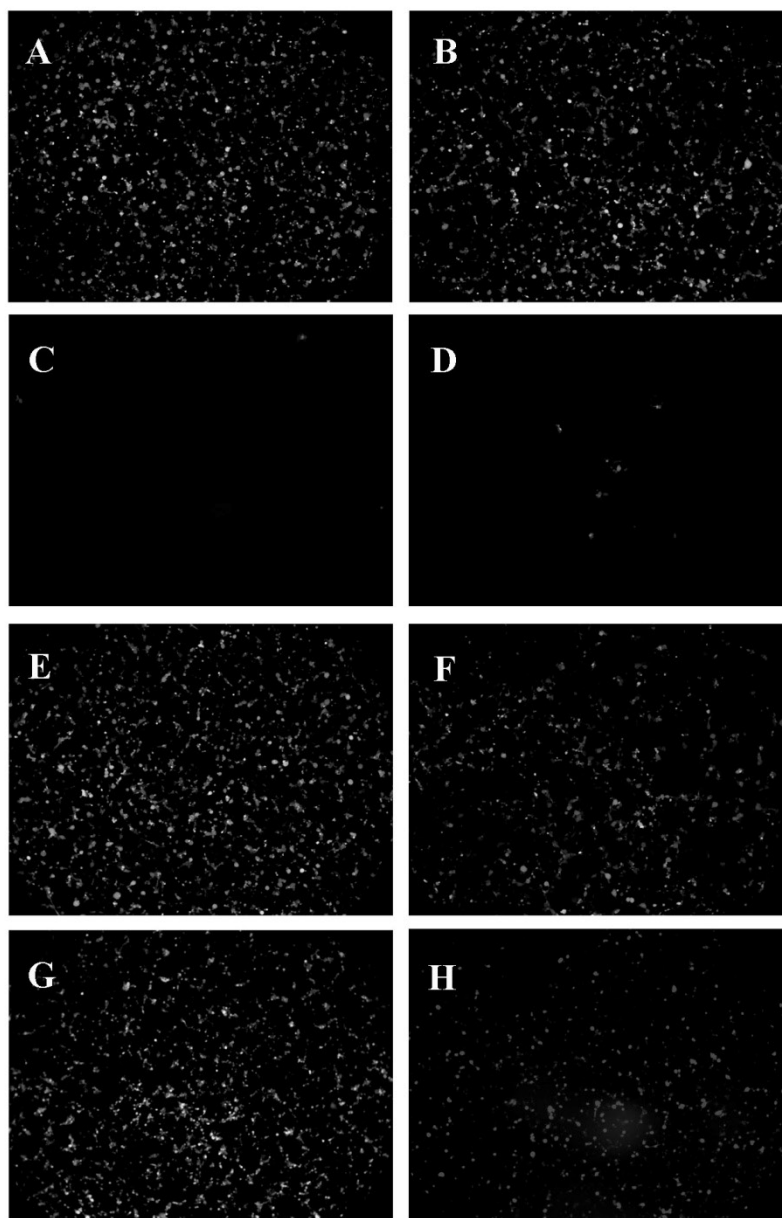


**Figure III-4. Inhibitory effects of metalloporphyrins on virus replication at high MOI.** Monolayers of RK13 cells were infected with EHV-1 RacL11 at an MOI of 5. At 4 hours post infection the cells were treated with antivirals. At indicated times post infection, cells and supernatant were harvested, and the total infectious virus production was measured by plaque assay. Viral titers as mean PFU/ml at each time point are shown in a logarithmic vs. linear scale. The error bars represent the average of three independent experiments.

intracellular and extracellular titers of EHV-1 are generally about equal at each time point (Schimmer and Neubauer, 2003). The viral titers of treated versus untreated virus were decreased by approximately two logs throughout infection. For example, at 12 hpi, the untreated virus titer was  $3 \times 10^6$  PFU/ml, while porphyrin treated virus titer was  $1 \times 10^4$  PFU/ml. Such large difference in virus yield cannot be explained by the porphyrin inactivation of the extracellular fraction alone. From previous experiments (Figure III-3), it is expected that the extracellular fraction of the treated viruses is not infectious, since free virus is easily inactivated by the porphyrins at this concentration. However, to definitively prove porphyrin effect on intracellular fraction of infectious virus, the experiment should be conducted with virus titers determined for intracellular and extracellular fraction individually.



**Figure III-5. Inhibitory effects on virus replication as shown by reduction at low MOI.** Monolayers of RK13 cells were infected with EHV-1 RacL11 at an MOI of 0.1. At 4 hours post infection the cells were treated with antivirals. At indicated times post infection, cells and supernatant were harvested, and the total infectious virus measured by plaque assay. Viral titers as mean PFU/ml at each time point are shown in a logarithmic vs. linear scale. The error bars represent the average of three independent experiments.



**Figure III-6. Antiviral effects at 48 hpi on RK13 cells infection with RacL11 virus at MOI 5i.** A). Cells were pretreated with compound for at 37°C 1 hour before infection. B). Untreated cells. C). Virus pretreated with compound for 1 hr at 4°C before infection. D). Cells were infected in the presence of the compound for 1 hour at 4°C to allow virus to attach. E). Following 1hr at 4°C, infected cells were overlaid with antiviral media and incubated for 1hr at 4°C. F). Following 1hr at 4°C, infected cells were overlaid with antiviral media and incubated for 1hr at 37°C. G). Antiviral compounds were added at 2 hpi following 1 hr at 4°C and 1 hr at 37°C. H). Acyclovir treatment.

### **Qualitative Assessment of Antiviral Effect during Early Stages of Viral Infection at MOI 5**

Infected cells were exposed for 10 minutes to the antiviral compounds added at various times post-infection, immediately following attachment, once attached, as well as during and post fusion to determine the relative antiviral efficacy of each compound at the binding, penetration, or post-entry infectious life cycle (Figure III-6). The RK13 cells were subjected to the Cu(II)TPPS4 or Fe(III)TPPS4 antivirals in the media for 1 hour, and then, washed thoroughly to remove any residual extracellular antiviral and infected with EHV-1 RacL11 at MOI of 5 (Figure III-6). The pre-treatment of cells with antivirals had no detectable effect on virus infection at 48 hours post infection as was evident by no change in the amount of green fluorescence of pre-treated and untreated infected cells (Figure III-6, A and B). Treating the cells for longer time and with higher concentration of metalloporphyrin prior to infection did not yield any detectable inhibition of virus infection. Mixing the virus and antiviral and either incubating the solution for 1 hour or using it to immediately infect the cells lead to no infection (Figure III-6, C and D). When the antiviral containing media was used to overlay the infected cells once the virus was allowed to attach to the cellular envelope for 1 hour at 4°C, a temperature not permissive for viral fusion events, there was no effect detected on viral infection at an MOI of 5. (Figure III-6, E). Metallated tetraporphyrins Cu(II)TPPS4 and Fe(III)TPPS4 at 20 µg/ml concentration prevented attachment of the EHV-1 RacL11 at MOI of 5 to the RK13 cells monolayers, but once the virus had attached the compounds had no effect on the virus.

