REPLICATION-COMPETENT NON-HUMAN VIRUSES FOR USE IN CLINICAL GENE THERAPY: AN INVENTORY STUDY

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The reliability of the information compiled for this study and its conclusions are the sole responsibility of the authors. The information contained in this report does not necessarily represent the views of the COGEM or the Leiden University Medical Center.

SAMENVATTING

Alle levende organismen worden blootgesteld aan een verscheidenheid van virussen. Virussen infecteren gewoonlijk één of een beperkt aantal gastheersoorten. In zeldzame gevallen vinden infecties buiten de natuurlijke gastheersoorten plaats. Dit kan zich in deze nieuwe gastheer manifesteren als een nieuwe ziekte. In zeer zeldzame gevallen kan het virus zich blijvende vestigen in de nieuwe gastheersoort. In dit licht is voorzichtigheid geboden wanneer niet-humane virussen, of hiervan afgeleide genetisch-gemodificeerde virussen, therapeutisch worden toegepast in de kliniek. Toediening van niet-humane virussen aan mensen kan leiden tot een selectie voor virussen met een verhoogde replicatie-efficiëntie in mensen. Dit zou immers kunnen leiden tot adaptatie van het virus en vergrootte kans op overdracht van het therapeutisch virus naar derden, bijvoorbeeld familieleden of medisch personeel. Dit is evident ongewenst. De mogelijke gevolgen van de verspreiding van het therapeutisch virus vanuit de behandelde patiënt naar derden zijn moeilijk in te schatten en uiteraard in belangrijke mate afhankelijk van (de eigenschappen van) de gebruikte virussen. Om inzicht te krijgen in welke niet-humane virussen worden ontwikkeld voor toekomstig klinische gebruikt heeft de Commissie Genetische Modificatie (COGEM) opdracht gegeven een bureaustudie uit te voeren naar de volgende vragen:

- Welke niet-humane virussen worden ontwikkeld voor therapeutisch gebruik, en hoe ver zijn deze ontwikkelingen gevorderd?
- Wat zijn de belangrijkste biologische eigenschappen van de gebruikte virussen die relevant zijn voor de milieurisico-evaluatie voor het gebruik van zulke virussen?

Dit rapport beschrijft de uitkomsten van deze inventariserende studie naar de niet-humane virussen die therapeutisch worden gebruikt, of worden ontwikkeld voor dit gebruik, in mensen. In de meeste van deze studies wordt toepassing beoogd als therapeutisch agens voor de behandeling van kanker. De klassieke vaccins vallen buiten het aandachtsgebied van deze studie.

De studie is beperkt tot niet-humane virussen en virale vectoren die in staat zijn zich te vermenigvuldigen in niet-gemodificeerde cellen van de natuurlijke gastheer. Replicatiedefectieve virussen, dat wil zeggen virussen die voor hun replicatie afhankelijk zijn van specifieke helperfuncties, zijn uitgezonderd van deze studie. De reden de replicatiedefectieve virussen uit te sluiten is gelegen in het gegeven dat deze door de afhankelijkheid van specifieke helperfuncties intrinsiek biologisch ingeperkt zijn.

Op basis van informatie uit wetenschappelijke publicaties, congresverslagen, van websites van bedrijven die werkzaam zijn in dit veld, en na consultatie van een

aantal nationale en internationale experts zijn 27 niet-humane virussen geïdentificeerd die worden ontwikkeld voor klinische toepassing. Voor elk van de virusfamilies is een korte samenvatting gegeven van de biologische eigenschappen en het voorgestelde gebruik. Op basis van deze gegevens is een *relatieve milieurisicoscore* toegekend aan elk van de virussen. De mogelijke score varieert van '*Verwaarloosbaar*' tot '*Zeer Hoog*'. Aan twee van de virussen werd een 'Verwaarloosbaar' score toegekend (i.c. kanariepokkenvirus en *Autographa californica* baculovirus) en drie virussen een '*Hoge*' *relatieve milieurisicoscore* (te weten: Seneca Valley virus, murine leukemia virus and Maraba virus). Een complicerende factor in deze laatste groep is dat er op onderdelen slechts beperkte informatie beschikbaar is, waardoor deze virussen uit voorzorg hoger zijn ingeschaald. Het lijkt waarschijnlijk dat bij deze virussen de classificatie met het beschikbaar komen van additionele informatie naar beneden kan worden bijgesteld. Geen van de virussen werd in de '*Zeer Hoog*' categorie geplaatst.

De auteurs vinden het essentieel dat een milieurisicoanalyse wordt uitgevoerd voordat niet-humane virussen klinisch worden toegepast, zelfs als het toe te passen virus niet-genetisch gemodificeerd is. Het is niet duidelijk of een milieurisicoanalyse voor dergelijke virussen hoeft te worden uitgevoerd onder de huidige regelgeving. De auteurs geven een aantal aanbevelingen om de regelgeving ten aanzien van het klinische gebruik van niet-genetisch gemodificeerde niet-humane virussen te verduidelijken. Zij hopen dat de informatie in dit rapport zal bijdragen aan de gedachtewisselingen over de mogelijk ongewenste gevolgen die geassocieerd kunnen zijn aan het klinisch toepassen van dergelijke virussen. Verduidelijking van de positiebepaling en de regelgeving kunnen verdere ontwikkelingen van niet-humane virussen als klinisch-toepasbare entiteiten stimuleren.

SUMMARY

All living organisms are exposed to viruses. In general, viruses infect only a single host species or a restricted set of host species. In very rare cases productive infection may occur outside the natural host(s) species. This may be manifested by a distinct disease in the new host. Although very rare, this could lead to establishment and persistence of the virus in the new host. In view of the knowledge that virus adaptation can occur, the intensive use of non-human viruses and their genetically modified variants as therapeutic agents in clinical therapy may be cause for concern. The administration of non-human viruses (either systemically or intratumoral) to humans may have an associated risk of adapting the virus to increase replication in humans. Theoretically this could result in horizontal transfer of adapted viruses to the recipient's relatives or medical personnel. This is evidently undesirable.

The risks associated with spread of the administered therapeutic virus from patients are unknown. Therefore the Commission on Genetic Modification (COGEM) commissioned a study which aims at resolving the following questions:

- What non-human viruses are being developed for use as therapeutic agent, and at what stage have the development arrived at?
- What are the key biological parameters to consider when assessing the risks of the pre-clinical and clinical activities with such viruses?

This report presents the result of this literature study on non-human viruses that are used, or developed to be used, as therapeutic agents for use in humans. The prime indications for such clinical use are found in cancer treatment. Classical vaccines fall outside the scope of this study.

The study is limited to those non-human viruses and viral vectors that are replication-competent in unmodified cells of their natural host. Replication-defective viruses, i.e. viruses and viral vectors that require special helper functions for their replication, and are therefore replication incompetent in unmodified cells of their natural host, are excluded in this survey. The reason for excluding the latter viruses and vectors is that they have an additional level of containment since they must re-acquire the genetic information to allow functional complementation of the missing function.

Based on articles in published scientific literature, congress reports, as well as from websites from companies involved in developing such products, and on the information obtained upon consultation of a number of national and international experts, 27 virus species have been identified that are evaluated pre-clinically or clinically. For each of the virus families a brief account of virus' biology is presented. Where pertinent, information is presented on their past, current, and anticipated

clinical use. Based on these data, each of these viruses was assigned to one of five *relative environmental risk* categories, ranging from '*Negligible*' to '*Very High*'. The use of two viruses were assigned to the '*Negligible*' category (canary pox virus and the *Autographa californica* baculovirus), and three viruses to the '*High*' category (viz. Seneca Valley virus, murine leukemia virus and Maraba virus). In the latter group, a complicating factor is the absence of publicly available information key aspects of the biology of these viruses. Therefore the scores of these viruses have cautiously been increased and these viruses have been placed in the group of viruses with a '*High*' relative environmental risk. None of the viruses were placed in the '*Very High*' group.

The authors deem it essential that an environmental risk assessment is performed before clinical use of non-human viruses is permitted, even if these viruses are not genetically modified organisms in the formal sense. Such assessment may not be formally required to date. The authors make a number of recommendations for clarifying the pertinent regulation for clinical use of non-modified non-human viruses. The information contained in this report should stimulate and facilitate discussions on the potential hazards that may be associated with such use. Clarification of the position of the regulatory authorities may stimulate further clinical developments of viruses as powerful therapeutic agents.

INTRODUCTION AND OUTLINE OF THIS STUDY

Already in the 19th century, the observation was made that occasionally cancer patients who contracted an infectious disease went into brief periods of clinical remission. In the case of leukemia, it was well recognized that contraction of influenza sometimes produced beneficial effects. These early observations predate the formal demonstration that influenza was, in fact, caused by a virus.

These observations led to the initiation of formal clinical studies in the 1950's. In these clinical studies a range of human viruses was administered to cancer patients, including Hepatitis B virus, West Nile virus, adenovirus, and mumps virus. While some anecdotal evidence of antitumor efficacy was obtained, the side effects were severe. In an effort to reduce the side effects and to circumvent the inhibitive effects brought about by neutralizing immunity, several non-human viruses entered the stage, including Newcastle disease virus and vesicular stomatitis virus. However, after the advent of new cytostatic drugs and the problems experienced in attenuating some candidate viruses, the oncolytic virus approach was largely abandoned.

The rise of gene-therapy as a clinically feasible approach for treating human diseases led to a revival of the oncolytic-virus approach. Moreover the recombinant DNA technology offers effective systems for reverse genetics in many virus families. This has allowed designing of new and potentially more efficacious oncolytic viruses, and even arming the replicative oncolytic viruses with therapeutic transgenes. Again, the absence of immunity in the human population has stimulated the evaluation of non-human viruses as clinically applicable oncolytic agents.

The revival of the use of non-human viruses as oncolytic agents brought back the bio-safety issue. In addition to the question how the oncolytic virus affects the patient, the fact that the agent is infectious and can potentially be shed from the patient, yields the possibility of horizontal transfer of the viral anti-cancer therapeutics. The use of such viruses requires procedures to reduce such transfer and minimize the risks associated with it.

So far, to the best of our knowledge, no specific guidance exists for estimating and evaluating the environmental risks associated with the clinical use of non-human viruses as therapeutic agent e.g. in cancer therapy. This seems at odds with the specific regulation that is in place in xenotransplantation.

The critical shortage of human donor material that is available for transplantation has triggered much interest in the use of animals as an alternative source of cells, tissues and whole organs for transplantation into humans, that is, xenotransplantation. The two main sources of clinically relevant cells and tissues are pigs and non-human primates (NHPs). NHPs would be the preferred choice since

they are most closely related and physiologically and immunologically most similar to humans. For a number of reasons, including ethical considerations, their small anatomical size, and their limited availability, they have been rejected as donor animals. Also their use is associated with the risk of transmission of a number of pathogens that are infectious for humans. Pigs are considered to be the most suitable alternative source. The pigs have large anatomical and physiological similarity with humans such as the size of the organs and the circulatory system. They are readily available and have relatively short reproduction cycles and large litter sizes. In addition, pigs can be bred in genetically homogeneous-specific pathogen-free (SPF) herds in a controlled environment.

A major concern for use of porcine material is the risk of infections of the human recipients upon exposure to the porcine graft. Xenotransplantation is considered a serious public health concern because it has the potential to transmit porcine pathogens. The main concern has been for transmission of porcine endogenous retroviruses (PERVs) that are present in the pig genome in multiple copies. Xenotransplantation is therefore a potential route by which zoonotic viruses may enter the human population. Since then, the risk of transmission posed by PERVs has remained a topic of concern and discussion for public health authorities. This led to a precautionary approach and instigation of moratoria on clinical trials involving xenotransplantation in many countries.

To establish the status of the use of non-human replication-competent viruses for therapeutic applications such as oncolytic-virus therapy, we have performed a literature search and discussed the topic with a number of national and international experts. This resulted in a table that summarizes the stage to which the research has progressed for each of the viruses identified. Viruses that are developed for use as conventional vaccines are excluded from this study. For the development of such viruses clear guidelines and criteria have been established.

For each of the pertaining virus families, the literature is succinctly described with the following topics:

- Pathogenesis, Disease associations, and Epidemiology,
- Host range and Tissue tropism
- Current status and stage of the research activities
- Available information in humans or humanized systems
- Bio-selection and Genetic Modifications
- Horizontal transmission and establishment in the human population
- Environmental risk factors and Biosafety considerations

A finalizing summarizing table provides an integral status update on the use of non-human viruses. This status update may help judging the necessity of new regulation

and facilitate discussions on how to develop rational risk evaluations in this blooming field.

The study has further benefitted from feedback, insights and helpful discussions with the members of the COGEM Project Steering Committee that comprised the following members:

- *Dr. Raoul de Groot (Utrecht University, Utrecht)*
- *Dr. Ben P. Peeters (Central Veterinary Institute of Wageningen UR, Lelystad)*
- *Dr. Victor van Beusechem (VU Medical Center, Amsterdam)*
- *Drs. Derrick Louz (GMO Office of National Institute for Public Health and the Environment; RIVM, Bilthoven)*
- *Dr. Erik Schagen (COGEM, Bilthoven)*

The authors would like to express their gratitude to the members of the Project Steering Committee and all colleagues that have contributed to these studies by providing input and for the stimulating discussions.

METHODS

Based on published research articles, congress reports, as well as on the information obtained upon consultation of a number of national and international experts, we compiled a list of non-human viruses developed as therapeutic agents. For the survey we performed in depth cross-reference searches and accessed several online resources to extract relevant data.

The following online sources (Internet sites) have been accessed (until September 2010) by broad search term/keyword "animal virus AND oncolytic":

http://www.ncbi.nlm.nih.gov/pubmed
http://clinicaltrials.gov/
http://clinicaltrialsfeeds.org/
http://www.google.com/
http://en.wikipedia.org
http://www.genetherapynet.com/
http://www.fda.gov/default.htm
http://www.ema.europa.eu
http://www.cancer.gov/

The following colleagues have been consulted on non-human therapeutic viruses in general, and their pertinent field of expertise, in particular:

- *Prof. Dr. Stefan Kochanek (University of Ulm, Ulm, Germany)*
- *Prof. Dr. Pierre Boulanger (Université de Lyon, Lyon, France)*
- *Prof. Dr. Norman Maitland (University of York, York, UK)*
- *Prof. Dr. Leonard Seymour (University of Oxford, Oxford, UK)*
- *Prof. Dr. Emmanuel Wiertz (University Medical Center Utrecht, Utrecht, Netherlands)*
- *Prof. Dr. Akseli Hemminki (University of Helsinki, Helsinki, Finland)*
- *Dr. Rik Bleijs (GMO Office / Gene Therapy Office, Bilthoven, Netherlands)*
- *Dr. Marjolein Kikkert (Leiden University Medical Center, Leiden, Netherlands)*
- *Dr. Leif Lindholm (Got-A-Gene, Gothenburg, Sweden)*
- *Dr. Kerry Fisher (University of Oxford, Oxford, UK)*
- *Dr. Frans Rijsewijk (Fed. University of Rio Grande do Sul, Porto Alegre, Brazil)*
- *Dr. Caroline Brown (WHO Regional Office for Europe, Copenhagen, Denmark)*

With the results of these surveys we have compiled the list of viruses described in tables 1 and 2.

RESULTS AND CONCLUSIONS

The use of oncolytic viruses for the treatment of patients with cancer is not a new one. To date, a wide variety of viruses is being explored for use as oncolytic agent. The hope of the safe and efficacious use of viruses as anti-cancer agents has been fuelled by the (largely) anecdotal evidence of antitumor efficacy and the history of safe use of viruses as live-virus vaccines. Excellent and extensive historic overviews of the clinical experience with this approach were written by Sinkovics and Horvath (1993, 2008).

There has been a continued and expanding interest in the use of non-human viruses for this purpose. There are two main reasons for this interest. The first is the (virtual) absence of immunity against non-human viruses in the human population. This may provide the viral oncolytic agent a head start, by allowing the therapeutic virus to be administrated without the risk that the pre-existing neutralizing immunity inactivates the vector and frustrates transduction of tumor cells. The other reason is that many non-human viruses have not been associated with diseases in humans. In addition recombinant-DNA technology and classical attenuation schemes offer the prospects of overcoming limitations imposed on their use by pathogenicity in humans, should this occur.

So far there has not been a comprehensive inventory of non-human viruses considered to be used in clinical gene therapy. To fill this gap, the Netherlands Commission on Genetic Modification (COGEM) commissioned a study that should provide such overview. In this report the results of this study precipitate. This report identifies 27 different non-human virus species that are being considered for (future) clinical application. These include DNA viruses (Table 1) as well as RNA viruses (Table 2). For each of the viruses a summary is provided of the salient details of the virus biology, their pathology and the current status of the research aiming at developing these viruses as clinically applicable entities.

These viruses are in various stages of development. At least 7 of these have already been evaluated in formal clinical studies (canary pox virus, infectious bursal disease virus, Newcastle disease virus, Seneca Valley virus, Sindbis virus, Semliki Forest virus, murine leukemia virus), and several others have been announced to follow soon. From these data it is reasonable to assume that the next few years will witness a rising number of clinical trials in which non-human viruses are used as therapeutic agents.

Genome	Family	Genus	Species	Pathogenicity (in natural host)	Preclinical studies		Clinical studies	Factors that *increase* or **decrease** the relative environmental risk of the virus	Relative environmental risk
DNA					*In vitro*	*In vivo*			
ds- linear	***Poxviridae***	Orthopoxvirus	**Raccoonpox virus**	-	**+**	**+**	-	**No known pathology in mammals, safely used as vaccine, only mild pathology seen in accidental human infections**	Low
		Leporipoxvirus	**Myxoma virus**	+	+	+	-		Medium
		Yatapoxvirus	**Yaba-like disease virus**	+	+	-	-		Medium
		Avipoxvirus	**Canarypox virus**	+	+	+	+	**No productive infection in human cells**	Negligible
	Herpesviridae	Rhadinovirus	**Bovine herpesvirus 4**	+	+	+	-		Medium
			Herpesvirus Saimiri	+	+	+	-		Medium
		Varicellovirus	**Bovine herpesvirus 1**	+	+	-	-	**Virus circulates world wide, no known pathology in humans despite intensive contact with cattle.**	Low
			Suid herpesvirus 1	+	+	-	-	*Aujeszky's disease-free status can be jeopardized*	Medium
ds circular	***Baculoviridae***	Baculovirus	***Autographa californica* baculovirus**	+	+	+	-	**No productive infection in human cells**	Negligible
ss linear	***Parvoviridae***	Parvovirus	**Rodent parvovirus H-1 & Minute virus of mice**	+/-	+	+	planned	**Causes mainly subclinical infections in rodents**	Low
			Feline panleukopenia virus	+	+	-	-		Medium

Table 1: Non-human DNA viruses

This table provides a summarizing overview of current oncolytic DNA-virus candidates, their progress towards clinical applications, and the arbitrary environmental risk associated with their use in humans. The arbitrary relative environmental risk is scaled from Negligible, Low, Medium, High, and Very High. The scale represents the authors' estimate of the environmental risks assigned to the clinical use of the viruses based on the aggregate of factors described in the family chapters. The wild-type viruses are assessed, and the default classification is 'Medium'. The default class was adjusted if there are factors that positively or negatively affect the risk for the environment. If the publically available information is found inadequate, the score was increased to provide caution.

Genome	Family	Genus	Species	Pathogenicity (in host)	Preclinical studies		Clinical studies	Factors that *increase* or **decrease** the relative environmental risk of the virus	Relative environmental risk
RNA					*In vitro*	*In vivo*			
ds	***Birnaviridae***	Aribirnavirus	**Infectious bursal disease virus**	+	+	+	+		Medium
	Reoviridae	Orbivirus	**Bluetongue virus 10**	+	+	-	-	**Transmission via insect vector**	Low
ss - strand	***Paramyxoviridae***	Avulavirus	**Newcastle disease virus**	+	+	+	+		Medium
		Respirovirus	**Sendai virus**	+	+	+	-		Medium
	Rhabdoviridae	Vesiculovirus	**Vesicular Stomatitis virus**	+	+	+	Planned in 2010	**Transmission via insect vector** *Aerosol-mediated transmission to humans*	Medium
			Maraba virus	Unknown	+	+	-	**Transmission via insect vector,** *Very little information available on natural host and disease associations*	High
ss +strand	***Coronaviridae***	Coronavirus	**Mouse hepatitis virus**[1]	+	+	+	-		Medium
			Feline coronavirus[1]	+	+	-	-		Medium
	Picornaviridae	Enterovirus	**Bovine enterovirus**	+	+	+	-		Medium
		Senecavirus	**Seneca Valley virus**	Unknown	+	+	+	*Very little information available on natural host and disease associations*	High
		Cardiovirus	**Encephalomyocarditis virus**	+	+	+	-		Medium
	Togaviridae	Alphavirus	**Sindbis virus**	+	+	+	-	**Transmission via insect vector**	Low
			Semliki Forest virus	+	+	+	-	**Transmission via insect vector**	Low
	Retroviridae	Gammaretrovirus	**Moloney murine leukemia virus**	+ (oncogenic)	+	+	+[2]	*Little information on amphotropic MuLV in primates, amphotropic MuLV demonstrated to be oncogenic in non-human primates.*	High
		Spumavirus	**Foamy virus**	-	+	+	-	**No known disease associations in host and non-host species**	Low

Table 2: Non-human RNA viruses

This table provides a summarizing overview of current oncolytic DNA-virus candidates, their progress towards clinical applications, and the arbitrary environmental risk associated with their use in humans. The arbitrary relative environmental risk is scaled from Negligible, Low, Medium, High, and Very High. The scale represents the authors' estimate of the environmental risks assigned to the clinical use of the viruses based on the aggregate of factors described in the family chapters. The wild type viruses are assessed, and the standard classification is 'Medium', and the default class was adjusted if there are factors that positively or negatively affect the risk for the environment. If the publically available information was found inadequate, the score was increased to provide caution.

[1]) The virus requires artificial modification of the host range as the wild type virus is unable to infect human cells.

[2]) Recruiting (A Study of a Replication Competent Retrovirus Administered to Subjects With Recurrent Glioblastoma, Tocagen Inc.)

However, there is a finite risk associated with the widespread clinical use of non-human viruses. Cancer patients frequently have a compromised immune function. This may allow the therapeutic viruses to replicate for prolonged periods of time and eventually to become adapted for efficient replication in humans. If the virus would have, or would acquire, the capacity to spread from the patient to health-care workers or the patients' relatives the virus can become established in the human population. While the authors are not aware of any examples of outbreaks of viruses as a consequence of intentional exposure of humans, recent history have provided several examples of spontaneous cross-species transfer. Examples are discussed in detail by Louz et al., (2005) and include e.g. the recent H1N1 'Swine Flu' influenza A virus, the SARS-corona virus, and in the more distant past, the HIV lentiviruses.

It is the risk of the transfer of non-human viruses to human hosts, which led many countries, including the Netherlands, to impose a moratorium on the clinical use of xenotransplantation, and more specifically the clinical use of porcine tissues. Pigs carry several copies of so-called porcine endogenous retroviruses (PERVs) in their genome. These PERVs can be transcriptionally activated and lead to formation of infectious retroviruses (reviewed by Louz et al., 2008). If porcine tissues are used for xenotransplantation, the PERVs can infect the host. This transfer is facilitated by the long-term immune suppression that is required in a xenotransplantation setting. While there is world-wide consensus on the moratorium on xenotransplantation, there seems no specific guidance for developing clinical strategies involving the deliberate administration of non-human viruses with a therapeutic intent. The formulation of guidelines to ensure appropriate assessment of patients and environmental risks may be appropriate to date. Such guidelines could come either as 'points-to-consider' for the investigators or as more formal regulation. In this regard it is relevant to recognize that many of the viruses that are being developed are not by definition 'genetically modified organisms'. Hence their use may not demand the environmental risk assessments that are part of the formal national or international (e.g. European) GMO regulations. The EMEA ICH Considerations document 'Oncolytic viruses' (EMEA/CHMP/ICH/607698/2008) does not provide guidance relating the management of environmental risks: while it advises to consider barrier contraception for the duration of the clinical trial as a standard precaution to prevent person-to-person transmission it does not address other environmental risks. It merely states *'Many of these considerations might fall under the heading of environmental release/risk and regional authorities should be contacted for details.'*

Nevertheless, the GMO guidelines may provide a scaffold for formulating guidelines for environmental risk assessments for the use of non-GMO non-human viruses for clinical trials. The assessment of patient-safety aspects may adopt some of the quality guidelines that have been established for evaluating human vaccine safety. The authors have devised a flowchart (Figure 1) that may provide guidance on defining the elements that need to be covered in the environmental risk assessment (ERA).

While we are not aware of the existence of such flowchart that outlines riskfactors for use in ERAs of GMO viruses, the elements that need to be considered in the ERA are very similar for GMO viruses and non-GMO viruses.

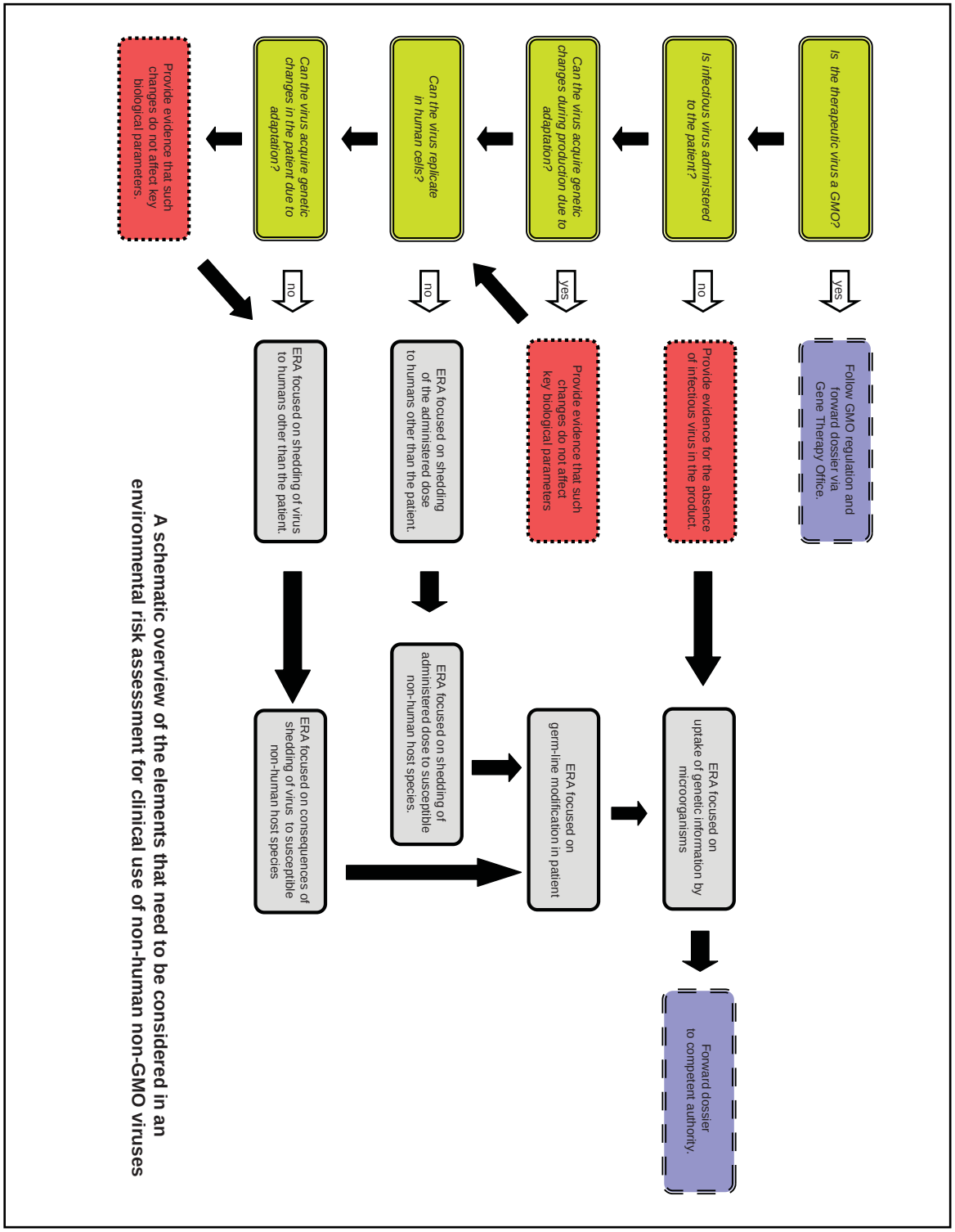

Figure 1. Outline of ERA elements for non-GMO viruses

Represented are the elements that should be considered in the ERA. The decisive questions are indicated in green, essential information requested is indicated in red, and the individual aspects to be covered in the ERA are indicated in grey. The decisive answers are indicated with white arrows, forced choices, e.g. the routes imposed if the alternative option is invalid, are indicated with black arrows. The routes end at forwarding the dossier at the competent authorities. For non-GMO viruses, there is uncertainty which authority this should be.

To evaluate the environmental risks involved in clinical use of non-human non-genetically modified viruses it is suggested that the ERA provides information on the following questions:

Virological and biological parameters:

- Is the virus replication-competent in human cells?
- Does the virus produce infectious progeny virus in human cancer cells?
- Does the virus produce infectious progeny virus in human non-cancerous cells?
- Does the virus cause viremia in humans?
- Is the replication of the virus restricted in humans, in such a way that it provides a level of biological containment?
- Is the therapeutic virus an attenuated derivative of a naturally circulating virus?
- Can the therapeutic virus interfere with other viruses that may be present in the treated patient (for example through recombination, (re)activation, immunosuppression) as to cause adverse effects for health or spread?
- Is there evidence of prior exposure in humans and what were the consequences?

Parameters relating to intended clinical use:

- Can the virus be shed from the patient with the proposed clinical use?
- Can the virus shed from the patients infect susceptible host species?
- Can the virus adapt itself in such a way that the capacity of the virus to spread beyond the treated patient is positively affected?

Parameters relating to potential consequences:

- What can be the potential consequences of unintended exposure of non-target humans?
- What can be the biological consequences of unintended exposure of the non-human susceptible hosts?
- What are the potential economical consequences of unintended exposure of the non-human susceptible hosts?

Parameters relating to risk management:

- Is a vaccine available that can be used for prophylactic treatment in humans?
- Is a vaccine available that can be used for prophylactic treatment in the normal host species?
- Are effective antivirals available that can be used for treatment of virally infected humans?

Box 1: Issues to be covered in the ERA

In addition, we listed a number of the key questions on the therapeutic viruses that specifically relate to the intended use (Box 1). These questions should raise awareness to topics that, if pertinent, should be covered in the ERA.

A large number of different virus species is being developed. Within these virus species there can be marked differences in relevant parameters between different isolates and serotypes. In addition, from many of these viruses, attenuated derivatives were isolated that are sometimes used as vaccines. Vaccine strains can

have properties that differ significantly from the wild-type viruses. During this desk study, it was noted that in published literature there is often limited and insufficient information provided on the precise nature of the viruses used. This frustrates the use and the extrapolation of these data.

The road for developing a particular virus to a clinically applicable product is usually a long one. A virus that made a remarkably fast progress toward clinical use is Seneca Valley virus (SVV). This virus was identified only in 2002 as a contaminant in the cell culture medium. It is the sole member of a new genus in the *Picornaviridae.* There is limited information on this virus and there are considerable uncertainties on its natural host and reservoir. The human population largely lacks neutralizing immunity against SVV. The virus was found to exhibit strong cytolytic activity in several human tumor cell lines. Notwithstanding the scanty information on its natural biology in published literature, the virus was developed as oncolytic agent. Already four clinical trials have been initiated, including one in children. All these trials were approved in the United States. It would be very interesting to study if and how environmental concerns have played a role in the evaluation process that led the American authorities to allow these clinical studies.

For all virus species identified in this study we have assigned a relative environmental risk. This score is based on several factors, including the capacity of the virus to replicate productively in human cells, the potential of amplification and shedding of the virus, and the potential of the virus to be transferred horizontally. In addition, the potential consequences of shedding on the natural host species are taken into account. This score is determined among others on the basis of the occurrence of this virus, its pathogenicity, and the availability of a vaccine. If limited information is available, which leads to a high level of uncertainty, the viruses are classified cautiously. This led to five categories for *relative environmental risks* 'Negligible, Low, Medium, High, and Very High'. The 'Medium' category was used as start point in the classification, and the assignment was scaled up or down based on factors that strongly affect the relative environmental risk. Essential factors are listed in the virus chapters, and the most important of these are included in Tables 1 and 2. None of the viruses were classified in the 'Very High' relative environmental risk category, while three viruses are classified as 'High'. Furthermore, two viruses are scored as the 'Negligible'. This concerns *Autographa californica* Baculovirus and the Canarypox virus.

The availability of registered vaccines may be advantageous to protect subseceptible hosts in the (unlikely) situation that shedding leads to infection on non-human host species. In table 3 the viruses are listed for which veterinarian vaccines are available. Note that the available vaccines do not necessarily protect against the serotypes, strains, or isolates that are being developed for use as therapeutic virus in humans.

Virus species
Canarypox virus
Bovine herpesvirus 1
Myxoma virus
Suid herpesvirus 1
Feline panleukopenia virus
Infectious bursal disease virus
Blue-tongue virus 10
Newcastle disease virus

Table 3: Viruses for which veterinarian vaccines are available
Data were extracted from the websites: http://www.cbip-vet.be/nl/nldrugsearch.php and http://www.ema.europa.eu/ema/.

Viruses placed in the 'High' relative environmental risk category

Three viruses are placed in the 'High' risk category; Maraba virus, Seneca Valley virus and Murine Leukemia virus. The relative score is rated as 'High' due to the limited information that is available on the virus, its natural host(s), and its disease associations.

Maraba virus is a Rhabdovirus isolated from sand flies in Brazil. Its natural mammalian host (if any) is unknown, but this virus is known to cause severe pathology in newborn mice. Despite the fact that the virus probably requires an insect host for transmission between susceptible mammals, we classify the relative environmental risk as '**High**' based on the scanty information on the biology and pathology of Maraba virus.

Seneca Valley virus is a Picornavirus that was described only in 2002 after being isolated as a contaminant in cell culture medium of cultured cells. While there is some evidence that it was derived from infected pigs, the information of its natural host and disease associations is limited. The virus was found to be strongly cytolytic in a range of human tumor cells, while barely affecting non-transformed cells. Based on these findings the virus was developed as clinically applicable oncolytic agent in record time, and so far 3 clinical trials have been initiated, including one in children (clinical trial ID: NCT01048892). Nevertheless, the limited information on the susceptible of other mammalian species, the uncertainties on its natural host, and the absence of shedding data in published literature led us to classify the Seneca Valley virus '**High**' in the relative environmental risk score.

Murine Leukemia Virus is a murine Retrovirus that has been studied very intensively for its capacity to induce leukemia in newborn and immune suppressed mice, hence their designation as 'RNA tumor viruses'. To allow efficient infection of human cells, the envelope protein of the conventional MuLV, which infects primarily rodent cells, needs to be replaced with an envelope of a virus that has the capacity to infect human cells. This 'envelope-pseudotyping' is essential for clinical gene therapy with MuLV. Often the envelope of an 'amphotropic' retrovirus isolate is used for

pseudotyping. Replication-defective derivatives of MuLV have been used frequently as gene transfer vector in clinical gene therapy. While generally safe and well tolerated, this has led to a number of cases where T-cell leukemia's developed in the recipient as result of insertional mutagenesis and activation of proto-oncogene expression. The use of replication-competent MuLV-derived vectors in cancer gene therapy is a novel approach. The uncertainty surrounding the effects of a replication-competent amphotropic MuLV led us to classify '**High**' in the relative environmental risk score.

Viruses placed in the 'Negligible' relative environmental risk category

At the low end of the spectrum of relative environmental risks the *Autographa californica* Baculovirus and the Canarypox virus are placed. Their relative risk is scored as '**Negligible**'.

The ***Autographa californica* Baculovirus** has a very narrow host range that is limited to a single moth species, the Alfalfa Looper *Autographa californica*, which occurs in the western halve of the North-American continent. While the virus transduces cells of many mammalian species, no baculovirus genes are expressed and the viral genome is not replicated. In human blood the insect-cell produced viral particles are rapidly inactivated by human complement. The very narrow host range, the virus incapacity to replicate in mammalian cells, and therefore the limited capacity of the virus to be shedded from the patient led us to classify the relative environmental risk of baculoviruses as '**Negligible**'.

ALVAC-based Canarypox viruses have a host range that is very narrow and limited to canaries (*Seriunus sp.).* Also the ALVAC-strain used in clinical studies is strongly attenuated in its natural host. Thus ALVAC-based canary pox vectors are unlikely to become a threat to the environment. Since the virus does not replicate in primate cells, the virus does not amplify and shedding is limited to maximally the administered dose. The very narrow host range, the virus incapacity to replicate in human cells, and therefore the limited capacity of the virus to be shed from the patient led us to classify the relative environmental risk of canary-pox virus as '**Negligible**'.

The viruses placed in the 'High' and 'Negligible' relative environmental risk categories have been scored according to the parameters listed in Box 1 in a binary fashion (Yes / No / Not known). The score forms are added in Appendix 1.

So far, no clinical studies involving therapeutic administration of non-human viruses have been performed in the Netherlands. However, several Dutch patients have been treated abroad, e.g. in Germany, with Newcastle Disease virus. In view of the considerable research efforts in this field, and the interest in oncolytic virus therapy

in Dutch cancer research, it is reasonable to anticipate the initiation of such trials in the Netherlands in the near future.

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RECOMMENDATIONS

- It is reasonable to anticipate that clinical use of non-human therapeutic viruses will be explored in the Netherlands in the near future. For activities involving genetically-modified viruses the legal framework permitting such activities is embedded in national (Dutch) and international (EU) regulations. The competent authorities are defined, and the procedures are well established and widely known in the field. The procedures for obtaining permission for clinical application of genetically-modified viruses require a proper step-wise environmental risk assessment. The authors consider it essential to perform a similar step-wise environmental risk assessment before initiation of clinical application of viruses that are not considered genetically-modified organisms.

- The clinical application of non-genetically modified viruses does not fall under the regulation pertaining to genetically-modified organisms. Therefore it is unclear to the authors if there is any environmental risk assessment formally required before such use can be initiated. The authors recommend delineating the current regulatory framework pertaining to therapeutic clinical application of infectious non-genetically modified non-human viruses.

- Knowledge of the regulatory framework pertaining to the therapeutic clinical application of infectious non-genetically modified non-human viruses is essential for prospective users of the technology. Therefore we recommend active dissemination the regulatory framework pertaining to the use of non-genetically modified viruses to parties working in this field. The Dutch Gene Therapy Office (*Du:* het Loket Gentherapie), as well as the Dutch Society of Gene Therapy (NVGT), could take an active role in this proces. Proactive elucidation of the regulatory framework could prevent the need of taking ad-hoc measures when intended use is announced in the near future.