To further evaluate the effect of metallated tetraporphyrins on virus entry events, the compounds were added to the virus infected cells when the virus had already attached to the cells and the fusion events of the virus and cellular envelopes were just initiated by

raising the temperature to 37°C. About 25% decrease in the number of infected cells was observed at 48 hours post infection (Figure III-6, F). If the antivirals were added post fusion events, no effect on the virus infection was detected at an MOI of 5 (Figure III-6, G). Acyclovir at 2 µg/ml was used as control antiviral to show its effect of significant inhibition of virus infection at post-entry events of virus life cycle (Figure III-6, H). An acyclovir concentration of 2 µg/mL was selected based on reports of the *in vitro* susceptibility of HSV-1 and several EHV-1 isolates to this concentration, a breakpoint concentration defining *in vitro* susceptibility of the virus to acyclovir. Virus isolates are defined as sensitive to acyclovir *in vitro* at IC<sub>50</sub> of <2 µg/mL and resistant to acyclovir if their IC<sub>50</sub> is ≥ 2 µg/mL (Bacon et al., 2003; Safrin et al., 1994).

### **Quantitative Assessment of Antiviral Effect during Early Stages of Viral Infection**

The above experiment was repeated for MOI of 5, 1, 0.1 and 0.01. Virus or cells alone or the virus-infected cells during early events of virus infection were subjected to antiviral treatment (Figure III-7 A and B). The amount of surviving virus was measured by counting the number of infected cells as percent of untreated control using Fluorescent-Activated Cell Sorting (FACS). Pretreating the cells with either antiviral had no significant effect on virus at all MOIs. Treating the virus for 1 hour or infecting the cells in the presence of either compound inhibited the infection by 100%. Exposing the virus-infected cells to either antiviral, once the virus has attached to cell surfaces, but prior to the onset of fusion events or post completion of fusion events had decreased the viral survival from 100% to about 40% as MOI decreased from 5 to 0.01. Exposing the virus-infected cells to either antiviral during the fusion of the virus particle with cellular membrane decreased the virus survival by about 20% at an MOI of 5, and by 75% at MOI of 0.01, 0.1 and 1.

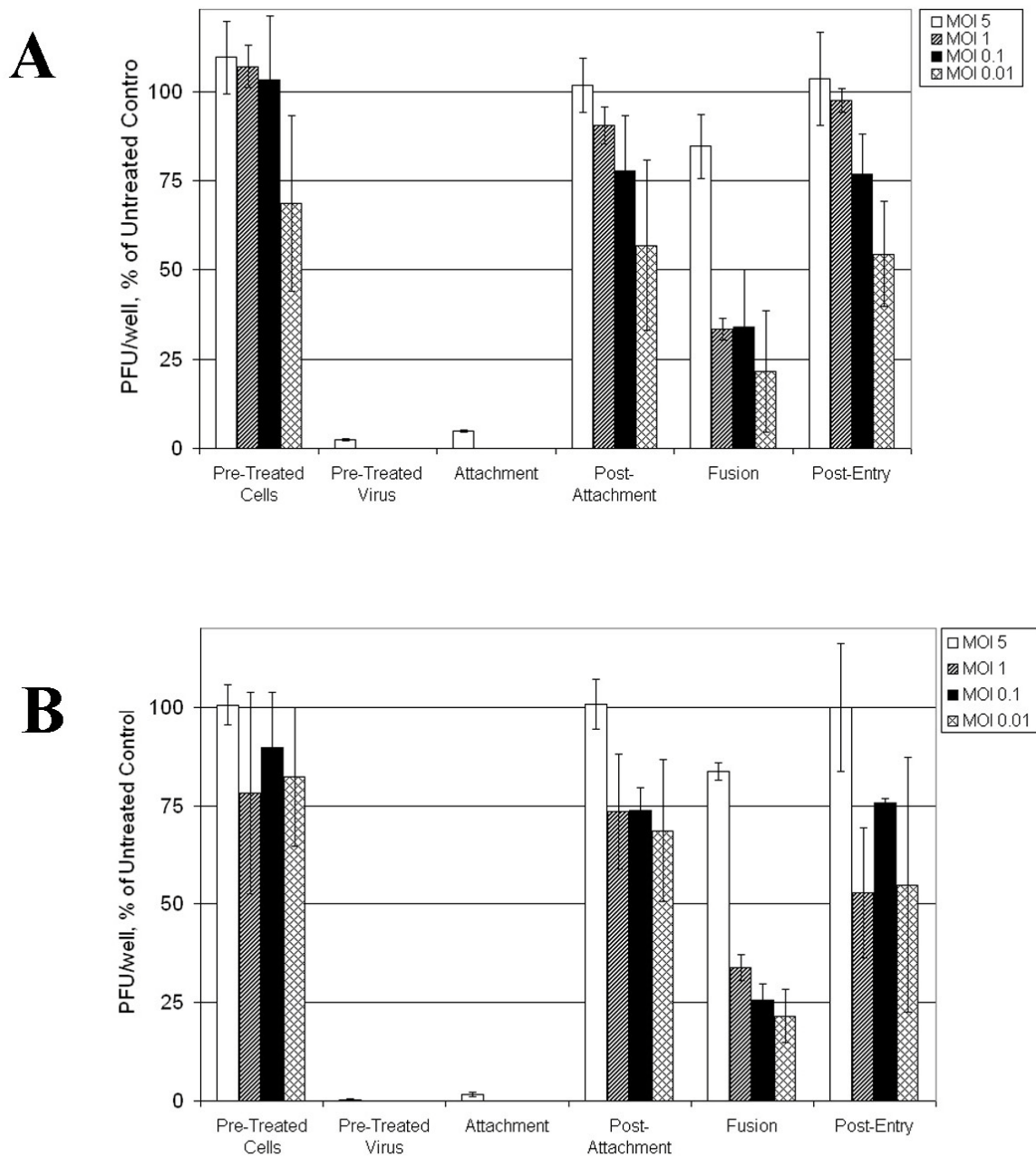


### **Antiviral Effect on Viral Cell-to-Cell Spread and Syncytium Formation**

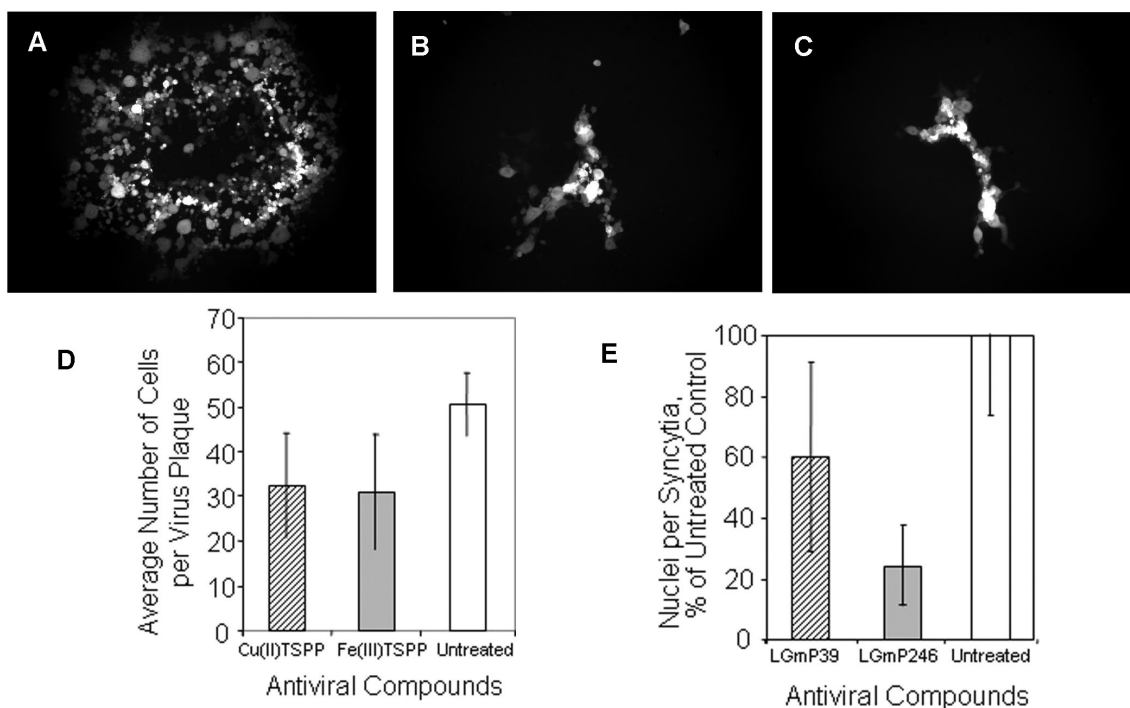
Although the entry of direct binding of free virions to target cells followed by fusion, entry and replication is an important initial route of infection, most pathogenic viruses prefer to move between cells without diffusion through the extracellular environment, but via cell-to-cell spread. EHV-1 RacL11 virus, being no exception, has natural propensity to mediate syncytium formation. To evaluate the effect of antiviral compounds on cell-to-cell spread and syncytium formation, RK-13 cells were infected with GFP- expressing virus, EHV-1 RacL11 and subjected to the antiviral treatment from 3 to 72 hours post infection. Presence of metalloporphyrins decreased the average number of infected cells per virus plaque from 50 to 30 cells/plaque (Figure III-8, D). The distribution of the number of nuclei per syncytia (polykaryocyte) followed a bell-curve shape with the majority of polykaryocytes within one standard deviation from the mean. The average number of nuclei per polykaryocyte for Cu(II)TPPS4 was  $3.6 \pm 1.9$ . The average number of nuclei per polykaryocyte for Fe(III)TPPS4 was  $1.2 \pm 0.8$ . The number of nuclei per polykaryocyte of untreated virus was  $6 \pm 1.3$ . The size of polykaryocytes decreased by 75% when treated with Fe(III)TPPS4 and by 40% when treated with Cu(II)TPPS4 as compared to that of untreated virus infection. Therefore, exposure of the viral infection to metalloporphyrins had reduced the plaque size by 40% and reduced the extent of cell-to-cell fusion by 40-75%.

### **PEG-mediated Fusion of Antiviral Compound Treated EHV-1**

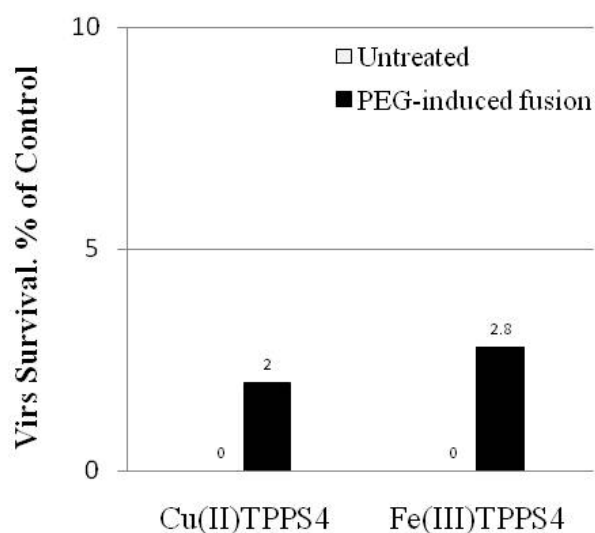
Polyethylene glycol (PEG) is a membrane-fusing reagent that is used to restore infectivity of the virus blocked at entry by binding of neutralizing compounds to its envelope. PEG-mediated fusion experiments were conducted to evaluate whether the



**Figure III-7. Time of addition experiment.** A) Cu(III)TPPS4. B) Fe(II)TPPS4. Quantitation of antiviral effects on free virus as well as attachment, fusion and post entry events of the virus life cycle.



**Figure III-8. Inhibition of Virus Spread.** Antiviral effect of porphyrin on plaque formation of GFP-expressing virus, EHV-1 RacL11 on RK13 (rabbit kidney) cell monolayers 72 hpi: untreated (A) and treated with 15  $\mu$ g/ml Cu(II)TPPS4 (B) or Fe (III) TPPS4 (C), and corresponding, reduction of plaque size (D) and the extend of cell to cell fusion (E).