- Rather than defining a set of strict goals and binding criteria, the authors suggest formulating points-to-consider in such environmental risk assessment of therapeutic clinical application of non-genetically modified viruses. Such points-to-consider list could be used to define the criteria in consultation with investigators. Ingredients for the ERA are summarized in Figure 1 and Box 1 of this report. This approach may increase the appeal of the Netherlands for performing clinical studies with non-human, non-GMO viruses if goals and criteria would more widely be announced.

DESCRIPTION OF THERAPEUTIC VIRUSES

Family *Poxviridae*

Poxviruses (members of the family Poxviridae) are viruses that can, as a family, infect both vertebrate and invertebrate animals. Poxviridae comprises two subfamilies: Chordopoxvirinae (8 genera) and Entomopoxvirinae (3 genera). Within subfamily Chordopoxvirinae, four genera of poxviruses may infect humans: Orthopoxvirus (variola virus, vaccinia virus, cowpox virus, monkeypox virus, smallpox (eradicated)), Parapoxvirus (orf virus, pseudocowpox, bovine papular steatites virus), Yatapoxvirus (tanapox virus, yaba monkey tumor virus), and Molluscipoxvirus (molluscum contagiosum virus (MCV)). The most common are vaccinia (seen on Indian subcontinent) and molluscum contagiousum, but monkeypox infections are rising (seen in west and central African rainforest countries). Viruses that belong to genus Avipoxvirus (e.g. Canarypox virus) and Leporipoxvirus (e.g. Myxoma virus) do not infect humans. However, their importance rose recently due to their applicability in medical research such as development of a carrier vaccine (Canarypox virus) or as viral oncolytic agent (Myxoma virus).

Poxviridae viral particles (virions) are generally enveloped. They are very large, brick-shaped viruses about 300 x 200 nm (the size of small bacteria). The virion carries a single, linear, double-stranded segment of DNA (about 200 kbp in size). This DNA genome is enclosed within a "core" that is flanked by 2 "lateral bodies". The surface of the virus particle is covered with filamentous protein components. The entire particle is enclosed in an envelope derived from the host cell membranes.
Replication of the poxvirus involves several stages. The first step involves bind to a receptor on the host cell surface; the receptors for the poxvirus are thought to be Glycosaminoglycans (GAGs). After binding to the receptor, the virus enters the cell, uncoats and replication occurs in the cytoplasm. Considering the fact that this virus is large and complex, replication is relatively quick taking approximately 12 hours.
Most poxviruses are host-species specific, but vaccinia is a remarkable exception. True pox viruses are antigenically rather similar, so that infection by one elicits immune protection against the others.

A range of pox viruses cause febrile illnesses in man and animals with a prominent vesicular rash. The most prominent of them was smallpox virus (variola) which caused a severe (deadly) disease in man but which has now been eradicated by intensive international vaccination (The last cases of variola major occurred in the Indian subcontinent in 1975. The last case of variola minor occurred in Somalia in 1977. The last cases of smallpox occurred in a Birmingham laboratory in 1979). In 1980, the world was declared to be free of smallpox.

Vaccinia virus played a crucial role in one of the greatest achievements in medicine: the eradication of smallpox. The precise origin of vaccinia virus is unknown, however, there is a speculation that the virus was originally isolated from horses or derived from the cowpox virus through serial passages under artificial conditions.

Due to its role in the eradication of smallpox, vaccinia virus has the longest and most extensive history of use in humans. This historical role has also led to a detailed understanding of vaccinia biology and pathogenesis. The fact that more people have been deliberately infected with vaccinia then with any other infectious agent means that we have unprecedented information on its behavior in humans, including information on risk groups, adverse effects and possible treatments. Currently, vaccinia virus is the most commonly used poxvirus in cancer therapies (clinical trials phase I, II and III) (Kirn and Thorne, 2009). Clinical data has now been published for one targeted and armed oncolytic poxvirus, JX-594 (Jennerex Biotherapeutics) (Park et al., 2008). This is a Wyeth strain vaccinia with inactivation of TK and transgenic expression of GM-CSF, under control of a synthetic early and late promoter (Kim et al., 2006). Importantly, biosafety issues have been addressed and it has been reported that no virus shedding has been detected from patients treated with JX-594 to date. In this respect, the lack of airborne spread of vaccinia is a crucial advantage for further medical-therapeutic and clinical use.

However, in order to avoid possible pre-existing host immunity to vaccinia virus vectors (arising from smallpox vaccination), other members of the Poxviridae family have been considered as recombinant vaccine vectors and as anti-cancer (oncolytic virus) therapeutics. Specifically, the newest viral oncolytic candidates include viruses that cause no disease in humans, yet still have natural tumor tropism. Current interest centers on nonhuman poxviruses such as Raccoonpox virus (Orthopoxvirus) and Myxoma virus (Leporipoxvirus). Another interesting candidate, Yaba-like disease virus (Yatapoxvirus), can cause zoonotic infections in humans (mainly caretakers handling infected monkeys).

Genus Orthopoxvirus
Raccoonpox virus

Pathogenicity/ Disease association/Host range/ Tissue tropism
Raccoonpox virus (RCNV) is a member of the genus Orthopoxvirus, and closely related to vaccinia virus and cowpox virus. Raccoonpox virus was first discovered in a screen of outwardly healthy raccoons in Maryland in 1961. Whereas natural habitat of raccoons is North America, the population has been introduced in Europe during mid-20th century (Germany, Russia). Although viral exposure of the wild population is probably widespread, the prevalence of raccoonpox disease in North America is not well known; there is little information on the naturally occurring disease in raccoons. It has no known pathology in any species (including raccoons), which

suggests that its "natural" host may still be undiscovered. Raccoons that have been experimentally inoculated with raccoonpox virus exhibited successful induction of protective immunity without any concurrent pathology (Thomas et al.; 1975). Furthermore, a study of Maryland, USA wildlife published in the 1970s found a seroconversion rate of 23% in wild raccoons but a search of the Wildlife database at the University of Guelph (Ontario, Canada) revealed no cases. At present there is only one case report of raccoonpox infection in a Canadian cat. This novel strain was isolated from the forepaw of domestic cat. Biopsy of the initial ulcerative lesion on the forepaw revealed ballooning degeneration of surface and follicular keratinoctyes.
Raccoonpox virus has been tested for its ability to infect and kill human tumor cells. The oncolytic capacity of this virus has been tested against established cancer cell lines from the NCI-60 cell panel. The NCI-60 is a set of 59 human cancer cell lines derived from diverse tissues; brain, blood and bone marrow, breast, colon, kidney, lung, ovary, prostate and skin. The cell lines were supplied by the NCI/NIH Developmental Therapeutics Program (http://www.sanger.ac.uk/genetics/CGP/NCI60/). Approximately 74% of human cell lines tested were permissive for raccoonpox infection. All murine cancer cells tested showed significant virus replication. According to this observation it can be concluded that raccoonpox virus has a broad tissue tropism with preference for cancer cells. In addition, the virus also demonstrated replication in three normal human cell types: HUVECs, HMVECs and astrocytes (Hast) in vitro. Importantly, pretreatment of these cell lines with type I IFN resulted in an up to four-log decrease in virus replication. The effect of IFN pretreatment on cancer cells infection was negligible. Thus, the replication of Raccoonpox virus in normal cell types is significantly dampened by pretreatment with IFN (Evgin et al. 2010).

Current status and stage of the research activities

The stage of the research activities is currently at the level of in vitro and in vivo studies and comprises of only one published report (Evgin et al. 2010). However, due to encouraging reports regarding safety and efficacy of vaccinia virus as oncolytic agent in the clinic to date, there is an increasing support for the idea that the poxvirus platform is a good starting point for the identification of additional poxvirus oncolytic agents. Since raccoonpox virus has no known significant pathology in any mammalian species, appears as naturally attenuated poxvirus, and it has inherent oncolytic activity, it is likely that this virus will be very soon added to the arsenal of viral anticancer agents under development for treatment of human tumors. At this stage it can be advised to further follow the research concerning the use of raccoonpox virus as oncolytic agent. It can be expected to advance as clinically applicable agent.

Available assessment information in humans or humanized systems

Currently there are no reports on Raccoonpox virus in-use as an oncolytic agent in humans. Raccoonpox virus has been used as a vaccine vector in mice, eliciting

excellent immunity to Yersinia pestis and rabies virus without any adverse effects of the vector (Rocke et al. 2009; Lodmell et al., 1991).

Raccoonpox virus has been developed as a vaccine vector for rabies protection in a variety of animals such as raccoons, prairie dogs, domestic dogs and cats. Importantly, none of the treated animals exhibited clinical side-effects. Recombinant raccoonpox vectors have been shown to induce protective immune response in domestic cats against feline panleukopenia virus and feline infectious peritonitis. Cats given 10^7 plaque-forming units (pfu) of a marked raccoonpox virus by an intranasal route did not exhibit any histopathological lesions up to 15 days post-vaccination.

Regarding in vivo experiments (humanized systems), in a recent study it was shown that in toxicity experiments, a single intraperitoneal injection of up to 5×10^7 pfu or intravenous dose of up to 10^8 pfu of raccoonpox virus did not yield measurable signs of illness in naïve immunocompetent (Balb/c) mice (Evgin et al. 2010).

Bio-selection and genetic modifications

At present there are no available reports on use of genetically modified raccoonpox virus for the purpose of developing anti-cancer viral agent. Published results regarding the exploitation of raccoonpox virus as oncolytic agent are restricted to wild type isolate from Canadian cat (Yager et al., 2006). To examine if the virus has significant effect on human cancer cell lines, the recombinant raccoonpox virus is constructed by inserting a GFP expression cassette into the viral TK locus for visualization purposes (Evgin et al. 2010).

Horizontal transmission and establishment in the human population

Animal poxviruses occasionally produce human skin disease often associated with systemic illness. Lesions are painful blisters and crusted eschars and are usually self-limiting and rarely fatal. Treatment is generally supportive.

However, there is no report in the literature of documented raccoonpox virus occurring in a domestic animals or even humans. It is possible that some of the anecdotal reports of unclassified orthopoxvirus infections were caused by raccoon, or some other wildlife-species pox infection.

Raccoonpox virus has also been unintentionally introduced into humans by way of laboratory accidents, and even with direct injection by needle-stick, there were a few symptoms beyond an injection site reaction and a small blister, suggesting further safety for eventual use in humans (Rocke et al. 2004).

Environmental risk assessment/Biosafety
Raccoonpox virus has demonstrated utility as a vaccine vector in wild animal vaccine for rabies virus and sylvatic plague (Yersinia pestis), suggesting its safety in multiple animal species (Osorio et al., 2003; Lodmell, 1991).

Raccoonpox virus (RCNV) has been assigned to the '**Low**' category in the relative environmental risk table since the virus has no known significant pathology in any mammalian species. Importantly, the virus has been utilized as a vaccine vector against rabies and has an excellent safety record in a variety of wild animal species. Raccoonpox virus has also been unintentionally introduced into humans by way of laboratory accidents, and even with direct injection by needle-stick there were a few symptoms beyond an injection site reaction and a small blister, suggesting further safety for eventual use in humans.

Genus Leporipoxvirus
Myxoma virus

Pathogenicity/ Disease association/Host range/ Tissue tropism
Myxoma virus is a causative agent of myxomatosis, a serious, life threatening, and systemic viral disease in European rabbit (*Oryctolagus cuniculus*). Myxoma virus causes a mild, benign infection in its well-adapted North and South American leporid hosts, the bush rabbit (*Sylvilagus bachmani*, *S. californicus*) and the jungle rabbit (tapeti; *Sylvilagus brasiliensis*), respectively.

Myxomatosis infection is mild and rare in wild hares that rarely exhibit clinical signs although they do have a limited serological response that indicates infection. It was first discovered in 1896 in Uruguay and was imported to Australia in 1951 to control its large rabbit populations - initially having the desired devastating effect. The disease was illegally introduced to France in 1952 and it appeared in Britain the following year. It quickly spread to both wild and domestic rabbit populations and within a few years had spread throughout Europe. Myxomatosis has been a threat to wild and domestic rabbits ever since.

Myxomatosis is typically spread by blood sucking insects and in particular the rabbit flea, (Spilopsyllus cuniculi). This flea is frequently found on wild rabbits and transmission in the absence of bites is unusual. All breeds of domestic rabbit can be affected.

The incubation period varies depending on the strain and its virulence and is typically at least five days. Typical skin lesions that are associated with disease in the domestic rabbit include edema of the eyelids, ears, nose, anus, and genitals; hemorrhage; and nodules on the ears, head, body, and legs at the site of infection that may become congested or necrotic. Severe swellings can lead to blindness and

distortion around the face within a day or so of the onset of symptoms, leading to difficulty with feeding and drinking. Bacterial respiratory infection often complicates the disease resulting in a fatal pneumonia.

Although myxoma virus is considered rabbit specific, it can replicate productively in a variety of human tumor cells in culture (Sypula et al., 2004). Furthermore, myxoma virus can productively infect certain non-rabbit cells in vitro including immortalized baby monkey kidney fibroblasts (BGMK) and primary murine cells genetically deficient in IFN responses. However, species-specific host cell surface receptors for poxviruses have never been identified and it is now believed that intracellular events downstream of poxvirus binding and entry determine whether a poxvirus infection will be "permissive" or "non-permissive". Hence, the host range and apparent species specificity of myxoma virus infection are linked directly to the intracellular environment and IFN responsiveness of the host cell.

Current status and stage of the research activities

The nonpathogenic nature of myxoma virus outside of the rabbit host, its capacity to be genetically modified, its ability to produce a long-lived infection in human tumor cells, and the lack of preexisting antibodies in the human population suggest that myxoma virus may be an attractive oncolytic agent against human malignancies.

The current literature and electronically available information, suggestst that that myxoma virus is an exciting new therapeutic oncolytic virus candidate and it is currently being developed as a new class of cancer therapies in man (part of the EU project 6th Framework Program, THERADPOX, project number LSHB-CT-2004-018700). At this stage it can be advised to further follow the research concerning the use of myxoma virus as oncolytic agent. It can be expected to advance as clinically applicable agent (Stanford and McFadden, 2007). Intended application: Patent application title: Use of myxoma virus for the therapeutic treatment of cancer and chronic viral infection; Inventors: Grant McFadden and John C. Bell; Agents: Wellstat Management Company. LLC; Patent application number: 20090317362

Available assessment information in humans or humanized systems

Regarding in vivo experiments (humanized systems), in a study by Lun et al. (2005) it was shown that myxoma virus has the ability to infect and clear tumors in immunocompromised mice. The purpose of this study was to determine if there was efficacy or toxicities of this oncolytic virus against experimental models of human malignant gliomas in vitro, in vivo, and ex vivo in malignant human glioma specimens. Importantly, the intracerebral administration of myxoma virus ($5x10^6$ PFUs) has been tested and proven to be safe in immunocompromised (nude) mice. Thus, intracerebral myxoma virus inoculation was well tolerated and produced only minimal focal inflammatory changes at the site of viral inoculation. In orthotopic glioma xenograft models, most myxoma virus-treated animals were alive (92%) and

apparently "cured" when the experiment was finished (>130 days). Interestingly, the data show a selective and long-lived myxoma virus infection in gliomas in vivo. Recent report by Stanford et al. describes the effectiveness of myxoma virus in combination with rapamycin for treating primary and metastatic mouse tumors in immunocompetent (C57BL6) mice. Combinatory therapy reduced both size and number of lung metastasis (Stanford et al. 2008). The use of multiple I.V. injections (systemic administration) of myxoma virus (10^8 PFUs) was well tolerated in mice. Currently there are no reports on myxoma virus in-use as an oncolytic agent in humans.

Bio-selection and genetic modifications

Published results regarding the exploitation of myxoma virus as a recombinant oncolytic virus describe the use of genetically modified myxoma virus (vMyxIL-12) that expresses human interleukin-12 (IL-12). IL-12 is produced by antigen presenting cells and acts as an important mediator of T-cell responses through promoting T type-1 immunity. In addition, the recombinant myxoma virus (vMyxgfp), which expresses EGFP, was also constructed and used to conduct experiments (Stanford et al., 2007). vMyxIL-12 replicates similar to wild-type virus. When tested in rabbit host, vMyxIL-12 did not cause myxomatosis, despite expressing the complete repertoire of myxoma virus proteins. According to described experiments and the data obtained, it seems that vMyxIL-12 is safe in all known vertebrate hosts, including lagomorphs (rabbits). Currently there are two myxoma virus strains used for experimental purposes: Moses (Standard Laboratory strain) that causes relatively flat skin lesions and Lausanne strain that is more virulent than the Moses strain and produces protuberant skin lesions.

Horizontal transmission and establishment in the human population

Myxoma virus species selectivity is so narrow that it was used to control the disastrous feral rabbit population in Australia in the 1950s (Fenner 1959). Importantly, it is nonpathogenic for all other vertebrate species tested including humans. Indeed, there has never been a report of myxoma virus infection in humans despite widespread release of the virus to control feral rabbit populations. (Fenner and Ratcliffe, 1966). Furthermore, from a practical perspective, testing in nonhuman primates and ultimately in clinical trials will definitively determine the safety and specificity of myxoma. The only time that myxoma virus was tested in humans, over 50 years ago, there was no infectivity or pathogenicity of any kind (Fenner and Woodroofe, 1965).

Environmental risk assessment/Biosafety

All rabbits, whether wild or domestic are at risk of myxomatosis. However, very intensive investigations have been done to determine the host range of the myxoma virus since this information was required by the Australian authorities before the decision could be made to release the virus. Numerous species of wild and domestic

animals and birds were tested for susceptibility to the virus and ultimately it was shown that under natural conditions the myxoma virus would produce disease in leporids (rabbits and hares) only. However, myxomatosis is a disease that knows no country borders. Actions taken in spreading disease in one country may lead to outbreaks of disease elsewhere. For instance, myxoma virus spread in Australia can survive on clothing (i.e. carrier rabbit flea) for many weeks making the virus easy to spread between countries by overseas visitors.

Myxomatosis was first introduced to Australia in 1950 to control the wild rabbit population. According to Australian government reports the original virus killed about 99% of infected rabbits. However rabbits that did not die produced a high level of antibodies and any does (female rabbits) that survived passed these antibodies on to the kittens. There is also an accumulation of genetic resistance that has built up within the wild rabbit population, so the figures are much lower today.

The Australian Government has made it illegal for any rabbit owner to vaccinate their pet against Myxomatosis. The argument is that immunity may pass to the wild rabbit population, which would reduce the effectiveness of the disease. However, in the 1950s, myxomatosis killed most rabbits, perhaps as much as 99% of the population. Today, myxomatosis is still having an effect but the kill rate is often less than 50%. There is evidence that wild rabbits have built their own immunity to the disease as shown from these quotes from a paper produced by the Australian Department of Primary Industries: "As the field strains of myxomatosis are no longer as virulent as the original releases the disease can no longer be considered a reliable control agent for rabbits." and "Releasing a virulent strain of myxomatosis does not guarantee that an outbreak will occur. There may have been a non-virulent strain previously endemic in the area giving immunity to the majority of rabbits present."

Current myxomatosis vaccine status of different countries:
Australia - No vaccine allowed
New Zealand - No vaccine is available/allowed
USA - No vaccine available
England - Yes, vaccine is available & legal
Netherlands - Yes, vaccine is available and legal (Lyomyxovax, manufacturer Merial)

Safety is always a concern when constructing recombinant poxviruses expressing a therapeutic transgenes (e.g. cytokines). Therefore, infectious myxoma virus expressing hIL-12 (vMyxIL-12) has been tested for the ability to induce myxomatosis in the host to which this virus is pathogenic. The recombinant virus exhibited attenuated pathogenicity in its natural host. This would indicate that vMyxIL-12 virus is safer than its wild-type counterpart, and if anything, might be self limiting in vivo. It is also possible that the IL-12 recombinant virus could produce a more potent and durable protective viral immunity in susceptible rabbit hosts. These possibilities

tackle issues regarding the control of undesirable wild rabbit overpopulation (as an environmental risk) and possible introduction of such recombinant virus into the wild.