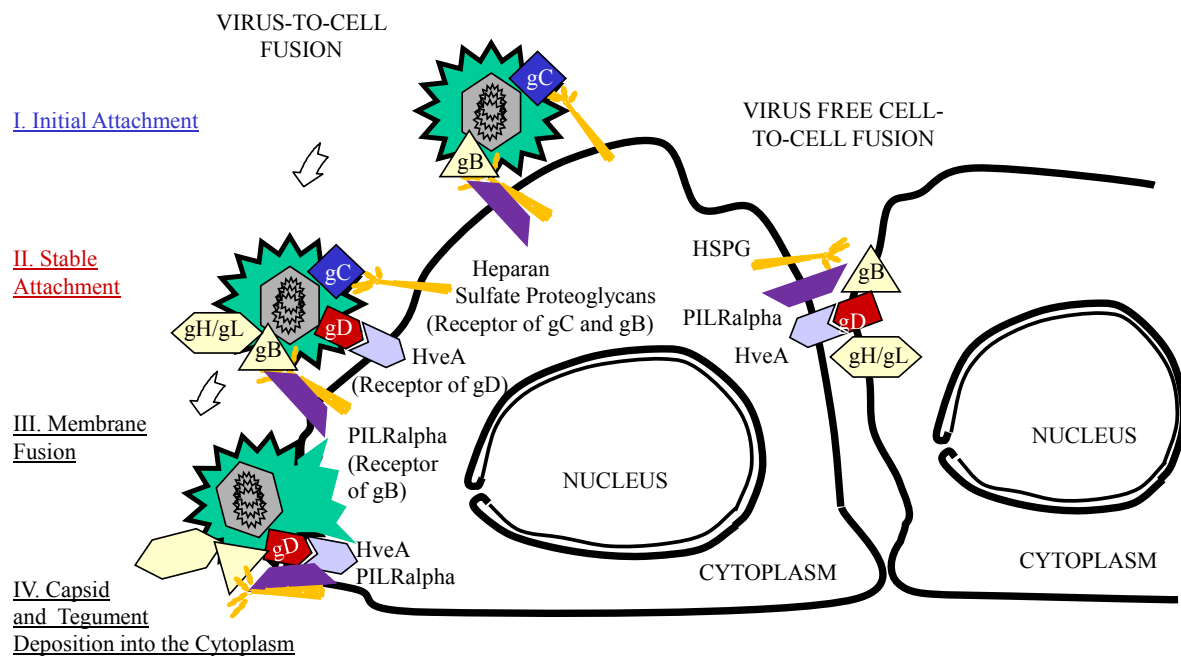


**Figure III-9. Polyethylene glycol induced fusion of antiviral compound treated EHV-1 RacL11/EGFP.** Virus survival is presented as percent of control.

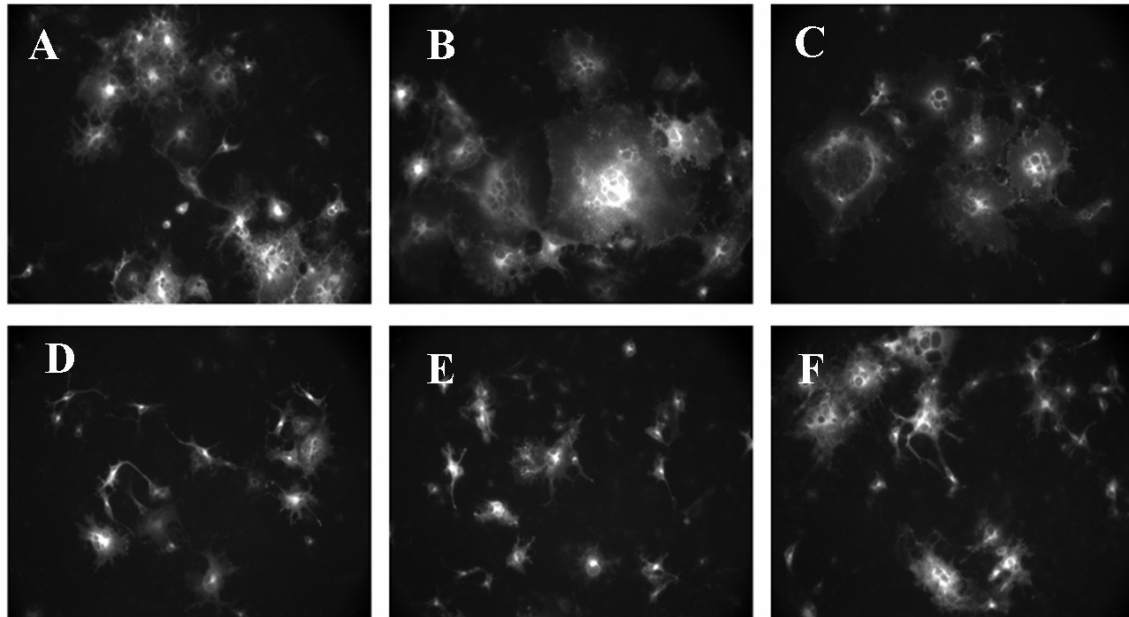
porphyrin renders virus particle permanently non-infectious, even if required entry step of fusion is bypassed by PEG (Figure III-9). The titers of infectious virus were only marginally increased, indicating that the antiviral permanently disabled the virus particle.