Despite the nonpathogenic nature of myxoma virus outside of the rabbit host, this virus has been classified in the '**Medium**' category regarding its relative environmental risk. Myxoma virus is a causative agent of myxomatosis, a serious, life threatening, and systemic viral disease in European rabbit. All rabbits, whether wild or domestic are at risk of myxomatosis, a disease that knows no country borders. Furthermore it has been shown that the exposure to the virus could produce a more potent and durable protective viral immunity in susceptible rabbit hosts. The clinical use of myxoma virus can have significant environmental implications due to the susceptibility of the rabbit population.

Genus Yatapoxvirus
Yaba-like disease virus

Pathogenicity/ Disease association /Host range/ Tissue tropism
Of the various genera of poxviruses, perhaps the least well characterized is the Yatapoxvirus genus. The Yatapoxvirus genus consists of the Yaba monkey tumor virus (YMTV), Tanapox virus (benign epidermal monkeypox, BEMP), and the Yaba-like disease (YLD) virus. Members of this genus have been responsible for zoonotic infections, forming cutaneous nodules in caretakers handling infected monkeys, and replicating virus has been recovered from these lesions. These viruses have not been extensively explored, nor has their host range been adequately defined. In addition, they may not be native monkey diseases; if not, natural host is unknown. Experimental inoculations of infectious material into nonprimate animal models have been unsuccessful, and humans, rhesus, and cynomolgus monkeys appear to be the most susceptible hosts. The geographic distribution of viruses that belong to Yatapoxvirus genus is unknown; however, serological evidence suggests that infection with these viruses is endemic in African and Malaysian monkeys (Downie, 1974). Interestingly, all three viruses do not cross-react with vaccinia virus antibodies. YMTV infection fully protected primates against Tanapox infection (Downie and Espana, 1973). YMTV was first isolated in 1958 from Rhesus monkeys in Yaba, near Lagos, Nigeria. It was found to induce transmissible benign tumors in Asiatic monkeys and humans and was difficult to grow in cultured cells. Tanapox virus was isolated from human skin biopsy specimens during an outbreak of an illness in 1957 and 1962 among natives living along the Tana River Valley in Kenya. The clinical manifestations included fever, headache, backache, and prostration. A pock lesion (generally solitary) appeared during the fever, from which Tanapox virus could be isolated.

Yaba-like disease virus (YLD) is closely related to Tanapox virus. It was first time isolated after it appeared simultaneously in macaques and handlers in labs in Oregon, Texas, and California (hence "OrTeCa") in mid 1960s, and slightly later in Maryland. In the first 3 labs the animals were from a single primate-importer, where the disease was also seen. Apparently, the disease has not occurred since original outbreak. In rhesus monkeys, the symptoms appear as typical pocks on face, arms and perineum, without general illness. Most affected people have only localized lesions, e.g., at sites of scratches on hands and arms, but some had regional lymphadenopathy, fever, and a few had generalized pocks.

Compared to Tanapox virus and YMTV, Yaba-like disease virus is the least characterized of the Yatapoxvirus genus. Yaba-like disease virus is almost identical to Tanapox disease, and the viruses were originally felt to be the same. Knight et al. differentiated Tanapox virus and Yaba-like disease virus by restrictive mapping (Knight et al., 1989). The YLD virus replicates efficiently in human cells and can be grown under normal conditions in monkey kidney (CV-1) cells. The host range of YLD virus was studied by infecting cell lines from various species with wild-type YLD virus. Productive infection was observed in at 48 h in CV-1 cells (monkey kidney) and 1299 cells (human non-small cell lung carcinoma). YLD virus did not propagate in RK-13 (rabbit kidney), CHO (Chinese hamster ovary), or MC-38 (murine colon carcinoma) cells. The results suggest a narrow host range of YLD virus in vitro. Although, DNA replication did not occur in cells that were not permissive, the expression of transgene (e.g. GFP) was demonstrated (Hu et al., 2001).

Current status and stage of the research activities

The stage of the research activities is currently at the level of in vitro studies and comprises of only one published report. The publication by Hu et al (2001) describes the expansion of YLD virus on various human cancer cells. The virus expanded 2- to 3-log-fold over 96 hours in the human tumor cell lines and appear to be less potent in comparison to vaccinia virus expansion that peaked at 48 hours with 2 logs higher virus yield. The experimental data also demonstrates the ability of the virus to reach 100% gene expression (GFP) efficiency at 48 hours post infection (Hu et al., 2001).

Available assessment information in humans or humanized systems

Currently there are no reports on use of Yaba-like disease virus as oncolytic agent in humans. Regarding in vivo experiments (humanized systems) YLD virus was tested in a murine model of ovarian cancer. A total of 10^8 PFUs of YLD-GFP was administered by intraperitoneal injection in immunocompromised (nude) mice. The in vivo efficiency of tumor gene delivery by YLD virus yielded up to 38% of tumor cells expressing GFP. As YLD virus does not replicate in murine cells, tumor specificity cannot be studied in this model. The toxicity of the intraperitoneal administration of yaba-like disease virus (10^8 PFUs) in immunocompromised (nude) mice has not been discussed in referred publication (Hu et al., 2001).

Bio-selection and genetic modifications
At present there are no available reports on use of genetically modified Yaba-like disease virus for the purpose of developing anti-cancer viral agent. Published results regarding the exploitation of this virus as oncolytic agent are restricted to a wild type YLD virus and a recombinant virus expressing GFP for visualization purposes (Hu et al., 2001).

Horizontal transmission and establishment in the human population
This virus was first recognized in monkey caretakers in 1965 and 1966, in primate centers in the United States, and was traced to a single source (Espana, 1971). YLD infection in caretakers produced a brief fever and the development of a few firm, elevated, round, necrotic maculopapular nodules (e.g., at sites of scratches), followed by complete resolution of the infection. During the outbreak, no human-to-human transmission has been recorded. The disease has not occurred since original outbreak.

Environmental risk assessment/Biosafety
Yaba-like disease virus species selectivity is very narrow as well as geographic distribution of susceptible species habitats (excluding animal/primates facilities and Zoos), thus, it is unlikely to become a threat to the environment.

The relative environmental risk assigned to Yaba-like disease virus is '**Medium**' due to the evidence of zoonotic infections in caretakers handling infected monkeys. Although human-to-human transmission did not occur during this outbreak, replicating virus has been recovered from skin lesions of infected individuals. Hence, virus shedding in the environment may occur.

Genus Avipoxvirus
Canarypox virus

Pathogenicity/ Disease association /Host range/ Tissue tropism
Canarypox is a member of the avipoxvirus genus of the Poxviridae family. The avipox virus is very host specific and only replicates and causes disease in avian species. The vector used extensively in recombinant vaccines is an attenuated canarypox strain that is used commercially to immunize canaries against canarypox. It is used as the recombinant vector under the name ALVAC. This canarypox virus was originally isolated from a single pox lesion on an infected canary. The canarypox was attenuated by 200 serial passages in chicken embryo fibroblasts. This attenuated strain is licensed in France as a vaccine in its natural host, canaries.

ALVAC and ALVAC-derived recombinants do not replicate in non-avian cell lines, including monkey, mouse, cat, and human. This was demonstrated by the results of a blinded study. Tissue cultures were infected, and 8 or 10 sequential passages of 7

days duration were done using human, monkey, and chicken embryo fibroblast cell lines. The chicken embryo fibroblast cells (avian cells) allowed the canarypox to replicate resulting in a 100-fold increase in titer with each passage. In contrast, in the human and monkey cell lines (non-avian cells) the virus was not detectable after 2 passages and did not replicate.

Current status and stage of the research activities

The large genome size of canarypox virus facilitates incorporation of heterologous DNA fragments. Single or multiple expression cassettes coding for antigenic proteins from pathogens or encoding tumor antigens can be inserted into the genome of a single ALVAC vector. Many vaccines against viral diseases have been generated: including feline leukemia virus, Japanese encephalitis virus, equine influenza virus, rabies virus, West Nile virus, canine distemper virus, measles virus and HIV-1. All of these ALVAC recombinant vaccines have demonstrated both outstanding immunogenicity and safety in the animals tested. (Taylor et al., 1995),

Available assessment information in humans or humanized systems

Canarypox-based vaccines have been generated to induce immune responses against human tumor antigens. ALVAC-CEA vectors have been clinically evaluated. So far these studies have underscored the clinical safety of this vector. Other clinical studies with Canarypox viruses encoding gp100, MAGE-1, MAGE-3, and p53, are ongoing. (Tartaglia et al., 2001, Bos et al., 2007)

Environmental risk assessment/Biosafety

ALVAC-based viruses have host range that is very narrow. Also the virus is strongly attenuated in its natural host. Thus ALVAC-based canary pox vectors are unlikely to become a threat to the environment.

ALVAC-based Canarypox viruses have host range that is very narrow and limited to canaries (*Seriunus sp.).* Also the ALVAC-strain used in clinical studies is strongly attenuated in its natural host. Thus ALVAC-based canary pox vectors are unlikely to become a threat to the environment. The virus incapacity to replicate in human cells ultimately limits the possibility of virus shedding from the patient. Hence, the relative environmental risk of the canary pox virus is assigned as '**Negligible**'.

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Family *Herpesviridae*

Herpesviruses are enveloped viruses with large DNA genomes (ranging from 124 kbp to 295 kbp), classified as pathogens of humans, and other vertebrates. At present, there are 90 known herpesviruses (ICTV Virus taxonomy report 2009) of which the complete genome sequences of 56 herpesviruses have been published. The family of Herpesviridae is divided in into three subfamilies: Alphaherpesvirinae (36 species), Betaherpesvirinae (12 species) and Gammaherpesvirinae (30 species) (for more details refer to Fields BN Virology 2007). Among 90 known herpesviruses, there are 8 herpesviruses that are classified as human pathogens. The most commonly isolated from human population are herpes simplex virus 1 and 2 (HSV-1 & 2), varicella zoster virus (VZV) (Alphaherpesvirinae); human cytomegalovirus (HCMV, Betaherpesvirinae) and Epstein-Barr virus (EBV, Gammaherpesvirinae).

Although, herpesviruses are highly host specific and share a long-standing co-evolution with their hosts, there are cases of a transfer of viruses between distantly related hosts (Ehlers B et al. JV 82, 2008). There are only two examples where there is a clear evidence for herpesviruses causing zoonoses (e.g. CeHV-1 from macaques to human; HSV-1 from human to primates). CeHV-1 causes mostly very mild infections in its natural host; however it presents a clear threat for people working with macaques. If antiviral treatment is not initiated, the patient develops severe neurological disorders with high mortality reaching up to 80% (Cohen et al. 2002). To date, only one case of human-to-human transmission of CeHV-1 has been documented (CDC 1989).

Herpesviruses in oncolytic virotherapy

Among a wide variety of viruses that have been closely studied for their ability to infect and destroy tumor cells both in tissue culture and in animal models of cancer, members of the herpesvirus family have generated much interest in the field of oncolytic virotherapy.

Over the past decade, an alphaherpesvirus, human herpes simplex virus type 1 (HSV-1), has been developed as a gene therapy vector and anti-cancer agent for the treatment of human cancers (Glorioso et al. 1995; Markert et al. 2000). HSV-1 was the first virus used to show that genetic mutation could render a virus oncolytic (Martuza et al. 1991). To date, a plethora of vectors based on HSV-1 is available and a number of them have been used in Phase I and II clinical trials (Markert et al. 2000; Rampling et al. 2000; Kemeny et al. 2006). Many vectors exist based on other members of herpesviruses among which are human herpes simplex virus type 2 (HSV-2; an alphaherpesvirus) (Fu et al. 2006), and Epstein-Barr virus (EBV; a gammaherpesvirus) (Hellebrand et al. 2006).

Although herpesviruses seem to be vectors of choice due to their capacity to accommodate large transgenes (inserts of 150 kb), the main concern regarding the use of human herpesviruses as vectors for gene therapy relates to their inherent pathogenicity, potential host inflammatory responses to infection, and the risk of recombination with or activation of endogenous herpesviruses. However, it should be noted that an attractive "safety" feature of this virus family is their genome stability (McGeoch and Cook; 1994). The error rate for DNA viruses has been calculated to be 10^{-8} to 10^{-11} errors per incorporated nucleotide. With this low mutation rate, replication of DNA viruses will generate mutants rather rarely.

Currently used herpesvirus vectors as oncolytic agents include viruses classified as human pathogens, e.g. HSV-1, that require genetic manipulation to render them harmless to humans but still capable to preferentially replicate in human tumor cells. In addition, the presence of preexisting immunity to those viruses (in 40-90% of general population) hampers efficient systemic delivery of oncolytic HSV-1 vector.

Consequently, the use of wild-type herpesviruses that are not human pathogens is emerging as an alternative approach. In the last 5 years, only a few non-human herpesviruses have been scrutinized for oncolytic properties. These include bovine herpes virus 1 and pseudorabies virus (both belong to subfamily of *Alphaherpesvirinae*), as well as bovine herpesvirus 4 and saimiri virus (both belong to subfamily of *Gammaherpesvirinae*).

Genus Varicellovirus
Bovine herpesvirus 1

Pathogenesis/Disease association/Epidemiology/Host range/Tissue tropism
Bovine herpesvirus type 1 (BoHV-1) is a wide spread pathogen in cattle. Primary infection is accompanied by various clinical manifestations such as infectious bovine rhinotracheitis (IBR), abortion, infectious pustular vulvovaginitis (IPV) and systemic infection of neonates. Acute infection of an adult causes lesions on mucosal surface and conjuctivitis, followed by the establishment of life-long latency in nervous sensory ganglia. The severity of the disease caused by BoHV-1 is influenced by several factors such as the virulence of the BoHV-1 strain, resistance factors of the host, especially the age, and potential concurrent bacterial infection. Reactivation of the virus from latency induces bovine respiratory disease also known as "shipping fever", through transient immunosuppression. This phenomenon is mostly associated with a stress response. Without secondary bacterial infection, the symptoms are typically cleared within 2-6 days.

As a member of the Herpesviridae family BoHV-1 most likely co-evolved with its host species, thus well adapted to its bovine host. It is also prevalent all over the world. Under particular circumstances such as introduction of BoHV-1 in a naïve

herd, the virus is causative agents of a severe and highly contagious respiratory disease (IBR). Abortion and fatal systemic disease in neonates (naïve calf) are the most severe consequences of respiratory infection with virulent strain.

BoHV-1 is a worldwide disseminated pathogen displaying significant differences in regional incidence and prevalence with regards to the geographical positions and the breeding managements of the considered regions. The latency reactivation cycle has deep epidemiological impact since it is responsible for the maintenance of BoHV-1 in a cattle population.

Although the natural host of BoHV-1 is the domestic cow, this virus has the capacity to cross the species barrier. Field data and experimental infections have brought evidence of possible infections of several ruminant species. In nature, BoHV-1 was detected in acutely and latently infected sheep. Under experimental conditions, successful infections were obtained in domestic goat. Among wild life, the Red deer exhibited a limited susceptibility to BoHV-1 and rabbits can be infected experimentally via the intraconjuctival or the intranasal route. Direct nose to nose contact is the preferential transmission route of BoHV-1. Natural port of entry is the mucous membrane of either upper respiratory or genital tract of cattle, thus BoHV-1 exhibits a natural tropism for respiratory and genital epithelial cells.

BoHV-1 infection of permissive cells is initiated by a three steps entry process. The first interaction involves low affinity virus attachment between virus envelope proteins (glycoprotein B and/or glycoprotein C) to cell surface heparin sulfate sugar moieties. This is followed by the stable binding of glycoprotein D to cellular receptor nectin-1 (a member of immunoglobulin superfamily). The subsequent virus penetration occurs by fusion of the virion envelope with plasma membrane involving four viral glycoproteins: gD, gB and the heterodimer formed by gH/gL.

Although BoHV-1 belongs to the subfamily *Alphaherpesvirinae*, it is unable to bind nectin-2 (receptor for HSV-1), but is capable of recognizing the poliovirus receptor CD155. HSV-1 does not bind CD155. In addition, CD155 receptor is associated with tumor cell migration and invasion (Sloan, 2004).

Current status and stage of the research activities

Current status of research activities is at the level of in vitro studies. In early 60's, it has been reported that BoHV-1 is cytopathic in various transformed human cell lines in vitro, including HeLa, KB, HEp-2 and Had-1 cell lines (Hammon et al. 1963). Furthermore, human melanoma cell line MelJuSo was found to be permissive to BoHV-1 infection. The virus infection resulted in high expression of all major viral glycoproteins at the surface of infected cells. Thus, BoHV-1 productively infects and replicates in those cells (Koppers-Lalic et al. 2003).

Recently, it has been demonstrated that BoHV-1 replicates and induces cytophatic effect in a wide subset of immortalized (MCF-10A, HME-1, FOB, RWPE-1) and transformed cells (A549, MDA-MB-468, U2OS, PC-3), thus decreasing cellular viability, but is significantly restricted in normal cells (Rodrigues et al. 2009).

Available assessment information in humans or humanized systems
Not available

Bio-selection and genetic modifications
BoHV-1 can be easily adapted to culture in human cell lines (HeLa, MelJuSo). The presence of field isolates with one or more point mutations or with genome segment deletion (BoHV-1 ROF3 isolate; F. Rijsewijk pers. comm.) has been reported. There are numerous genetic modifications that have been introduced in the genome of BoHV-1 for variety of studies. To date, no viral mutants except BoHV-1 expressing a GFP under IE viral promoter (Rodrigues, 2009) have been tested in the context of viral oncolyisis.

Horizontal transmission and establishment in the human population
BoHV-1 has a very narrow-host range and to date, there are no reports on its pathogenic profile in human. In addition, there is no seroconversion known in human routinely working with BoHV-1 or working with infected cattle (IDL-Lelystad, pers.comm). Although BoHV-1 replication is normally restricted to bovine cells, the experiments performed in vitro show that BoHV-1 is capable of productive replication and killing of a variety of human cancer cell types. In addition, a panel of 6 non-transformed and non-immortalized human cell lines was used to perform in vitro assessment of BoHV-1 ability to replicate in normal human cells (Rodrigues et al. 2009). Three cell lines were refractory to the virus, whereas the other cell lines exhibit some cytopathic effect only at high multiplicities of infection (MOI 5 and 10). At present there are no reports on in vivo (in-host) genome recombination between animal herpesvirus and human herpesvirus, or if the animal herpesvirus is capable of reactivating human virus gene expression in in-vivo settings.

Environmental risk assessment/ Biosafety
BoHV-1 is responsible for significant losses caused by disease and trading restrictions in the cattle industry. Therefore, control programs were rapidly developed in many European countries and in North American region. Pending on the level of seroprevalence towards BoHV-1, eradication programs are based either on the detection and the culling of seropositive animals (e.g. Switzerland), or on the repeated vaccination of seropositive herds (e.g. the Netherlands). Because of the inability of vaccines to prevent BoHV-1 infection and the establishment of latency, BoHV-1 control programs may require a long time period before complete eradication can be achieved.

Bovine herpesvirus 1 (BoHV-1) is classified as '**Low**' in the relative environmental risk assessment. This assessment is based on the fact that the virus has a very narrow-host range and it is circulated in cattle population worldwide. To date, there are no reports on its pathogenic profile in human. However, it should be noted that BoHV-1 is responsible for significant trading restrictions in the cattle industry and it can pose a threat to countries that declared BoHV-1 free status through extensive eradication programs.