### **Virus-Free Cell Fusion System**

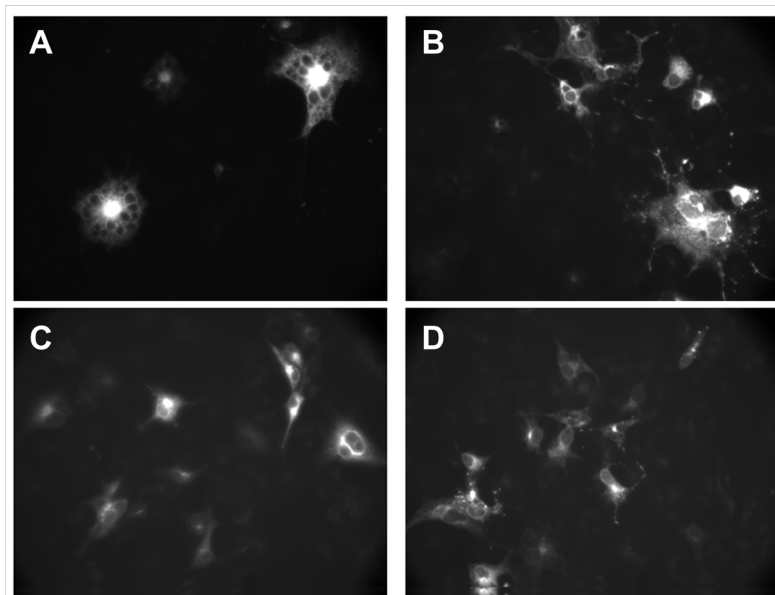
To further evaluate the effect of antivirals on fusion during cell-to-cell spread, a virus-free cell fusion system was used. Four envelope glycoproteins of the herpesviruses, gB, gD, and gH/gL complex have been shown capable of inducing cell-to-cell fusion in the absence of any other viral components, when they are expressed from the plasmid vectors transfected into the cells (Browne et al., 2001). Glycoproteins were amplified from the EHV-1 and HSV-1 genomes and cloned into the pcDNA<sup>TM</sup>3.1 vector under the control of CMV early promoter, so they can be easily expressed in the mammalian cells to high levels. Co-transfection of these glycoproteins results in syncytia formation in the absence of active virus infection. The expression of all four glycoproteins together on the same cellular membrane is required to induce syncytium formation. COS7 cells were used for the assay, since they can be transfected with much higher efficiency, then RK13 cells. Expression of HSV-1 gB, gH/gL, and gD resulted in formation of polykaryocytes of about 3 to 20 nuclei each (Figure III-11, A-C). Significant reduction of fusion was observed if the transfected cells were exposed to Cu(II)TPPS4 (Figure III-11, D-F) or Fe(TPPS4) at 75 µg/ml at 6 hours post transfection. Metallo-porphyrin treated cells formed polykaryocytes of about 2 to 6 nuclei/cell. The virus-free cell fusion experiment was repeated for EHV-1, a less established system then HSV-1. In the absence of antiviral, the fusion observed was about 3 to 15 nuclei/cell (Figure III-12, A-B), while post Cu(II)TPPS4 treatment, the amount of cell-to-cell fusion has reduced to 2 to 4 nuclei/cell (Figure III-12, C-D).



**Figure III-10. Diagram of the viral envelope glycoproteins and their corresponding cellular envelope receptors.**



**Figure III-11. Virus-free cell fusion system.** COS7 were cells co-transfected with HSV-1 gB1511, gH/gL, and gD-V5. At 6 hours post transfection, cells were either mock treated (A-C) or treated with 75 µg/ml of Cu(II)TPPS4 ( D-E). The cells were than stain with V5 antibody for visualization of syncytia formation (65X magnification).



**Figure III-12. Virus-free cell fusion system.** COS7 cells co-transfected with EHV-1 gB-V5, gH-V5, gL-V5, and gD-V5. A-B). No antiviral added. C-D). Antiviral (Cu(II)TPPS4) added at 6 hours post transfection at 75 µg/ml (65X magnification).

## **DISCUSSION**

### **Chemical Structure and Function**

Two synthetic compounds were selected for detailed studies based of their virucidal and antiviral properties against EHV-1 as well as their low cytotoxicity in cell culture assays. These compounds, Cu(II)TPPS4 and Fe(III) TPPS4, were derived by insertion of Cu(II) or Fe(III) into 5,10,15,20 –tetrakis(4'-sulfophenyl) porphyrin (H2TPPS4) respectively. H2TPPS4 is a sulfonated derivative of tetraphenylporphyrin and has been shown to be active against HIV-1, HSV-1 and HSV-2 (Vzorov et al., 2002). Modification of porphyrins to extend their chemical structure by sulfonation resulted in improvement of antiviral properties. Metalloporphyrins, Cu(II)TPPS4 and Fe(III)TPPS4, inactivated all free virus particles at the concentrations as low as 10 µg/ml. Addition of the metal at the meso position significantly increased antiviral function of sulfonated tetraporphyrin compound on virus infection, irrespective of the metal compound added. The addition of metal, copper or iron chelate also resulted in significant decrease of cytotoxicity due to elimination of photodynamic properties. The parental compound, H2TPPS4, had extremely narrow therapeutic range, as expected due to it phototoxic properties. Metalloporphyrins had sufficiently safe therapeutic index, with iron metalloporphyrin reaching a two-fold higher therapeutic index then its copper counterpart.

Current legislation demands that new drugs go through extensive toxicity testing before they are released starting with cytotoxicity testing *in vitro*. Cytotoxicity is a complex event *in vivo*, with a wide spectrum of effects, from simple cell death to subtle functional change of the cell leading to complex metabolic aberrations. To prove absence of toxicity would require not only to show cell viability and minimal changes in its growth

and phenotype, but also a subtle analysis of minor metabolic changes or alterations in cell to cell signaling, that are beyond the scope of this study. The assay used in this study was chosen as it is a baseline in modern drug testing, providing sufficient information to estimate potential therapeutic range of the compounds.

### **Antiviral Effect of Metalloporphyrins: Reduction of Infectious Virus Production**

Antiviral compounds did not affect the relative growth rate of the virus, but decreased the production of infectious virus progeny on RK13 cells dramatically, by two logs throughout infection. In our experiments, the antiviral effect on the sum of the extracellular free virus in the supernatants and the intracellular infectious virus was evaluated. Such large difference in virus yield cannot be explained by the metalloporphyrin inactivation of the extracellular free virus alone, because the intracellular and extracellular titers of EHV-1 are generally about equal at each time point (Schimmer and Neubauer, 2003), therefore two-times and not two-log difference would have been seen. Thus, the degree of porphyrin effect on virus infection would depend on ability, quantity and kinetics of porphyrin diffusion across cellular membranes. While, the parental compound, H2TPPS4, is known to enter the cells, further experiments would be needed to show if there is cytoplasmic localization of metalloporphyrins and the effect of metalloporphyrins on intracellular infectious virus alone.

### **Antiviral Effect of Metalloporphyrins: Inhibition of Early Stages of Viral Infection**

EHV-1 entry into the cells is a carefully orchestrated process of recognition of cellular receptors, triggering of fusion, fusion, and release of capsid into the cytoplasm. Pretreating cells with antivirals prior to infection had no effect on infection. Therefore, it is



likely that metalloporphyrins do not interact with the cell membrane or receptors directly, and remain extracellular, and thus, were unable to interfere with infection once removed by washing the cells. Since the parental compound is able to localize inside the cell, a small amount of metalloporphyrins is expected to enter the cell, but the antiviral concentration intracellularly may be too low or metalloporphyrin binding sites quickly saturated to have any detectable antiviral effect on virus infection. Alteration of cellular membrane by virus infection may render cell envelope more permissive to metalloporphyrins explaining the inhibitory effect on intracellular virus fraction seen as drastic decrease in virus production in the presence of metalloporphyrins.