Genus Varicellovirus
Suid herpesvirus 1

Pathogenesis/Disease association/Epidemiology/Host range/Tissue tropism
Suid herpesvirus 1 (SuHV-1) also commonly known as Pseudorabies virus (PRV) or Aujeszky's disease (AD) virus is a causative agent of Aujeszky's disease in swine. This is highly contagious and economically significant disease of domestic pigs where clinical signs vary with age of the animal. The mortality rate decreases with increasing age; it may be as 1-2% in adult pigs and as high as 100% in animals less than a week old. If the signs of central nervous system infection are present in piglets (less than a week old), the animal usually dies within 24 to 36 hours. Signs of viral infection observed in adults are usually demonstrated by mild symptoms of respiratory disease. If infected, pregnant sows abort or give birth to weak, trembling neonates. In general, morbidity and mortality associated with SuHV-1 infection varies with the age of the pig, overall health status of the animal, viral strain, and infectious dose.

SuHV-1 outbreaks occur in swine population world-wide, resulting in substantial economic losses for affected countries. The disease can be found in parts of Europe (East and South), Southeast Asia, Central and South America including Mexico. Two European countries, Germany and the Netherlands, mounted successful agricultural campaigns to eradicate SuHV-1 from their swine populations. Systematic eradication program in Germany resulted in Aujeszky's disease-free status in 2001.

In addition to infection of its natural host, SuHV-1 infects a broad range of vertebrates. These include cattle, sheep, dogs, cats, goats, chickens, raccoons, possums, skunks, rodents, rabbits, guinea pigs and rarely horses (Pomeranz et al. 2005).

SuHV-1 has a tropism for both respiratory and nervous system tissue of pig and viral particles enter sensory nerve endings innervating the infected mucosal epithelium. The virus entry into permissive cells is mediate by cellular receptors such as nectin-1 and CD155. The process of virus binding to the cells and internalization follows similar step as described for BoHV-1.

Current status and stage of the research activities

Current status of research activities is mainly at the level of in vitro studies. Although non-pathogenic in humans, SuHV-1 has been evaluated as an oncolytic agent for human tumor cells (Boldogkoi Z 2003). It has been reported that the virus successfully infected a panel of tumor cell lines including human neuroblastoma, a glioblastoma and a hepatoma. SuHV-1 could replicate and egress mature virions (Boldogki Z 2002). At high virus concentration, SuHV-1 infects and kills glioblastoma cells, whereas at low concentrations is unable to efficiently infect rapidly dividing tumor cells. Furthermore, SuHV-1 exhibited a high affinity for normal human fibroblasts that raises a concern regarding the specificity for human tumor tissue (Wollmann G 2005).

Available assessment information in humans or humanized systems

The efficacy of SuHV-1 as an oncolytic virus has been assessed in immunocompromised mouse–tumor models (Boldogkoi Z 2002) and in immunocompetent mice (Shiau Ai-Li 2007). The assessment of SuHV-1 as therapeutic anti-cancer agent in human subjects is not available.

Bio-selection and genetic modifications

SuHV-1 has a large genome containing numerous accessory genes that can be replaced with foreign genes, as well as a large capacity for harboring lengthy exogenous DNA sequences or multiple transgenes. To date, a few genetically-modified SuHV-1 variants have been tested for their ability to infect cultured human cells (Boldogki Z 2002). Recently, a conditionally replicating glycoprotein E-defective SuHV-1 mutant carrying glycoprotein D and HSV-1 TK genes under the transcriptional control of the HER-2/neu promoter, has been tested in vitro. HER-2/neu is a cell surface proto-oncogen that is over-expressed in various cancers. The results show that the recombinant selectively replicated in and lysed HER-2/neu-overexpressing human bladder, mouse bladder and hamster oral cancer cells (Shiau Ai-Li 2007). Serial passage adaptation might enhance the ability of SuHV-1 to infect selected tumor cells but since this virus can have lethal consequences after retrograde transport into the brain of rodents and other mammals, the health risk to humans that normally are not affected by SuHV-1 might increase considerably. Given that normal human fibroblasts appear to be susceptible to SuHV-1 infection then human cancer cell lines tested, thus, regarding safety concerns it would be more desirable to use genetically modified, conditionally replicating SuHV-1 viral vectors. Furthermore, similar to HSV-1, SuHV-1 has a native TK gene, which can potentially be utilized in prodrug-activation strategies.

Horizontal transmission and establishment in the human population

SuHV-1 is usually transmitted between pigs by the respiratory route (nose-to-nose). During acute infection, the virus is present for more than two weeks in the tonsillar epithelium and other bodily fluids (milk, urine, and vaginal secretion). After primary

infection virus establishes the latent stage in the trigeminal ganglia and infected pigs become latent carriers of virus. The virus reactivates due to stress-associated conditions such as transport, crowding and farrowing.
It is interesting to note, that higher-order primates and humans are, for unknown reason, not susceptible to SuHV-1 infection. Although anecdotic in nature, three cases of Aujeszky's disease symptoms in human have been reported (Mravak S 1987). Previous case-reports have been disputed, since cultivation of the virus from human subjects and attempts to transmit the infection to volunteers have failed (reports from 1914, 1940 and 1963; in German).

Environmental risk assessment/ Biosafety
Globally, pork producers suffer substantial economic loss due to Aujeszky's disease. Successful vaccination programs provide protection against the disease but are not sufficient to prevent the virus infection. At present, infections caused by SuHV-1 are coming under control thanks to extensive agricultural management that includes frequent vaccination, surveillance, and control and culling of known infected SuHV-1 herds.

Importantly, other than pigs, most species are dead-end hosts, but sheep and cattle my occasionally excrete some virus (saliva). Domesticated and wild-life animals listed usually become infected through close contact with infected pigs. Infected animals die within 1 to 2 days. Infection of other wild-life carnivores, such as bears and wild felines, has been reported to occur after consumption of raw infected meat. (Pomeranz et al. 2005). Importantly, infection of other species always results in sever neurological symptoms and death.

Under experimental conditions SuHV-1 is able to infect rhesus monkeys and marmosets. Humans are not susceptible to SuHV-1 infection. Although, Aujeszky's disease is usually spread directly between the animals, the virus can remain infectious for as long as seven hours in the air, if relative humidity is at least 55% and it may travel up to two kilometers as an aerosol. It can also be transmitted in vomits and in carcasses. Under favorable conditions, the virus can survive for several days in contaminated animal bedding and water. The use of SuHV-1 as oncolytic vector in human subjects would require a solid environmental risk assessment. Currently there is no information regarding SuHV-1 virus shedding from human subjects.

Suid herpesvirus 1 (SuHV-1) is known to cause Aujeszky's disease in swine, but it can infect a broad range of animal species. Despite the fact that the disease in pigs is usually manifested by mild respiratory symptoms, the infection of other animal species results in severe neurological symptoms and death. The virus is usually spread directly between the animals by aerosol. Remarkably, higher-order primates and humans are, for unknown reason, not susceptible to SuHV-1 infection. Our findings led us to classify the relative environmental risk for SuHV-1 as '**Medium**'.

Genus Rhadinovirus
Herpesvirus saimiri

Pathogenesis/Disease association/Epidemiology/Host range/Tissue tropism
Herpesvirus saimiri (HVS; Saimiriine Herpesvirus 2; SaHV-2), a member of the genus Rhadinovirus (γ2-herpesvirus), naturally infects squirrel monkeys (*Saimiri sciureus*) of South America and perhaps also *Cebus* (e.g. capuchin monkeys) and *Ateles* (e.g. spider monkeys) species as supported by serologic evidence (Fickenscher and Fleckenstein, 2001). As natural host of herpes saimiri squirrel monkeys are infected via saliva usually within the first two years of life. Infected squirrel monkeys usually do not display any symptoms but in acute cases, the monkeys can have lip, tongue, or palate ulcers. Systemic disease and death are not common. Many of these squirrel monkeys develop lifelong latent infection with periodic shedding of the virus.

HVS persists in T lymphocytes of the natural host without any apparent disease. However, infection of other species of New World primates results in rapid development of lymphomas, lymphosarcomas, and leukemias of T cell origin (Jung and Desrosiers, 1994). In owl monkeys and marmosets, the virus can spread rapidly causing high morbidity and mortality (up to 100%). Affected owl monkeys and marmosets develop oral and cutaneous vesicles and ulcers, conjunctivitis, and occasionally tingling and pricking sensation of the skin, indicated by intensive scratching.

Although HVS infection of other species of New World primates can result in lymphoproliferative diseases, this can be completely eliminated by deletion of the transforming genes, STP and Tip (Duboise et al. 1998). Depending on the pathogenic properties and on the sequence divergence in the terminal non-repetitive genomic region, HVS strains were classified into the three subgroups A, B and C (Desrosiers and Falk, 1982). The major representative strains are A11 for subgroup A, B-S295C and B-SMHI for subgroup B, and C488 and C484 for subgroup C. Generally, viruses of subgroup B have the weakest and subgroup C strains the strongest oncogenic properties.

Herpesvirus saimiri is capable of infecting a range of human cell types in vitro with high efficiency. The viral genome persists as high copy number, circular, nonintegrated episomes which segregate to progeny upon cell division. Specifically HVS subgroup C strains such as C488 are able to transform human T-cells to stable growth in vitro (Biesinger et al. 1992). After HVS infection, a broad range of human epithelial, mesenchymal and haematopietic cell types become persistently infected. The pancreatic carcinoma line PANC-1 and human foreskin fibroblasts even produced infectious virus under selection conditions (Simmer et al 1991; Stevenson et al. 2000). These findings suggest that there is widely distributed cellular receptor

among various tissues that still needs to be identified. The receptor seems to be well conserved among the species since rabbit T-cells can also be infected and transformed by HVS strains (Ablashi et al. 1985).

Current status and stage of the research activities

The stage of the research activities regarding the use of herpesvirus saimiri as oncolytic agent is currently at the level of in vitro studies. The publication by Smith et al. (2005), describes the approach to investigate the ability of herpesvirus saimiri to specifically lyse selected human cancer cell lines. Upon infection with a replication-competent HVS, the pancreatic cancer lines MIAPACA and PANC-1 exhibited definite cytopathic effects. In contrast, the colonic carcinoma cell lines SW480 and HCT116 were phenotypically unaltered. In addition, stable cell lines could not be generated from PANC-1 infected cultures, in marked contrast to cultures of cells from other human tissues. In addition, treatment with acyclovir inhibited virus replication but paradoxically increased cytopathic effect. The authors suggest that herpesvirus saimiri may have potential as an oncolytic agent for the treatment of pancreatic cancer.

Available assessment information in humans or humanized systems

Currently there are no reports on use of herpesvirus saimiri as oncolytic agent in humans. Regarding in vivo experiments (humanized systems) in a recent study by Smith et al. (2005) it was shown that HVS can efficiently infect and develop a persistent episomal infection in a range of human tumor xenografts via intratumoral injections. Wild-type SW480 (colorectal), MiaPaCa (pancreatic), A673s (Ewing's sarcoma) or MCF7 (breast) carcinoma cells were used to induce tumor xenografts. Virus was injected into three different sites of each tumor ($1x10^8$ PFU/ml). HVS infected solid tumors to a high efficiency and allowed long-term gene expression (e.g. eGFP) following infection of selected tissues. In addition, HVS genomes were amplified from the host mouse liver and spleen samples but were not detected in any other host tissues.

In addition, the dissemination of HVS-based vectors in vivo following intravenous and intraperitoneal administration has also been assessed (Smith et al. 2005). Bioluminescence imaging of an HVS-based vector expressing luciferase demonstrates that the virus can infect and establish a persistent latent infection in a variety of mouse tissues. Moreover, the long-term in vivo maintenance of the HVS genome as a nonintegrated circular episome provided sustained expression of luciferase over a 10-week period. A particularly high level of transgene expression in the liver and the ability of HVS to infect and persist in hepatic stellate cells suggest that HVS-based vectors may have potential for the treatment of inherited and acquired liver diseases.

Bio-selection and genetic modifications

HVS has several properties that make it amenable to development as a gene delivery vector. HVS offers the potential to incorporate large amounts of heterologous DNA

and infects a broad range of human cell lines (Grassmann et al. 1989; Stevenson et al. 1999). Upon infection, the viral genome can persist by virtue of episomal maintenance and stably transfer heterologous gene expression. This allows the HVS-based vector to stably transduce a dividing cell population and provide sustained transgene expression for an extended period of time both in vitro and in vivo. HVS-associated lymphoproliferative diseases can be completely eliminated by deletion of the transforming genes, STP and Tip. (Duboise et al. 1998).The vectors utilized for research purposes possess these deletions and as such are incapable of transforming any cell type.

Horizontal transmission and establishment in the human population

The mode of transmission is still unknown but it has been suspected the virus can be spread by contact, fomites and aerosol (Wright et al. 1977). The species-specific molecular determinants for the cellular susceptibility to permissive or persistent infection are not known. As described in literature, the features of infection can vary considerably between closely related species (e.g. productivity of C488-transformed T-cells from macaques versus humans). Not even the macaque model seems optimal for the non-permissive persistence conditions in human T-cells (Knappe et al. 2000). Thus, it is difficult to predict the behavior of HSV in another species. Although the virus was shown to infect and transform human T-cells in vitro, currently there is no evidence that people can be infected or develop tumors as a consequence of exposure to herpesvirus saimiri.

Environmental risk assessment/ Biosafety

HVS might be useful as a gene vector for targeting amplification of functional human T-cells, even for therapeutic application if a series of biosafety aspects are clarified, especially given its relatively close relationship with Human Herpesvirus 8, the causative agent of Kaposi sarcoma. It has been shown that after reinfusion of transformed T-cells into the donor macaques, the animal did not develop lympho-proliferative disease, although the infused T-cells persisted for extended period (Knappe et al. 2000). Furthermore, the biological safety was improved by inserting the prodrug activating gene thymidine kinase (TK) of herpes simplex virus into the genome of HSV. TK-expressing transformed T-cells were efficiently eliminated in the presence of low concentration of ganciclovir.

Herpesvirus saimiri (HVS) is classified in the '**Medium**' category of the relative environmental risk classification. While HVS persists in T cells of the natural host (squirrel monkeys) without any apparent disease, the infection of other species of New World primates results in rapid development of lymphomas and T-cell leukemias. It is assumed that the virus can be transmitted by contact and aerosol. The species-specific molecular determinants for the cellular susceptibility to this virus are currently unknown. Although the virus was shown to infect and transform human T-cells in vitro, currently there is no evidence that people can be infected or develop

tumors as a consequence of exposure to the virus. Nevertheless, biosafety aspects require clarification, especially given its relatively close relationship with Human Herpesvirus 8, the causative agent of Kaposi sarcoma in humans.

Bovine herpesvirus 4

Pathogenesis/Disease association/Epidemiology/Host range/Tissue tropism
Bovine herpesvirus 4 (BoHV-4) belongs to the subfamily γ-herpesviridae, genus rhadinovirus. BoHV-4 was first isolated in Europe (Hungary, 1963, from calves with respiratory disease and keratoconjunctivitis) and later in the United States. The virus has been isolated from a variety of samples and cells from healthy cattle and from cattle with abortion, metritis (inflammation of the lining of the uterus), pneumonia, diarrhea, respiratory infection, and mammary pustular dermatitis. However, only a few investigators have successfully produced experimental disease.

After infection, BoHV-4 replicates in the epithelial cells of the upper respiratory tract and of the intestines. The virus may also replicate in peripheral blood leukocytes and may spread throughout the body by these infected cells. At that time BoHV-4 can be isolated from various tissues and organs, and the association of BoHV-4 with peripheral blood leukocytes could explain the diversity of tissues and organs from which the virus has been isolated (Thiry et al. 1992).

Like other herpesviruses, BoHV-4 establishes persistent infections in its natural host and in an experimental host, the rabbit. In experimentally infected rabbits, BoHV-4 may accelerate the atherosclerotic process (Lin et al. 2000). Although BoHV-4 has been demonstrated in many tissues, accumulated evidence suggests that the main site of persistence/latency in both natural and experimental hosts is cells of the monocyte/macrophage lineage (Donofrio et al. 2010). Based on this and other evidence, a pathogenetic model of persistent BoHV-4 infection along with bacterial co-infection has been postulated. Bacterially induced metritis in cattle persistently infected with BoHV-4 could possibly be intensified or become chronic following the recruitment of macrophages persistently infected with BoHV-4 from the bloodstream to the site of inflammation. Therefore, persistent infection represents a prerequisite for BoHV-4 potential pathogenicity.

BoHV-4 is distributed worldwide. BoHV-4 infections have been diagnosed in the United States (Mohanty et al. 1971; Naeem et al. 1989), in several countries of Europe, and also in different countries of Africa. A serological survey study revealed that 70% of the examined cattle in Zaire were BoHV-4 seropositive (Thiry et al. 1989). The BoHV-4 seroprevalence in cattle has also been determined in many European countries, including the countries surrounding the Netherlands (Metzler and Wyler, 1986). However, no information concerning the seroprevalence of BoHV-4 in Dutch cattle has been published until yet. In the northern part of Belgium 15%, and in

Wallony (Belgium) 29%, of the cattle older than 1 year were BoHV-4 seropositive (Van Malderen et al. 1987). In the former West Germany, the BoHV-4 seroprevalence in cattle was 18.4%, while 38% of the bulls used for artificial insemination had BoHV-4 antibodies (Truman et al. 1986). BoHV-4 antibodies have also been detected in cats (Kruger et al. 2000), but the epidemiological distribution of BoHV-4 in cats is unknown.

The replication of most γ-herpesviruses is restricted to their natural host species. BoHV-4 is one of the few exceptions to this rule. Indeed, it has been shown that BoHV-4 is able to replicate in a broad range of host species both in vivo and in vitro. In addition to cattle, isolates of BoHV-4 have been recovered from other ruminant species. Sporadic isolations were also reported in lions, cats, and owl monkeys. Experimentally, BoHV-4 was also shown to infect goats, guinea pigs, and rabbits. In vitro, BoHV-4 is able to replicate in primary cell cultures or cell lines from a broad spectrum of host species, such as sheep, goats, swine, cats, dogs, rabbits, mink, horses, turkeys, ferrets, chickens, hamsters, rats, mice, and monkeys (Peterson et al. 1988). Recently, several studies have shown that some human cell lines (human embryonic lung cells and giant-cell glioblastoma cell cultures) support BoHV-4 infection (Egyed, 1998).

Current status and stage of the research activities

The stage of the research activities is currently at the level of in vitro and in vivo animal model studies. In 2004, Gillet et al. (2004) evaluated the susceptibility of 21 human cell lines to BoHV-4 infection. These experiments revealed that human cell lines from lymphoid and myeloid origins were resistant to infection, whereas epithelial cells, carcinoma, or adenocarcinoma cells isolated from various organs were sensitive but poorly permissive to BoHV-4 infection. Despite their sensitivity to the infection, BoHV-4 did not induce apoptosis in those cell lines. To date, there is no data that can explain or suggest why OVCAR-3 (human ovary adenocarcinoma) and A549 (human lung carcinoma) are sensitive to BoHV-4–induced apoptosis, whereas the other cell lines tested are not. This restriction could be the consequence of the expression of a particular viral and/or cellular proteome. Furthermore, the authors show that the expression of some immediate-early or early BoHV-4 genes induces apoptosis of human carcinoma OVCAR and A549 cells through a pathway involving caspase-10 activity (Gillet et al. 2005). Thus, in vitro and in vivo experiments presented by two publications emphasize the potential of BoHV-4 as a new candidate for the development of a viro-oncoapoptotic vector.

Available assessment information in humans or humanized systems

Currently there are no reports on use of BoHV-4 as oncolytic agent in humans. Regarding the in vivo (humanized systems) experiments, the impact of BoHV-4 on healthy nude mice was investigated. The results obtained by Gillet et al. (2005) can be summarized as follows: (a) subcutaneous injection of 10^8 pfu of BoHV-4 to healthy

nude mice (n = 10) did not induce any clinical sign. (b) Excretion of the virus was investigated between day 1 and day 5 after viral inoculation. The virus was never recovered. (c) 48 hrs postinfection, infectious virus could not be detected at the site of injection nor in the spleen, the kidney, and the lung of inoculated mice. When the BoHV-4-infected A549 cells were injected into the mice, none of them (n=5) developed tumors over a period of 3 months after implantation. Furthermore, the effect of BoHV-4 injections into A549 xenograft tumors has been evaluated. A549 xenograft tumors growing in nude mice were injected with PBS or with BoHV-4. At day 21, all mice were euthanized and tumors harvested for analysis. Average tumor volumes were significantly smaller for BoHV-4 injected tumors compared with controls (PBS only).

Bio-selection and genetic modifications

At present there are no available reports on use of genetically modified replication-competent BoHV-4 for the purpose of developing anti-cancer viral agent. Published results regarding the exploitation of this virus as oncolytic agent are restricted to the BoHV-4 Vtest strain and a derived recombinant strain expressing EGFP, called Vtest EGFP XhoI (Gillet et al. 2004). The Vtest EGFP XhoI strain carries an enhanced green fluorescent protein (EGFP) expression cassette under the control of the human cytomegalovirus immediate-early promoter/enhancer inserted into a noncoding region of BoHV-4. BoHV-4 Vtest EGFP XhoI recombinant strain leads to EGFP expression in cells that are sensitive (supporting viral entry) and/or permissive (supporting viral replication) to BoHV-4 infection (Gillet et al. 2004).

Horizontal transmission and establishment in the human population

Transmission experiments with BHV4 are very scarce. It has been suggested that the natural route of infection may be through the respiratory and alimentary tracts, by inspired air, and recently it has been suggested that cattle may be infected by virus-infected-cells in milk (Donofrio et al. 2000).

The risk of a viral cross-species transmission usually depends on the prevalence of the virus in the environment as well as on the events permitting the exposure of the non-natural host to the virus. Importantly, the virus must have the ability to productively infect non-natural host. Regarding BoHV-4 transmission to humans, there are several points that have to be taken into consideration. BoHV-4 is highly prevalent in the cattle population and to date, no eradication scheme exists. Furthermore, BoHV-4 is frequently isolated from bovine serum which is abundantly used in food and pharmacological preparations as well as in laboratory work. BoHV-4 has been found in the milk of cows with mastitis as well as from apparently healthy cows, suggesting possible human exposure or contamination by oral route through milk ingestion (if the milk is not pasteurized). In case of direct contact with infected cattle, humans can be infected by animal excretes (e.g. saliva). However, to date there are no reported cases of human infection with BoHV-4.

Interestingly, Machiels et al. (2007) investigated the sensitivity of BoHV-4 to neutralization by naïve human sera in order to determine if humans exhibit innate anti-viral activities against this virus. Their findings demonstrate that human sera from naïve individuals, in contrast to the sera of naïve subjects from various animal species, neutralize BoHV-4 efficiently. The mechanism of this neutralization was demonstrated to be complement-dependent through activation of the classical pathway by natural antibodies raised against the carbohydrate moiety Galα1-3Galβ1-4GlcNAc-R (Galα1-3Gal epitope) (Galili et al. 1987). Human serum contains high level of IgG and IgM raised against Galα1-3Gal epitope as a presumed consequence of bacterial exposure. Thus, BoHV-4 neutralization mediated by anti- Galα1-3Gal antibodies and complement activation may play a role as a barrier to prevent cross-species infection from cattle to human.

Environmental risk assessment/ Biosafety

Although the replication of most γ-herpesviruses is restricted to their natural host species, BoHV-4 is one of the few exceptions to this rule. In addition to cattle, isolates of BoHV-4 have been recovered from other ruminant species and occasionally from lions, cats, and owl monkeys. The observation that human cells can support BoHV-4 infection raises the question of if the field strain of BoHV-4 could represent a danger for human health. The virus could replicate and destroy permissive cells as well as to protect persistently infected non-permissive cells from apoptosis. Importantly, the latter phenomenon might allow infected cells to accumulate mutations leading eventually to transformation.

To date there are no reported cases of human infection with BoHV-4 or human disease elicit by BoHV-4 probably due to the virus neutralization mediated by anti-Galα1-3Gal antibodies. However, it is doubtful if the innate-immunity will be sufficient to act upon the viral anti-cancer agent if administered intratumorally or intravenously since progeny virions released by human cells will be Galα1-3Gal negative (due to the ancestral inactivation of GT gene, no human cells express the enzyme required to add terminal galactose onto glycoproteins and glycolipids in a specific α1-3 linkage). As a consequence, the virus particle will be insensitive to anti-Galα1-3Gal antibodies.

The bovine herpesvirus 4 (BoHV-4) is assigned to the '**Medium**' category of the relative environmental risk classification. The virus is able to replicate in a broad range of host species both in vivo and in vitro. To date, there have been no reported cases of human infection with BoHV-4 probably due to the virus neutralization mediated by natural antibodies raised against the carbohydrate moiety (anti-Galα1-3Gal antibodies). Probably, the innate-immunity will be insufficient to act upon the viral anti-cancer agent since progeny virions released by human cells will be Galα1-3Gal negative. The effects of such modification on virus-host (human) interactions and possible consequences are unpredictable.

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Family *Baculoviridae*
Baculovirus (*Autographa californica*)

Pathogenesis / Disease association / Epidemiology / Host range / Tissue tropism
Baculoviruses are widespread pathogens of insects and invertebrates, ranging from shrimps to moths and butterflies. The most studied types are those which cause disease in common insect pests. The individual Baculovirus strains have a limited host range, which is usually restricted to one species. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is one of the best studied baculoviruses.

Current status and stage of the research activities
The use of baculoviruses as expression vectors have been first time explored in early 80's. Since then, the potential of the baculovirus-insect cell expression system has been fully exploited for the production of numerous recombinant proteins. Although the baculovirus host range is restricted to insects and invertebrates, the virus can be internalized by mammalian cells. Recombinant baculoviruses harboring mammalian promoters efficiently express reporter genes in a variety of mammalian cell lines (human, nonhuman primates, porcine, bovine, rodent, and rabbit origin) and fish cells. The broad range of mammalian cells permissive to baculovirus transduction, the nontoxic nature, large DNA-packaging capacity and easy production make baculoviruses attractive tool for gene therapy. Baculoviruses carrying tumor-suppressor or suicide genes can be applied in combinatory anti-cancer therapies. Furthermore, the high efficiency of gene transfer to mesenchymal stem cells implies applicability of baculoviruses for ex-vivo genetic modification of cells prior to transplantation into animals.

Available assessment information in humans or humanized systems
The enhanced transduction efficiency and wider tropism, led to increasing interest for baculoviruses as vectors for *in-vivo* gene delivery. Tissues that have been targeted include rabbit carotid artery, rat liver and brain, murine brain, skeletal muscle, cerebral cortex, testis, and liver.

Bio-selection and genetic modifications
The tropism and transduction efficiency of baculoviruses has been manipulated by modifying the envelope protein. Modification can be performed by fusing a gene of interest in frame with the gp64 gene under the control of the polyhedron or p10 promoter. The fusion protein, after expression as an additional copy, is translocated to the plasma membrane and incorporated into the viral envelope on virus budding. Baculoviruses carrying heterologous envelope proteins such as the VSV-G envelope protein, can exhibit enhanced (up to 100-fold) transduction efficiencies in comparison to unmodified baculoviruses.

Horizontal transmission and establishment in the human population
Baculovirus transduction is nontoxic to mammalian cells and does not hinder cell growth even at high MOI. Moreover, baculoviruses do not replicate in transduced mammalian cells. Baculoviruses are nonpathogenic to humans.

Environmental risk assessment/ Biosafety
Baculovirus DNA tends to be degraded in mammalian cells. However, it has been reported that a recombinant baculovirus containing two expression cassettes (pCMV-GFP and pSV40-G418) is capable of mediating stable expression (Condreay et al. 1999). Selected clones maintained stable expression of the reporter gene for at least 25 passages. Stably transduced derivatives have been selected from a substantial number of cell types. It appears that baculovirus DNA can integrate into the host cell genome as discrete single-copy fragments. Hence, the possibility that baculoviruses spontaneously integrate (part of) their genome into genomic DNA cannot be excluded. So far there is no direct evidence showing that integration of baculovirus DNA occurs in the absence of an antibiotic resistance gene and selective pressure. However, it would be advisable to study the state and fate of recombinant baculovirus DNA in mammalian cells to further establish the safety of baculovirus-derived gene therapy vectors.

Another issues regarding the use of baculoviruses is the lack of evidence supporting the absence of baculovirus endogenous gene expression. Although, no baculovirus gene transcripts have been found in human cells (Stanbridge et al. 2003), a study demonstrated that the baculovirus genomic early-to-late (ETL) promoter is active (Liu et al. 2006). The consequences of this expression for the host remain to be established. However, it is well established that baculoviruses genomes are not replicated in mammalian cells and that no virus progeny is produced. This limits the environmental risks associated with its use as gene therapy vector.

The *Autographa californica* Baculovirus has a very narrow host range that is limited to a single moth species. The virus incapacity to replicate in mammalian (human) cells limits the ability of virus shedding from the patient. Thus, we classify the relative environmental risk of baculoviruses as '**Negligible**'.

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Family *Parvoviridae*

The family *Parvoviridae* is subdivided into the subfamily *Parvovirinae* (vertebrae host) and *Densovirinae* (arthropod host). *Parvoviridae* infecting vertebrates are adeno-associated viruses (AAV; genus Dependovirus) and autonomous parvoviruses (genus Parvovirus and genus Erythrovirus, e.g. human parvovirus B19).

Parvoviruses are small viruses (20-25 nm diameter) with a non-enveloped icosahedral capsid containing a linear single-stranded DNA genome (approximately 5 kb) that replicates in the nucleus. A general feature of parvovirus replication is the requirement for cellular factors expressed during the S phase of the cell cycle. This explains why a common characteristic in the pathogenesis of these viruses is the infection of mitotically active cells and why clinical courses are more severe in developing hosts, in which many tissues are undergoing proliferation. Thus, parvoviruses are primarily teratogenic agents causing fetal and neonatal abnormalities, osteolytic syndromes, abortion, and malformations, although in adults they may attack tissues such as intestinal epithelium and hemopoietic system that undergo proliferation throughout life (latter characteristic is related to human parvovirus B19 infection).

Genus Parvovirus
Rodent parvovirus H-1 & Minute virus of mice

Members of the genus Parvovirus typically grow efficiently in cell culture, and are predominantly infect host species that are readily susceptible to experimental manipulation. This genus contains four distinct subgroups:

1. cluster of "rodent virus" species that contains distinct clades: Minute virus of mice (MVM), Mouse parvovirus 1 (MPV1), and a rat virus group that includes Rat minute virus 1 (RMV1), H1 (Toolan's) virus and Kilham or Rat virus (RV/KRV)
2. Rat parvovirus 1 (RPV1) brunch
3. Feline panleucopenia virus/Canine parvovirus (FPV/CPV) serotype (infecting various members of the Carnivora)
4. Porcine parvovirus (PPV)

Another parvovirus, named LuIII has been isolated from and can grow only in human cells. A single isolate, recovered from a human lung culture in 1971 (named LuIII), did not show an antigenic relationship with any previously known parvovirus. Comparisons based on sequence homology strongly suggest that is closely related to the rodent parvoviruses (e.g. MVM). However, the species of origin of this serotype is still unknown.

Unique characteristics of parvoviruses, such as non-pathogenicity in human, anti-oncogenicity and methods of efficient recombinant vectors production, have drawn more attention towards utilizing replication competent parvovirus-based vectors in cancer gene therapy. Although, more than 30 different parvoviruses have been identified so far, recombinant vectors derived from minute virus of mice (MVM) and rat H1 virus have been successfully tested in many preclinical models of human cancer. These two viruses will be further discussed.

Pathogenesis/Disease association

Rodent parvoviruses were first isolated from cell culture stocks in the 1960s, and since that time an ever-increasing number of serologically distinct viruses have been isolated and characterized from both rodent colonies - the virus's natural host - as well as cell culture stocks.

With the exception of hamster parvovirus (HaPV) and minute virus of mice immunosuppressive strain (MVMi), nearly all of the rodent parvoviruses (e.g. MVM and H1) result in sub-clinical infections, which means that the infected animals do not show any outward signs of being sick. The pathogenicity of these viruses is restricted to proliferating (developing) tissues, thus, the severity of infection decreases from the fetus to the adult. Viruses from the rodent groups can establish persistent infections associated with prolonged virus release from reservoirs that are currently unknown.

Host range/Tissue tropism

Rodent parvoviruses predominantly infect their species specific hosts namely mouse, rat and hamster. Although autonomous parvoviruses (e.g. MVM and H1) hosts are widely spread in the animal kingdom, some parvoviruses species are known to consist of several strains, possessing different pathological spectra (Hueffer and Parrish, 2003). Therefore, rodent parvoviruses serve as a valuable tool for studying virus-host interaction.

Lytic parvovirus replication can be only achieved if the following conditions are met: 1.) if cells originate from the right animal species (the range for a given parvoviruses is limited to one or a few distinct animal species), 2.) target cells must proliferate and 3.) target cells must be in not very differentiated state. Furthermore, parvoviruses show a preference for multiplying in malignantly transformed cells. This oncotropism is not due to better virus uptake by transformed cells, but to more efficient parvovirus replication in these cells. It has been shown that factors known to become deregulated following cell oncogenic transformation are required for parvovirus genome conversion (ssDNA into dsDNA) and genes transcription (Cornelis et al 2006).

Since number of conditions are requires for lytic replication of parvoviruses, mainly found among characteristics of transformed (or malignant) tissue, it is often noted that parvoviruses are called "viruses in search of a disease". For example rodent parvoviruses actually protect various animal models against oncogenesis (Cornelis et al. 2006; Rommelaere et al. 2005). Furthermore, it has been experimentally shown that rat H1 parvovirus efficiently kills rat and human glioblastoma and gliosarcoma cell lines while normal brain cells show resistance to viral cytopathic effects (Herrero y Calle et al. 2004).

Current status and stage of the research activities

Studies on animal cells revealed the capacity of parvoviruses to preferentially kill transformed cells (in vitro studies) and inhibit their tumor-forming abilities (in vivo studies). It has been shown that H1 parvovirus efficiently kills various human tumor-derived cells in culture (Faisst et al. 1989), and also causes regression of human mammary and cervical carcinoma cell transplants in immunocompromised mice (van Pachterbeke et al. 1993; Faisst et al. 1998). Recently, H1 parvovirus-mediated oncosuppression and selective targeting of transformed human cells, has been shown with brain tumor- and lymphoma-derived cells (Geletneky et al. 2005; Herrero y calle et al. 2004). Furthermore, infection of some, but not all, human glioma cell lines with the prototype mouse parvovirus (MVMp) has been shown to be cytotoxic (Rubio et al. J 2001). Indeed, MVMp preferentially infects astrocytoma with no significant effect on astrocytes or glial cells survival (Abschuetz et al. 2006).

On the basis of the promising in vitro and in vivo (preclinical) evidence of rat H1-induced oncolysis of glioma cells, currently there is ongoing preparation for a phase I/IIa clinical trial for the treatment of patients with recurrent glioblastoma multiforme. In the trial under name ParvOryx1, it is intended to administer H1 parvovirus preparations (GMP-grade virus) by intratumoral and intravenous injections. The primary goal of this trial is to assess the safety profile of H1 parvovirus in glioma patients after local or systemic injection. The trial is scheduled to start at the end of 2010 (Geletneky et al. 2010; Rommelaere at al. 2010). Further information regarding clinical trial can be obtained through the following source: Helmholtz Association of German Research Centres (2010, May 5). Viruses effective against brain cancer in animals: Human trials set to start. ScienceDaily (retrieved from: http://www.sciencedaily.com/releases/2010/05/100504095106.htm)

Available assessment information in humans or humanized systems

Rat H1 parvovirus has been tested in a immunocompetent rat glioma model, by injecting the virus into the tumor (intratumoral) or by multiple intravenous injections. Both administration routes were sufficient for remission of advanced intracranial gliomas, without detectable deleterious side effects. Importantly, in adult laboratory rat, intracerebral injection of H1 parvovirus did not cause any damage to brain or other tissue (Herrero et al. 2004).

The concept of using rat H1 parvovirus as an oncolytic agent in humans date back in 1965 when two young patients (under age of 14) diagnosed with advanced osteosarcoma received intramuscular injection of the virus (10^9 plaque forming units; PFUs). Although they developed viremia at days 4 and 9 after infection, there was no significant clinical side effects (Toolan et al. 1965). At day 10 post-infection, neutralizing antibodies were detected in the serum of both patients. In other experiments concerning human subjects with cutaneous metastases from different solid tumors, rat H1 parvovirus preparations were administered at different dose levels. Separate patients groups received two or three injections of virus (10^8, 10^9 or 10^{10} PFUs) at 10-day interval. The presence of the virus was demonstrated by transient viremia (in three patients) and seroconversion. Thus, the injections of rat H1 parvovirus did not lead to any pathological side effects (Le Cesne et al. 1993).

Bio-selection and genetic modifications

Since wild-type parvoviruses are often not potent enough to stop tumor progression, recombinant parvovirus vectors encoding immuno-stimulatory molecules have been engineered that allow the transduction of these genes into tumor cells (Cornelis et al. 2004). Engineered viral vectors may facilitate a double-edged strategy by combining viral oncolytic activity with the activity of immune cells stimulated by tumor-secreted transgene product (Wetzel et al. 2007). Vector development has focused on rodent parvoviruses that can infect human cells. Parvovirus-vectors with complete replacement of the viral coding sequence can direct transient or persistent expression of transgenes in cell culture. MVM-based and H1-based vectors with substitution of transgenes for the viral capsid sequences retain viral nonstructural (NS) coding sequences and express the NS1 protein. The latter serves to amplify the vectors genome in target cells, potentially contributing to antitumor activity (Maxwell et al. 2002). In addition, it has been demonstrated that MVM-based vector efficiently transduced cells and expressed interleukin 2 or IL-4 transgenes from cDNA substituted for capsid sequences in an infectious clone of the viral genome (Russell et al. 1992). Despite certain limitations in the production of recombinant rodent (autonomous) parvoviruses, recent reports indicated successful production and transduction in vivo, leading to therapeutic efficacy against tumors in preclinical models (Haag et al. 2000; Geise et al. Gene 2002; Malerba et al. 2003).