Metallated tetraporphyrins interfere with ability of the virus to attach to the cell surface, either by directly binding to the free virus but not the cell, or by interfering with virus attachment to the cellular membranes in other ways. Since metalloporphyrins do not interact with cellular envelope as shown by no effect on infection by pretreating cells with antivirals, they most likely interact with the proteins embedded in the viral envelope and not with a viral envelope itself, which is derived from a cellular membrane.

Virus that attached to the cellular membranes but did not undergo fusion is also susceptible to the antivirals, but to a limited degree. The fusion of the viral envelope and cellular membrane during virus entry is significantly affected by the metalloporphyrins. The antiviral effect on viral replication events post fusion is evident, but also limited. At all stages of the virus life cycle, higher amount of virus used for infection resulted in more virus survival. Thus, more porphyrin compounds were available to interact with fewer virus particles, indicating a saturation effect on the virus-porphyrin interaction. Complete

inhibition of the virus at each stage of virus entry may be potentially reached if the porphyrin to virus ratio is increased further.

PEG experiments suggest that interaction with porphyrin renders the virus particle permanently non-infectious, even if required entry step of virus-induced fusion is bypassed by PEG-induced fusion. The study in which the virus is subjected to the antivirals, and then, purified by ultracentrifugation and used for infection would help to show if the virus is infectious once antivirals are washed off or does the porphyrin permanently disables the virus by direct binding. To conduct this experiment we would need to determine if the porphyrin is still present in association with the virus post purification by ultracentrifugation. However, there is no suitable detection method for metalloporphyrins, short of mass spectrometry and other analytical techniques.

### **Antiviral Effect of Metalloporphyrins: Reduction of Cell-to-Cell Spread and Syncytia Formation**

Metalloporphyrins exhibited inhibitory effects on virus spread, reducing the plaque size and the extent of cell-to-cell fusion. It is highly unlikely that inhibition of viral replication could account for the mechanism(s) by which compounds achieved almost five-fold inhibition of spread in RK13 cells. Acyclovir by comparison, reduces replication, but has no effect on the spread of the virus via either virus-to-cell fusion or cell-to-cell fusion. EHV-1 expresses a number of membrane glycoproteins that function in both entry of virus particles and movement of virus from an infected cell to an uninfected cell or cell-to-cell spread.

Multiple, viral proteins are involved in cell-to-cell spread and syncytia formation, such as gH/gL, gB, gK, UL20, UL11, and gE/gI (Cheshenko and Herold, 2002; Foster et al., 2008; Navarro et al., 1992; Schimmer and Neubauer, 2003). While, gB, gD, gH/gL are

required for virus-to-cell fusion during entry, complex of gE/gI is required for efficient cell-to-cell spread, yet not required for entry fusion events (Polcicova et al., 2005).

### **Antiviral Effect of Metalloporphyrins: Interaction with Viral Glycoproteins**

The cell-to-cell and the virus-to-cell fusion require direct protein–protein interactions among gD, gB, and gH/gL. Both metalloporphyrins decreased the syncytium formation in a virus-free cell fusion system, indicating that the porphyrins have direct effect on at least one of the four glycoproteins. Glycoprotein B mediates attachment and is required for virus entry, cell-to-cell spread and syncytia formation and is the primary candidate for direct inhibition by metalloporphyrins (Cassiani-Ingoni et al., 2005). Cytoplasmic domain of gB is a potential target for interaction with metalloporphyrins, based on its known function in cell-to-cell spread (Cassiani-Ingoni et al., 2005).

### **SUMMARY**

The continuous circulation of EHV-1 in equine populations despite regular vaccinations and apparent ineffectiveness of currently available vaccines in preventing or ameliorating neurological manifestations of EHV-1 infections as well as limitations of EHM treatment options, necessitates the discovery of new antiviral drugs that can effectively manage this important infection of horses.

Glycoprotein conformational changes and interactions induce structural alterations in the membrane that leads to membrane fusion. Tetraporphyrins block these early phases of viral infection, exerting maximal effect when the virus is exposed to the antiviral at the time of initial infection or at any time through the end of the attachment and fusion processes. Tetraporphyrins also considerably curtail the spread of the virus via virus-to-cell and cell-to-cell fusion. Our results suggest that tetraporphyrins may be used as effective

virucidal and antiviral agents to prevent and to treat disseminated disease and neurological outcomes of EHV-1.

Future work should focus on further delineating the mechanism of antiviral and virucidal effects of these compounds, their cellular distribution, in depth toxicity studies, and their effect against *in vivo* EHV-1 infections of mice, specifically, the efficacy of these drugs to prevent acute viral infections in respiratory and myeloencephalopathy EHV-1 mouse models.

## CHAPTER IV. CONCLUDING REMARKS

### FINAL COMMENTS

EHV-1 antiviral properties and their pharmacological characteristics make porphyrins auspicious candidates for the treatment of EHV-1 infections. Porphyrin compounds were shown to specifically inhibit free virus particles as well as membrane fusion phenomena required for virus entry and virus spread, and the antiviral activity was enhanced by modification of the chemical structure of the porphyrin compounds via primary sulfonation and metallation. An initial screening of almost 100 porphyrin and platinum compounds as well as a detailed screening of 18 different compounds revealed that Cu (II) tetrasulfonated phenylporphyrin and Fe (III) tetrasulfonated phenylporphyrin possessed strong virucidal and antiviral activities against EHV-1. These compounds, Cu(II)TPPS4 and Fe(III)TPPS4, were derived by insertion of Cu(II) or Fe(III) into 5,10,15,20 –tetrakis(4'-sulfophenyl) porphyrin (H2TPPS4) respectively.

The selected two compounds were used to synthesize new compounds of extended chemical structure. Additional sulfonation of compounds did not lead to improvement in their antiviral properties against free virus nor virus attached to cell surfaces. Addition of fluoride or naphthalene groups, also did not improve antiviral properties.

Glycoprotein conformational changes and interactions induce structural alterations in the cellular membrane that leads to membrane fusion. Antivirals block the early phases of viral infection, exerting maximal effect when the virus is exposed to the antiviral at the time of initial infection or at any time at the end of the attachment and throughout fusion processes. Polyethylene glycol experiments suggest that interaction with porphyrin renders virus particles permanently non-infectious. Cu(II)TPPS4 and Fe(III)TPPS4 antiviral

compounds did not affect the relative growth rate of the virus, but decreased the production of infectious virus progeny on RK13 cells dramatically, by two logs throughout infection, most likely by direct inactivation of infectious virus progeny. Thus, the degree of porphyrin effect on virus infection at the stages post entry would depend on ability, quantity and kinetics of porphyrin diffusion across cellular membranes.