The well-defined tropism makes autonomous parvoviruses a valuable model for studies of virus-cell interactions and gene therapy research. Some parvovirus species are known to consist of several strains, possessing different pathological spectra (Hueffer and Parrish, 2003). MVM serves as a good model of parvovirus strain-specific variation. The prototype (MVMp) and immunosuppressive (MVMi) strains efficiently propagate in cultured mouse fibroblasts and T lymphocytes, respectively. Their host ranges are reciprocally restricted, although they are serologically indistinguishable and exhibit only ~3% DNA sequence divergence (Tattersall and Bratton, 1983). It is not known what the adaptive cell-type range of MVM or other

autonomous parvoviruses is. It has been shown that forced culture (persistent infection) of MVMi in mouse fibroblasts can lead to an acquired tropism (Ron et al. 1984). The data points out that increased divergence of initial viral population can increase chances of successful selection of adoptive changes. Application of multiple cycles of infection in a permissive host could create the high genetic heterogeneity that has been reported for the parvoviruses (Lopez-Bueno et al. 2006). MVM parvovirus has been shown to readily adapt to new host environments both in vitro (Ron et al. 1984) and in vivo (Lopez-Bueno et al. 2003). Bio-selection apparently leads to a shift in predominant virus variants in MVM population as a result of environmental constraints such as a new host or treatment with antibodies against MVM capsid. Recently it has been reported that the extension of MVM tropism as a result of quasispecies enrichment consequently yields virus-host range switch of MVMp from mouse to rat fibroblasts (Etingov et al. 2008).

Horizontal transmission and establishment in the human population

Parvoviruses are among the most common infectious agents of laboratory rodents and major impediments to rodent-based research. Recognition of the increased diversity of rodent parvoviruses presents new challenges for determining the impact of parvovirus infection on research and for detecting, preventing, and eliminating infection.

There are no reported hazards to human health associated with rodent parvovirus infections that occur in wild life or laboratory animals (rodents). However, it should be noted that members of genus Parvovirus display ability to generate virus variants with extended tropism as a result of environmental constraints. MVM parvovirus has been shown to readily adapt to new host environments both in vitro (Ron et al. 1984) and in vivo (Lopez-Bueno et al. 2003). At present, it is not known if this tendency to acquire new tissue tropism through forced culture is also applicable to rat H1 parvovirus and if this could further result in changes favorable for adaptation to a new host. In clinical study with patients groups received two or three injections of rat H1 parvovirus no pathological side effects were observed (Le Cesne et al. 1993). This report does not indicate detection of any parvovirus shedding from treated patients nor any subsequent studies on prolonged presence of parvovirus in those patients.

Environmental risk assessment/Biosafety

Rodent parvoviruses become a problem when they infect research rodents. Rodents are the most commonly used species of research animal. The destructive potential of rodent parvoviruses lies in their ability to alter cellular growth kinetics and immune response, two factors often under examination in laboratory animals. Rodent parvoviruses have been identified in the urine, feces, saliva, nasopharyngeal region, and lungs of infected animals, indicating virus spread through bodily excretions. Parvoviruses can survive almost indefinitely at -80oC and can only be destroyed following extensive exposure to harsh disinfectants and ultraviolet radiation. If such

a virus is introduced into an animal facility, it can silently infect a few animals, even just one, and in a matter of days be transferred throughout the animal facility and beyond.

The relative environmental risk assigned to rodent parvoviruses (e.g. MVM and H-1) is '**Low**'. These viruses predominantly infect their species specific hosts namely mouse, rat and hamster. They are among the most common infectious agents of laboratory rodents and nearly all of the rodent parvoviruses result in sub-clinical infections. Rodent parvoviruses have been isolated and characterized from rodent colonies indicating their wide spread in natural host population. Rodent parvoviruses only become a problem when they infect research rodents.

Feline panleukopenia virus

Pathogenesis, Disease associations, and Epidemiology,

The Feline panleukopenia virus is a member of the autonomous parvirus group. Feline panleukopenia virus is a causative agent of highly contagious disease of cats. Although it can affect cats of all ages, feline panleukopenia is primarily a disease of kittens. The disease in kittens is often associated with high mortality rate. All members of the cat family (*Felidae*) are susceptible to the virus infection. FPV is a severe, highly contagious disease that is oftentimes fatal. It occurs worldwide, but is rarely seen as a clinical entity due to the effectiveness of vaccination in preventing the disease. Young, unvaccinated kittens present most commonly with this disease.

Host range and Tissue tropism

Unvaccinated feral cat colonies and other wild felids also serve as reservoirs of infection for the domestic cat population FPV exposure and infection can occur in several ways. The major route of transmission is direct contact between a susceptible host and an infected animal or its secretions. The virus is shed in all body secretions of infected animals for up to six weeks. Once introduced into the environment, the virus is very hardy and can persist for years. Fomites, including contaminated instruments, cages and bedding, are also an important route of viral exposure. Mechanical transmission of FPV via arthropod vectors is probable as well. Lastly, this virus also can cross the placenta to infect the fetuses *in utero*. Neither FPV, nor the closely related canine parvovirus can efficiently infect human cells (Maxwell et al., 2001).

Current status and stage of the research activities

So far only a single study proposed and evaluated the use of FPV (Maxwell et al., 2001). To allow infection of human cells an integrin-binding RGD motif was engineered into the VP2 protein, which is the major constituent of the FPV capsid. This virus was capable of infecting a panel of human tumor cells albeit with varying efficiency. The rhabdoidsarcoma cell line Rh18A was most efficiently transduced for

unknown reasons. Given the very limited research efforts with this virus, it seems unlikely that this virus will be clinically applied in the near future.

Available information in humans or humanized systems

There is no information available on the replication of FPV in human cells or humanized system. This is mainly due to the fact that human cells are refractory to infection.

Environmental risk factors and Biosafety considerations

Given the fact that excellent vaccines are available to protect cats against panleukopenia, the feline panleukopenia virus is categorized as '**Medium**' relative risk for the environment. However, it should be noted that applications in humans will require the use of host-range modified viruses since human cells resist infection with wild type FPV.

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Family *Birnaviridae*
Genus Aribirnavirus

Infectious Bursal Disease virus

Infectious Bursal Disease virus **(**IBDV) belongs to the genus Avibirnavirus of family *Birnaviridae*. It is a double stranded RNA virus that has a segmented genome that consists of two segments, A and B, which are enclosed within a nonenveloped icosahedral capsid. The genome segment B (2.9 kb) encodes viral protein I (VP1), the putative viral RNA polymerase. The larger segment A (3.2 kb) encodes viral proteins VP2 to VP5. The VP2 protein contains important neutralizing epitopes and can elicits protective immune responses. Most of the amino acid changes between antigenically different IBDVs are clustered in the hypervariable region of VP2. Thus, this hypervariable region of VP2 is the obvious target for the molecular techniques applied for IBDV detection and strain-variation studies.

Pathogenicity /Disease association/ Host range / Tissue tropism
IBDV is a causative agent of infectious bursal disease (IBD) or Gumboro disease, which affects the bursa of Fabricius, an important organ in the young chicken's developing immune system. IBD occurs in both layer and broiler birds and although it has been found in turkeys, these birds do not show overt signs of infection. IBDV destroys immature B-lymphocytes in the bursa of Fabricius causing immune suppression. IBD is a highly contagious disease of young chickens with a mortality rate of up to 40%. Mortality rates vary with virulence of the strain involved, the challenge dose as well as the flock's ability to mount an effective immune response. Infection with less virulent strains may not show overt clinical signs but the birds may die of infections by agents that would not usually cause disease in immunocompetent birds.

The virus infects lymphoid cells and especially those of B-lymphocyte lineage. Young birds at around two to eight weeks of age that have highly active bursa of Fabricius are most susceptible to disease. The underlying mechanism for IBDV tropism is far from being resolved. After ingestion, the virus destroys the lymphoid follicles in the bursa of Fabricius as well as the circulating B-cells in the secondary lymphoid tissues. Acute disease and death is due to the necrotizing effect of these viruses on the host tissue. Surviving birds can recover but remain immunocompromized, which increases their susceptibility to other pathogens. Chickens recovered from IBDV infections have small, atrophied, cloacal bursas due to the destruction and lack of regeneration of the bursal follicles.

Passive immunity protects against disease, as does previous infection with avirulent strains. In broiler farms, breeder flocks are immunized against IBD so that they would transfer protective antibodies to their progenies which would be slaughtered

for consumption before their passive immunity wears out. The presence of maternal antibody will modify the clinical course of the disease. Due to the specificity of disease, vaccination during outbreak will not be effective.

IBD is economically important to the poultry industry worldwide due to increased susceptibility to other diseases and negative interference with effective vaccination. Virulence of field strains of the virus varies considerably. Very virulent strains that cause high mortality and morbidity were first detected in Europe. These spread throughout the Old World in the last decade and in 1999 were identified in South America. The vv strains have not (yet) been detected in the USA. Control is best achieved by improved biosecurity and vaccination.

Current status and stage of the research activities (the proposed or intended application)

A single institute is actively developing IBDV based therapies, i.e. VectorLogics (VLI) Inc, is a privately-held biotechnology firm, based in Birmingham, Alabama and Rockville, Maryland. VectorLogics Inc. is developing two therapeutic vaccine product candidates utilizing an attenuated IBDV virus. The first product candidate (VLI-03A) is a proprietary recombinant IBDV vector with proven safety and anti-HBV/HCV activity that is being developed as a orally-administered therapeutic vaccine to treat advanced chronic HCV infections. The second product candidate (VLI-03B) is comprised of a recombinant IBDV vector engineered to deliver and express interferon alpha (IFN-α) in the liver for the local treatment of recurrent liver cancer (hepatocellular carcinoma; HCC). The recombinant IBDV used in this treatment modality is adapted to grow in Vero cells. IBDV is stated to be superior to other vector candidates because (i) it has anti-HBV/HCV activity, (ii) it induces the regeneration of the liver, and (iii) as a B-lymphotropic virus IBDV is shielded from virus-neutralizing antibodies allowing long-term maintenance of the therapy. To this end, in collaboration with University of Maryland Biotechnology Institute (UMBI), VectorLogics is developing second generation IBDV vectors. VLI intends to develop a well-characterized drug candidate that will result in an orally administered IBDV therapeutic vaccine vector expressing IFNα for the long-term adjuvant therapy of intra-hepatic recurrence of hepatocellular carcinoma. (website VectorLogics Inc.)

Available assessment information in humans or humanized systems

Interesting proof-of-concept for effective IBDV co-infection therapy was obtained in human subjects and published by Csatary and colleagues (1999). The group reports three cases of women with chronic liver inflammation caused by hepatitis B (two) and C (one) viral infections that progressed into decompensated liver disease. Those patients were superinfected (intranasal or oral administration) with massive doses of an attenuated variant (MTH-68/B) of the apathogenic IBDV. Clinical symptoms and biochemical abnormalities were resolved in two patients following few months of virus treatment. Cirrhosis was stabilized and significant clinical improvement was

achieved in the third patient--who before the virus therapy was moribund with recurring, diuretic-resistant ascites, variceal bleedings, portal encephalopathy and renal failure. The authors state that these are the first recorded cases of decompensated chronic viral hepatitis which went to long-lasting remission or were stabilized by superinfection with an apathogenic virus.

Furthermore, the safety and efficacy of infectious bursal disease virus (IBDV) co-infection therapy were reported in 42 acute hepatitis patients (HBV and HCV) in a phase II clinical trial (Csatary et al. 1998). The encouraging evidence was that progression to chronic infection was marginally better in IBDV-treated patients than in the controls. The clinical efficacy of IBDV co-infection in hepatitis patients is rather difficult to explain, since the natural hosts of IBDV and HCV (birds and humans, respectively) are separated for several hundred million years. The results of the IBDV co-infection therapy has been extensively reviewed by Bakács and colleagues (2004). Of note is that in the publications regarding the clinical studies remarkable little information was provided on the quality of the IBDV vaccine virus batch.

Bio-selection and genetic modifications

There are two distinct serotypes of the virus, but only serotype 1 viruses cause disease in poultry. At least six antigenic subtypes of IBDV serotype 1 have been identified by in vitro cross-neutralization assay. Viruses belonging to one of these antigenic subtypes are commonly known as variants, which were reported to break through high levels of maternal antibodies in commercial flocks, causing up to 60 to 100 percent mortality rates in chickens. With the advent of highly sensitive molecular techniques, such as reverse transcription polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP), it became possible to detect the vvIBDV, to differentiate IBDV strains, and to use such information in studying the molecular epidemiology of the virus.

In the 1980's, the United States confronted an increase amount of downgrades in the slaughter plant. Further investigation revealed that birds are suffering from respiratory and other secondary infections i.e. *Staphylococcus* and *E. coli*. The underlying reason appears to be the non-responsiveness to the IBD, ND and IB vaccinations given. Intensive diagnostic testing revealed the presence of an IBD virus that is different from the classic Type 1 isolate that has been diagnosed for years.

At the same time that the US is dealing with variant IBD viruses, Europe, Africa and Asia start seeing acute cases of IBDV related to the appearance of the vvIBDV. These illnesses were being diagnosed in flocks at a later age and in farms that are on very good vaccination, biosecurity and management. The vvIBDV strains so far identified are 849 VB and DV86. Few reasons that might have "pushed" the evolution of the virus: extreme vaccination pressure (e.g. the use of cloned intermediate IBD vaccine that confers very narrow protection; vaccinating chicken (breeders) with inactivated

classic-type virus only results in chicks hatching with maternal antibodies that are limited to the classic type virus), improper cleaning and disinfection, and increased farm birds population.

Regarding genetic-modifications of IBDV, the only information available concerns the website of vector Logics. In principle, it should be possible and practical to select, adapt or modify viruses for different therapeutic usages. An obvious concern in such a treatment would be the occasional virus mutant that is more virulent to the host than is the original strain. In case of IBDV, this should be carefully considered due to reports of acute cases of IBD related to the appearance of the vvIBDV.

Horizontal transmission and establishment in the human population

IBDV is shed in the feces of infected chickens and it is transferred from house to house by fomites. It is very stable and difficult to eradicate from premises. The virus is highly contagious and it spreads by direct contact between infected and susceptible birds. It can also be transmitted by equipment, people, air, vehicle etc. Mice and darkling beetles can carry the organism for a very long time. IBD does not transmit from hen to progeny. Affected birds can start shedding the virus 24 hours post-infection. It takes about 2 to 4 days for the disease to occur.

At present there is neither evidence nor likelihood of zoonotic transmission of IBDV. IBDV is widespread in chicken population worldwide, but it is unknown in humans. Given the fact that, a large numbers of progeny virus are produced that are shed in the feces of infected chickens, it seems likely that human population with agricultural background has already been well exposed to this virus.

Consistent with this is the use of IBDV preparation in a clinical trial for the treatment of 42 acute B and C hepatitis patients (Csatary et al. 1998). However, this study does assess virus spreading and does not address the risks associated with this.

Environmental risk assessment/Biosafety

There is no treatment for IBD. Depopulation and rigorous disinfection of contaminated farms have achieved limited success. Live vaccines of chick-embryo or cell-culture origin and of varying virulence can be administered by eye drop, drinking water, or subcutaneous routes at 1-21 days of age. The immune response can be altered by maternal antibody, and the more virulent vaccine strains can override higher levels of antibody. Most of the economic devastation associated with IBD is due to its immunosuppressive effects that lead to poor vaccination response, secondary bacterial, viral and protozoan infections and poor performance. The virus is now recognized in every poultry-producing country in the world. First diagnosed in 1962, the virus has since then changed and manifested itself in different forms that made it even a bigger threat to the industry.

The virus was assigned to the **'Medium'** Relative Environmental Risk category based on the uncertainty surrounding the pathogenicity on poultry of the Vero-cell adapted strain that is being developed for clinical use. Also there is a chance that the virus may adapt to more efficiently infect humans while residing in patients. Shedding of IBDV from treated patients can theoretically reach the natural host species. This may be facilitated by the anticipated persistence in mammalian hosts and by the physical stability of the virus when shedded.

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Family *Reoviridae*

Reoviridae are currently classified into nine genera. The genomes of these viruses consist of 10 to 12 segments of dsRNA, each generally encoding one protein. The mature virions are non-enveloped. Their capsids, formed by multiple proteins, have icosahedral symmetry.

Orthoreoviruses The orthoreoviruses (reoviruses) are the prototypic members of the virus *Reoviridae* family. They have been isolated from the human respiratory and enteric tracts and are not associated with serious human disease. Reoviruses have broad geographic distribution, and virtually all mammals, including humans, can serve as host for infection. There are three types of human Orthoreoviruses isolated from a human host: type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D).
In the last decade, the human reovirus T3D, has been evaluated as oncolytic agent in experimental cancer therapy. The combination of the apathogenicity in humans and the preferential replication in, and cytolysis of, transformed cells lead to the initiation of at least 13 clinical trials in Canada, the US, and the UK using human reovirus T3D. So far the application of replicating wild-type reoviruses has been safe with no adverse effects reported.

Rotaviruses Rotavirus is the most common cause of acute gastroenteritis in infants and young children worldwide.

Orbiviruses The members of Orbivirus genus within the *Reoviridae* family are arthropod-borne viruses and are responsible for high morbidity and mortality in ruminants. Bluetongue virus (BTV) which causes disease in livestock (sheep, goat, cattle) has been in the forefront of molecular studies for the last three decades and now represents the best understood orbivirus at the molecular and structural levels. BTV, like other members of the family, is a complex non-enveloped virus with seven structural proteins and a RNA genome consisting of 10 variously sized dsRNA segments.

Genus Orbivirus
Bluetongue virus 10

Pathogenesis/Disease association/Epidemiology/Host range/Tissue tropism
Bluetongue (BT) is a disease of domestic and wild ruminants caused by bluetongue virus (BTV) that is only transmitted by insects known as midges. Signs of clinical disease vary among different species. Infection is unapparent in the vast majority of species. Cattle, goats and elk, for example, are affected by a very mild, self-limiting infection.

BT can cause serious illness and death in sheep as well as deer and potentially other wildlife. There is no cure and death may occur within seven days. In sheep, the symptoms of BT include: fever, reddening of the lining of the mouth and nose, swelling of the lips tongue and gums, difficulty swallowing and breathing, a swollen, purple-colored tongue (hence, the name bluetongue), and lameness.

BT can be found anywhere the midges that carry the virus are present. BT is commonly found in the U.S., Mexico, Australia, Africa, the Middle East, the Indian subcontinent and China. BT is also spreading northwards in Europe.

The range of domestic animals that can be infected with BT virus includes: domestic cattle, bison, mules, goats and sheep. BT virus can also affect wild animals such as: bighorn sheep, elk, mountain goats, deer, and most other even-toed hoofed animals.
BTV is transmitted from animal-to-animal by specific species of biting *Culicoides* midges that are limited geographically. The virus spread is usually restricted to late summer and early fall, since conditions must be warm enough for the BT virus to multiply within the midge (13°C to 35°C). Midge activity ceases with the first hard frost.

Current status and stage of the research activities
The stage of the research activities is currently at the level of *in vitro* studies and comprises of only one published report. The publication by Hu J. et al (2008) describes the comparison of normal and cancer cells susceptibility to bluetongue virus-10 infection. The experimental data demonstrates the ability of BT virus to induce cytolysis of human cancer cells with no effect on normal human cells.

Available assessment information in humans or humanized systems
Currently there are no reports on use of bluetongue virus as oncolytic agent in humans or in any other *in vivo* studies.

Bio-selection and genetic modifications
At present there are no available reports on use of genetically modified BT virus for the purpose of developing anti-cancer viral agent. Published results regarding the exploitation of BT virus as oncolytic agent are restricted to cell culture adapted BT virus isolated in Hubei, China named BTV-HbC$_3$ (Hu et al. 2008).

Horizontal transmission and establishment in the human population
There is no risk to human health associated with BT virus infections in animals. However, it should be noted that members of *Reoviridae* family display a high mutation rate, thus their tendency to change or revert could result in adaptation to human host.

Environmental risk assessment/ Biosafety

There is no effective treatment for BT virus in animals. Vaccines are available for certain types of the disease and are used in Africa and Asia. Some countries in the European Union have implemented compulsory vaccination for a certain type of BT (serotype 8) which is emerging as a disease. The virus does not survive in the environment outside a midge or its animal host. It cannot be spread through contact with animal carcasses and products such as meat and wool.