The cell-to-cell and the virus-to-cell fusion require direct protein–protein interactions among gD, gB, and gH/gL. Both metalloporphyrins decreased the syncytium formation during virus infection and in virus-free cell fusion system, indicating that the porphyrins have direct effect on at least one of the four glycoproteins. Cytoplasmic domain of gB is a potential target for interaction with metalloporphyrins, based on its known function in cell-to-cell spread.

### **CURRENT AND FUTURE RESEARCH CHALLENGES**

A number of experiments can be performed to add further detail to our understanding of EHV-1 infection and its inhibition by metalloporphyrin compounds. Purifying the antiviral treated virus and quantifying infectious particles remaining, would determine if the effect of the porphyrins on the virus particle is a permanent inactivation or not. Adsorption of <sup>35</sup>S-labeled HSV-1 and detection of bound virus by liquid scintillation radioisotope counting could be considered for precise quantitation of virus able to bind to the cells following antiviral treatment in the presence of varied antiviral concentrations. Further experiments would be needed to show if there is cytoplasmic localization of metalloporphyrins and to estimate the effect of metalloporphyrins on intracellular infectious virus alone. Surface Plasmon Resonance (SPR) using a Biacore 2000 instrument can be considered to study the interaction between the antiviral and virus particles. SPR

aids the study interactions between a wide range of molecules including proteins, nucleotides, pharmaceuticals, and surface active agents. In order to limit the spread of the infection *in vivo*, a consideration should be made at what dose of medication the horse is to be treated, the treatment regiment, achievable blood concentration and the cost of the compound administration, as well as potential side-effects such as hypersensitivity.

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## APPENDIX: EHV-1, EHV-4, HSV-1, AND HSV-3 GENE NOMENCLATURE

EHV-1 ORF	EHV-4	HSV-1	VZV	Function	Product
1	1	UL56		possibly vesicular trafficking	type 2 membrane protein, UL56 family
2	2			unknown	type 2 membrane protein, membrane protein V1
3	3			unknown	potentially envelope-associated, myristylated tegument protein CIRC
4	4	UL55		unknown	nuclear protein UL55
5	5	UL54	4	RNA metabolism and transport, RNA-binding protein, shuttles between nucleus and cytoplasm, inhibits pre-mRNA splicing, exports virus mRNA from nucleus, exerts most effects post-transcriptionally	expression regulator
6	6	UL53	5	virion morphogenesis, membrane fusion	envelope glycoprotein K, type 3 membrane protein, contains a signal peptide, 4 transmembrane domains
7	7	UL52	6	DNA replication	helicase-primase primase subunit
8	8	UL51	7	virion morphogenesis	tegument protein UL51
9	9	UL50	8	nucleotide metabolism	deoxyuridine triphosphatase
10	10	UL49A	9A	virion morphogenesis, membrane fusion	type 1 membrane protein, contains a signal peptide, complexed with envelope glycoprotein M
11	11	UL49	9	virion morphogenesis, possibly RNA transport to uninfected cells	tegument protein VP22
12	12	UL48	10	regulation, virion morphogenesis, transactivates immediate early genes	transactivating tegument protein VP16
13	13	UL47	11	possibly regulation, modulates transactivating tegument protein VP16, RNA-binding protein	tegument protein VP1314
14	14	UL46	12	possibly regulation, modulates transactivating tegument protein VP16	tegument protein VP1112
15	15	UL45		possibly membrane fusion, membrane protein, tegument-associated	membrane protein UL45, type 2
16	16	UL44	14	cell attachment, binds cell surface heparan sulphate, binds complement C3b,	envelope glycoprotein C, type 1 membrane protein, contains a signal peptide to block neutralization
17	17	UL43	15	possibly membrane fusion	envelope protein UL43, type 3 membrane protein, 11 transmembrane domains
18	18	UL42	16	DNA replication, dsDNA-binding	DNA polymerase processivity



				protein	subunit
19	19	UL41	17	cellular mRNA degradation, mRNA-specific RNase	tegument host shutoff protein
20	20	UL40	18	nucleotide metabolism	ribonucleotide reductase subunit 2, small
21	21	UL39	19	nucleotide metabolism	ribonucleotide reductase subunit 1, large
22	22	UL38	20	capsid morphogenesis, complexed 1:2 with capsid triplex subunit 2 to connect capsid hexons and pentons	capsid triplex subunit 1
23	23	UL37	21	virion morphogenesis, complexed with large tegument protein	tegument protein UL37
24	24	UL36	22	capsid transport, complexed with tegument protein UL37, ubiquitin-specific protease (N-terminal region)	large tegument protein
25	25	UL35	23	capsid morphogenesis, possibly capsid transport, located externally on capsid hexons	small capsid protein
26	26	UL34	24	nuclear egress, type 2 membrane protein, interacts with nuclear egress lamina protein	nuclear egress membrane protein
27	27	UL33	25	DNA encapsidation, interacts with DNA packaging terminase subunit 2	DNA packaging protein UL33
28	28	UL32	26	DNA encapsidation, possibly capsid transport	DNA packaging protein UL32
29	29	UL31	27	nuclear egress, interacts with nuclear egress membrane protein	nuclear egress lamina protein
30	30	UL30	28	DNA replication, DNA polymerase catalytic subunit	
31	31	UL29	29	DNA replication, possibly regulation	single-stranded DNA-binding protein, contains a zinc-finger
32	32	UL28	30	DNA encapsidation	DNA packaging terminase subunit 2
33	33	UL27	31	cell entry, cell-to-cell spread, possible membrane fusogen, binds cell surface heparan sulphate	envelope glycoprotein B, type 1 membrane protein, contains a signal peptide
34	34		32	unknown	protein V32
35	35	UL26	33	capsid morphogenesis, serine protease (N-terminal region), minor scaffold protein (remainder of protein, clipped near C terminus)	capsid maturation protease, N-terminal protease domain acts in capsid maturation and is a capsid protein, C-terminal domain is the minor capsid scaffold protein
35.5	35.5	UL26.5	33.5	capsid morphogenesis, clipped near C terminus	Major capsid scaffold protein
36	36	UL25	34	DNA encapsidation, located on capsid near vertices, possibly stabilizes the capsid and retains the genome	DNA packaging tegument protein UL25
37	37	UL24	35	unknown	nuclear protein UL24
38	38	UL23	36	nucleotide metabolism,	thymidine kinase
39	39	UL22	37	cell entry, cell-to-cell spread, possible membrane fusogen,	envelope glycoprotein H, type 1 membrane protein, contains a

				complexed with envelope glycoprotein L	signal peptide
40	40	UL21		virion morphogenesis, interacts with microtubules	tegument protein UL21
41	41	UL20		virion morphogenesis, membrane fusion	envelope protein UL20, type 3 membrane protein, 4 transmembrane domains
42	42	UL19	40	capsid morphogenesis, 6 copies form hexons, 5 copies form pentons	major capsid protein
43	43	UL18	41	capsid morphogenesis, complexed 2:1 with capsid triplex subunit 1 to connect capsid hexons and pentons	capsid triplex subunit 2
44/47	44/47	UL15	42/45	DNA encapsidation contains an ATPase domain	DNA packaging terminase subunit 1
45	45	UL17	43	DNA encapsidation, capsid transport, capsid-associated product	DNA packaging tegument protein UL17
46	46	UL16	44	possibly virion morphogenesis product	tegument protein UL16
48	48	UL14	46	virion morphogenesis	tegument protein UL14
49	49	UL13	47	protein phosphorylation, PK family product	tegument serine/threonine protein kinase
50	50	UL12	48	DNA processing, maturation and packaging of DNA	deoxyribonuclease
51	51	UL11	49	virion morphogenesis envelope-associated	myristylated tegument protein
52	52	UL10	50	virion morphogenesis, complexed with envelope glycoprotein N	envelope glycoprotein M, membrane fusion type 3 membrane protein, 8 transmembrane domains
53	53	UL9	51	DNA replication	DNA replication origin-binding helicase
54	54	UL8	52	DNA replication	helicase-primase subunit
55	55	UL7	53	virion morphogenesis	tegument protein UL7
56	56	UL6	54	DNA encapsidation dodecamer located at one capsid vertex in place of a penton	Minor capsid portal protein
57	57	UL5	55	DNA replication	helicase-primase helicase subunit
58	58	UL4	56	Unknown, colocalizes with regulatory protein ICP22 and nuclear protein UL3 in small, dense nuclear bodies	nuclear protein UL4
59	59	-	57	possibly virion morphogenesis product	protein V57
60	60	UL3	58	unknown colocalizes with regulatory protein ICP22 and nuclear protein UL4 in small, dense nuclear bodies	nuclear protein UL3
61	61	UL2	59	DNA repair	uracil-DNA glycosylase
62	62	UL1	60	cell entry, cell-to-cell spread complexed with envelope glycoprotein H	envelope glycoprotein L, contains a signal peptide
63	63	RL2	61	regulation, cellular protein degradation, latency contains a RING finger, disrupts ND10,	ubiquitin E3 ligase, ICP0

				proteasome-dependent degradation of several cellular proteins	
64/64.1	64	RS1	62	regulation	transcriptional regulator ICP4
65	65	US1	63	regulation, cell cycle regulation required for expression of a subset of late genes, host range determinant	regulatory protein ICP22
66	66	US10	64	unknown	virion protein US10
67	67	-	-	unknown, colocalizes with nuclear lamins, Virulence determinant	virion protein V67
68	68	US2	-	Unknown, possibly envelope-associated, interacts with cytokeratin 18	virion protein US2
69	69	US3	66	protein phosphorylation, apoptosis, nuclear egress tegument protein, phosphorylates nuclear egress lamina protein, mediates phosphorylation of HDAC1 and HDAC2 and other cellular and viral proteins, PK family	serinethreonine protein kinase US3
70	70	US4	-	cell-to-cell spread type 1 membrane protein, contains a signal peptide, gD family product	envelope glycoprotein G
71	71	US5	-	unknown type 1 membrane protein, contains a signal peptide	envelope glycoprotein J
72	72	US6	-	cell attachment type 1 membrane protein, contains a signal peptide, binds cell surface receptors, gD family	envelope glycoprotein D
73	73	US7	67	cell-to-cell spread type 1 membrane protein, contains a signal peptide, complexed with envelope glycoprotein E to form an Fc-receptor, gD family	envelope glycoprotein I
74	74	US8	68	cell-to-cell spread type 1 membrane protein, contains a signal peptide, complexed with envelope glycoprotein I to form an Fc-receptor, gE family	envelope glycoprotein E
75	75	US8A	-	Unknown, type 2 membrane protein product	membrane protein US8A
76	76	US9	65	axonal transport type 2 membrane protein, tegument-associated, localizes envelope proteins	membrane protein US9

Proteins that are not conserved in both HSV-1 and VZV are encoded by genes 1, 2, 3, 15, 34, 59, 67, 68, 70, 71, 72 and 75. Poorly conserved are genes 48, 62, 63, 73 and 76. The best conserved proteins have counterparts in all mammalian herpesviruses and include those involved in DNA replication and packaging and capsid structure, such as those encoded by genes 42, 43, 47/44 and 57.

## **VITA**

Galena V. Rybachuk was born in August, 1974, to Viktor Pavlovich Rybachuk and Nellie Grigorievna Videnina in Kiev, Ukraine. She has a sister and a lifelong friend, Yunona Videnina. Galena spent her childhood in Kiev with her parents and sister and in Dneprodzerjansk, Ukraine, with her maternal grandmother, Anna Evtihievna Videnina. She completed elementary education and was accepted into the Natural Sciences Lyceum in September 1987, where she focused on the subjects of mathematics, physics and computer sciences. Upon graduation from the lyceum in 1991, she was accepted, into Kiev Polytechnic Institute, Department of Mathematics to pursue a Real Time Computer Systems major. In 1992, Galena was awarded a scholarship to attend Louisiana State University. She transferred to Louisiana State University to pursue majors of biochemistry and microbiology. In 1994, when Galena was granted a Howard Hughes Medical Institute Undergraduate Research Fellowship, she joined the laboratory of Dr. Konstantin Gus Kousoulas where she later pursued a doctoral degree in the research of molecular biology of herpesviruses. In 2001, Galena was accepted into the Louisiana State University School of Veterinary Medicine. She completed her Doctor of Veterinary Medicine degree in May of 2007 and is currently a practicing veterinarian at Durango Animal Hospital, Las Vegas, Nevada.