The BTV-10 has been assigned to the '**Low**' category in the relative environmental risk table. While BTV can be severely pathogenic in its natural hosts, the shedding of BTV from the patients is strongly limited by the requirement of an insect vector for transmission.

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Family *Paramyxoviridae*

Members of the *Paramyxoviridae* family are negative-sense single-stranded RNA viruses. The genome sizes are 15-19 kb in length containing 6-10 genes. Virions (150-350 nm) are enveloped and can be spherical, filamentous or pleomorphic. Fusion proteins and attachment proteins appear as spikes on the virion surface. Paramyxoviruses have two surface glycoproteins, a receptor-binding attachment protein and a fusion (F) protein. Attachment proteins of many paramyxoviruses (those belonging to the genera Respirovirus, Rubulavirus, and Avulavirus) have both hemagglutinin (H) and neuraminidase (NA) activities and are thus called hemagglutinin–neuramidases (HNs). HNs recognize sialic acid-containing cell surface molecules as receptors and, upon receptor binding, promote fusion activity of the F protein, thereby allowing the virus to penetrate the cell membrane. HNs also act as NAs, removing sialic acid from infected cells and progeny virus particles to allow efficient virus production. The sialic acid recognition of the HN proteins of many paramyxoviruses, as well as of other NA/sialidase derived from a variety of species, has been well studied.

The Paramyxoviridae family is divided into two subfamilies *Paramyxovirinae* and *Pneumovirinae*, encompassing six genera: Avula, Henipa, Metapneumo, Morbilli, Paramyxo, Pneumo, and Rubula. All genera except Avulaviruses infect humans.

A number of important human diseases are caused by paramyxoviruses. These include mumps, measles, parainfluenza, and respiratory syncytial virus (RSV). Paramyxoviruses are also responsible for a range of diseases in other animal species, for example canine distemper virus (dogs), phocine distemper virus (seals), cetacean morbillivirus (dolphins and porpoises) Newcastle disease virus (birds) and rinderpest virus (cattle). Some paramyxoviruses such as the henipaviruses are zoonotic pathogens, occurring naturally in an animal host, but also able to infect humans. In recent years, the Menangle, Tioman, Hendra, and Nipah viruses have emerged. Hendra virus (HeV) and Nipah virus (NiV) have caused outbreaks in livestock in Australia and Southeast Asia. These outbreaks have spread to humans as a result of direct contact with infected animals. Both viruses are contagious, highly virulent, and capable of infecting a number of mammalian species and causing potentially fatal disease. Due to the lack of a licensed vaccine or antiviral therapies, HeV and NiV are designated as biosafety level (BSL) 4 agents.

Some members of this virus family such as measles virus, mumps virus and the Newcastle disease virus (NDV), have been extensively used in cancer targeting. Although still being investigated in more detail, the specificity of these viruses to tumor cells likely stems from the inability of tumor cells to respond to type I interferons and to mount an anti-viral defense.

Genus Avulavirus
Newcastle disease virus

Pathogenesis/Disease association/Epidemiology / Host range / Tissue tropism
Newcastle disease virus (NDV) is the causative agent for one of the most serious infectious diseases of poultry (Alexander, 2000). The virus name comes from the site of the first reported disease outbreak among chickens, at a farm near Newcastle-upon-Tyne in England in 1926. Newcastle disease (ND) is an acute viral disease of domestic poultry and many other bird species. It is a worldwide problem that presents primarily as a respiratory disease, but depression, nervous manifestations, or diarrhea may be the predominant clinical form. Mortality is variable. Occurrence of a virulent form of the disease is reportable and may result in trade restrictions.

NDV isolates are classified into 1 of 3 virulence groups by chicken embryo and chicken inoculation as virulent (velogenic), moderately virulent (mesogenic), or of low virulence (lentogenic). Lentogenic strains are used widely as live vaccines in healthy chickens. Clinical manifestations vary from high morbidity and mortality to asymptomatic infections. The severity of an infection is dependent on virus virulence and the age, immune status, and susceptibility of the host species. Low virulence NDV is prevalent in poultry and wild birds, especially waterfowl. Of all domestic poultry, chickens are the most susceptible to the virus. Infection of domestic poultry with low virulence NDV contributes to lower egg productivity.

Disease onset is rapid, and signs appear throughout the flock within 2-12 days (average 5) after aerosol exposure. Spread is slower if the fecal-oral route is the primary means of transmission, particularly for caged birds. Young birds are the most susceptible. Observed signs depend on whether the infecting virus has a predilection for respiratory, digestive, or nervous systems. Respiratory signs of gasping, coughing, sneezing, and rales predominate in low virulence infections. Nervous signs of tremors, paralyzed wings and legs, twisted necks, spasms, and complete paralysis may accompany, but usually follow, the respiratory signs in neurotropic velogenic disease. Nervous signs with diarrhea are typical in pigeons, and nervous signs are frequently seen in cormorants and exotic bird species. Respiratory signs with depression, watery-greenish diarrhea, and swelling of the tissues of the head and neck are typical of the most virulent form of the disease, viscerotropic velogenic Newcastle disease (VVND, also called exotic Newcastle disease), although nervous signs may also be seen. Varying degrees of depression and lack of appetite are observed. A partial or complete interruption of egg production may occur. Eggs may be abnormal in color, shape, or surface, and have watery albumen. Mortality is variable but can be as high as 100%.

Epidemiology: Virulent NDV strains are endemic in poultry in most of Asia, Africa, and some countries of North, Central, and South America. Other countries, including

the USA and Canada, are free of those strains and maintain that status with import restrictions and eradication by destroying diseased poultry. Cormorants, pigeons, and imported psittacine species have also been sources of virulent NDV infections of poultry. In the last seventy years, several ND epizootics have struck the poultry industry in many countries in the form of panzootics. The first panzootic started in the mid-1920s and subsided only in the late fifties when vaccination became widespread. The second panzootic was believed to have started in the Far East in the early 1960s and spread to Europe through the Middle East. A South American origin of this panzootic has been proposed, as caged birds (e.g. psittacines) were implicated in transmitting ND to Europe and the United States. NDV infection of racing pigeons was regarded as the third panzootic since it spread many countries across the world. At least three different genotypes were responsible for the epizootics during the first panzootic, each showing geographical restrictions (genetic groups II–IV; grouping is based on restriction site analysis of the F gene). Two new genotypes of NDV emerged during the second panzootic. Strains that were believed to be transmitted by imported psittacine birds (genetic group V) and were responsible for the outbreaks in England and other European countries as well as California in 1970-71 differed from those that caused epizootics in the Middle East and Greece during the late 1960s (genetic group VI). Viruses related to the psittacine transmitted group have recently been implicated in isolated cases of ND in colonies of cormorants and in turkeys in the United States. Most countries in Western Europe had a long disease free period during the 1980s. However, since the early nineties, ND cases have been reported from these countries in increasing numbers (Alexander, 1995). The source of these infections has remained unknown. NDV strains derived from Denmark, Sweden, Switzerland and Austria were identical and could be placed into genetic group VI, where Middle-East strains of the late sixties are found. In contrast, isolates from Germany, Belgium, Netherlands, Italy and Spain represent a new genetic lineage (VII) hitherto undetected in Europe and most probably these strains originated from the Far-East.

Host range: Most avian species, domestic and wild; Chickens and turkeys are the most susceptible poultry, ducks and geese are the least; Humans

Tropism: Oncolytic NDV strains are cytotoxic to human tumor cell lines of ectodermal, endodermal, and mesodermal origin.

Current status and stage of the research activities

The first report that NDV may be useful as a cancer treatment was published in 1964. Already 20 years before this report NDV had been used in a vaccine to prevent Newcastle disease in birds. During that time, it was learned that NDV caused only minor illness in humans. The mild side effects caused by NDV in humans and its ability to replicate up to 10,000 times faster in human cancer cells than in most normal human cells, led to a more close look at NDV as a possible cancer treatment.

Available assessment information in humans or humanized systems

Preclinical Studies (*in vitro* and *in vivo* animal studies)

The ability of NDV to replicate efficiently in human cancer cell lines has been demonstrated in both laboratory studies and animal studies (Lorence et al., 1994a and 1994b; Reichard et al., 1992). Several of these studies have provided much of the evidence that lytic strains of NDV are also oncolytic (Reichard et al., 1992; Bar-Eli et al., 1996; Lorence et al., 1988, Zorn et al., 1994; Kirn et al., 1996). Strain 73-T, which is lytic, has been shown to replicate efficiently in human tumor cells (Rusell, 2002) and kill the following types of human cancer cells in vitro: fibrosarcoma, osteosarcoma, neuroblastoma, bladder carcinoma, cervical carcinoma, melanoma, Wilms tumor, and myeloid leukemia (Reichard et al., 1992; Lorence et al., 1988; Cassel & Garrett, 1965). However, this strain did not kill human B-cell lymphoma (i.e., Burkitt lymphoma) cells *in vitro* (Zorn et al., 1994). In addition, strain 73-T did not kill normal, proliferating human white blood cells or normal human skin fibroblasts *in vitro* (Reichard et al., 1992; Lorence et al., 1988), but it killed normal human lung fibroblasts at the same rate that it killed cancer cells (Zorn et al., 1994).

Lytic strain Roakin has been reported to kill human B-cell lymphoma cells and T cells transformed *in vitro* from a Hodgkin lymphoma patient four to five times faster than it kills normal, resting human white blood cells (Bar-Eli et al., 1996; Tzadok-David et al., 1995). This strain, however, has also been reported to kill normal, proliferating human white blood cells, though at a lower rate than it kills cancer cells (Bar-Eli et al., 1996). Lytic strain Italien (or Italian) has been shown to kill human squamous cell lung carcinoma, melanoma, breast carcinoma, and larynx carcinoma, but not cervical carcinoma cells *in vitro* (Ahlert & Schirrmacher, 1990). The replication efficiency of this strain in normal human cells was not tested.

Overall, these results show that there are some types of human cancer cells in which individual lytic strains of NDV do not replicate very well and that there are some types of normal human cells in which they replicate very efficiently. Nonetheless, these data and the absence of serious illness in individuals infected with NDV (Phuangsab et al., 2001; Csatary et al., 1999; Csatary et al., 1993; Kenney & Pagano, 1994; Batliwalla et al., 1998) are consistent with the view that NDV replicates much more efficiently in human cancer cells than it does in most types of normal human cells (i.e., DBTRG.05MG human glioblastoma, U87MG human astrocytoma, rat F98 glioblastoma cells, and mouse Ehrlich ascites carcinoma) (Zulkifli et al., 2009; Schneider et al., 2001; Sinkovics & Horvath, 2000).

NDV strain Ulster, which is non-lytic, has also been shown to replicate efficiently in human cancer cells *in vitro*, including cells of the following types of human tumors: colorectal carcinoma, gastric carcinoma, pancreatic carcinoma, bladder carcinoma, breast carcinoma, ovarian carcinoma, renal cell carcinoma, lung carcinoma, larynx

carcinoma, cervical carcinoma, glioblastoma, melanoma, B-cell lymphoma, and T-cell lymphoma. This strain does not replicate very efficiently in resting or proliferating normal human white blood cells (Schirrmacher et al., 1999). Other experiments have shown that NDV Ulster can kill infected cells (Schirrmacher et al., 2001) and it can replicate in human cancer cells whether they are actively proliferating or not (Schirrmacher et al., 1998).

The ability of lytic strains of NDV to kill human cancer cells *in vivo* has also been examined. In xenograft studies, human cancer cells were injected either subcutaneously or intradermally into athymic, nude mice (i.e., mice with defective immune system), and tumors were allowed to form. NDV was injected directly into the tumors, and tumor growth and animal survival were monitored. Intratumoral injection of strain 73-T was associated with complete tumor regression in 75% to 100% of mice bearing human fibrosarcoma, neuroblastoma, or cervical carcinoma tumors (Lorence et al., 1994a and 1994b; Reichard et al., 1992; Phuangsab et al., 2001). Intratumoral injection of 73-T was also associated with more than 80% tumor regression in 66% of mice bearing human synovial sarcoma tumors (Lorence et al., 1994b). In addition, intratumoral injection of 73-T was associated with 68% to 96% inhibition of tumor growth in mice bearing human epidermoid, colon, lung, breast, or prostate carcinoma tumors (Phuangsab et al., 2001).

Intratumoral injection of strain Italien was associated with complete tumor regression in 100% of mice bearing human melanoma tumors. The growth of metastatic tumors in these animals, however, was not affected, suggesting that the virus was unable to disseminate widely throughout the body (Schirrmacher et al., 1989, 1998 and 2001). Replication of strain 73-T in the above-mentioned neuroblastoma xenografts was demonstrated by showing an increase in the amount of virus that could be recovered from tumor samples over time. When this strain was injected into the thigh muscle of athymic, nude mice (e.g. control group), the amount of virus that could be recovered decreased with time, a finding consistent with the proposal that NDV replicates much more efficiently in cancer cells than in most normal cells (Lorence et al., 1994a).

Another study of intratumoral injection used the V4UPM strain of NDV in a nude mouse model of tumors produced by subcutaneous injection of human glioblastoma multiform cells (Zulkifli et al., 2009). All four mice with tumors from the U87MG cell line experienced sustained complete responses after one injection. However, no complete responses were observed in mice with tumors from the DBTRG.05MG cell line despite a similar *in vitro* cytotoxicity compared with U87MG.

In one study, mice bearing human neuroblastoma xenografts were given single intraperitoneal injections of strain 73-T, and 9 (75%) of 12 treated mice exhibited complete, durable tumor regressions (Phuangsab et al., 2001).

It is important to note that athymic, nude mice still make small numbers of T cells, and they produce interferons, natural killer cells, and macrophages (Schirrmacher et al., 1986; Budzynski & Radzikowski, 1994). The possibility that these residual components of the immune system, which may be activated by the presence of NDV, contributed to the antitumor effects observed in the xenograft studies cannot be ruled out.

Clinical Studies

The anticancer potential of NDV has been investigated in clinical studies in the United States, Canada, China, Germany, and Hungary. Almost all of those studies were phase I or phase II clinical trials with cancer patient that have been treated with lytic NDV strain(s). Findings from most of the studies have been documented. Overall, the results of these studies must be considered preliminary. Most studies enrolled only small numbers of patients, and historical control subjects, rather than actual control groups, which were often used for outcome comparisons. In addition, the evaluation of many studies is made difficult by poor descriptions of study design and the incomplete reporting of clinical data.

To date, most research into the treatment of human cancer by infection of patients with NDV has been conducted in Hungary (Csatary et al., 1971, 1993 and 1999; Csatary & Bakacs, 1999; Nelson, 1999; Moss, 1996; Sinkovics & Horvath, 1993). The Hungarian research effort has been led by a single group of investigators who advocate the use of NDV strain MTH-68, which is presumed to be lytic. Findings from these investigations have been published in the form of an anecdotal report that briefly describes results for 3 patients who had metastatic disease (Csatary, 1971); a single case report about a child who had glioblastoma multiforme (Csatary & Bakacs, 1999); a report of a small case series that included 4 individuals with advanced cancer and a report of a placebo-controlled, phase II clinical trial that included 33 patients in the NDV treatment group and 26 patients in the placebo group. The patients in the phase II trial had various advanced cancers. According to the investigators, MTH-68 treatment was beneficial for the majority of these patients.

The five patients described in the case report and the small case series were reported to have had either a complete remission or a partial remission following NDV therapy (Csatary et al., 1999). Two of the patients in the case series had advanced colorectal cancer, another had melanoma, and the fourth had advanced Hodgkin disease. These five patients were treated with NDV daily for periods of time that ranged from 1 month to 7 years. Inhalation and intravenous injection were the main routes of virus administration. One of the patients with colorectal cancer, however, was treated by means of intracolonic injection (i.e., via a colostomy opening) for 4 weeks. It is important to note that all five patients were treated with conventional therapy before the start of NDV therapy and that four of the five received conventional therapy either concurrently with NDV therapy or after it. Given the

small number of patients, the absence of control subjects, and the overlapping treatments, it is difficult to draw conclusions about the effectiveness of NDV therapy from these small studies. Nonetheless, taken as a whole the results of the available NDV studies suggest potential clinical value warranting further study with controlled clinical trials.

In the phase II trial (a Hungarian study), NDV was administered by inhalation only 2 times a week for a period of 6 months (Csatary et al., 1993). The 33 patients in the NDV treatment group had the following types of cancer: colorectal, stomach, kidney, pancreatic, lung, breast, ovarian, melanoma, bile duct, gallbladder, sarcoma, and ependymoma. The distribution of cancers among the 26 patients in the placebo group was as follows: colorectal, stomach, kidney, lung, breast, melanoma, bile duct, sarcoma, and bladder. Two complete responses and six partial responses were reported for patients in the NDV treatment group, whereas no responses were observed in the placebo group. In the NDV treatment group, ten patients were reported to have stable disease, compared with just two patients in the placebo group. In addition, more patients in the NDV treatment group than in the placebo group reported subjective improvements in their quality of life. Twenty-two (67%) of the patients in the NDV treatment group survived at least 1 year, compared with 4 (15%) of the patients in the placebo group. The 2-year survival proportions were 21% and 0% for patients in the NDV treatment group and the placebo group, respectively.

In a phase I trial that was conducted in the United States, another lytic NDV strain, PV701, was tested in patients with various advanced cancers (Pecora et al., 2002). In this trial, 79 patients whose tumors had not responded to conventional therapy were given intravenous injections of virus.

Four different treatment regimens were evaluated as follows:
1. A single dose of NDV given once every 28 days (17 patients).
2. A single dose of NDV given 3 times during a 1-week period, repeated every 28 days (13 patients).
3. Three injections of NDV given during a 1-week period, with the first injection containing a lower dose of virus than the remaining 2, repeated every 28 days (37 patients).
4. Six injections of NDV given during a 2-week period, with the first injection containing a lower dose of virus than the remaining 5, repeated every 21 days (12 patients).

The researchers found that the use of lower initial doses of virus allowed the administration of higher subsequent doses. A complete response was reported for one patient, and partial tumor regression was observed in eight patients. Thirteen patients had stable disease for periods of time that lasted from 4 months to more than 30 months. Five patients died during the trial: four due to progressive disease and

one due, possibly, to a treatment-related complication. Several patients experienced significant adverse side effects from NDV treatment, including fever, fatigue, dehydration, low blood pressure, shortness of breath, and hypoxia. Some patients who experienced these adverse effects required hospitalization. The researchers who conducted this trial have indicated that additional clinical studies are under way.

The effectiveness of treating cancer patients by repeated administration of a lytic strain of NDV depends on the ability of the immune system to produce virus-neutralizing antibodies. Virus-neutralizing antibodies would prevent NDV from reaching and infecting malignant cells, thereby blocking oncolysis. The Hungarian investigators have shown that anti-NDV antibodies are produced in MTH-68-treated patients, but they have not determined whether these antibodies are virus-neutralizing (Csatary et al., 1999).

Conclusion: Clinical trials of NDV have been performed (table 4) but so far yielded no reliable proof that NDV is effective as a cancer treatment. While most studies of NDV in cancer treatment have been small and without control groups, there have been enough promising results to call for continued research. However, the US Food and Drug Administration (FDA) has so far not approved Newcastle disease virus as a treatment for cancer (2010, current status).

Bio-selection and genetic modifications

Since the discovery of NDV in 1926, nine genotypes of class I viruses and ten of class II have been identified, representing a diverse and continually evolving group of viruses. The emergence of new virulent genotypes from global epizootics and the year-to-year changes observed in the genomic sequence of NDV of low and high virulence implies that distinct genotypes of NDV are simultaneously evolving at different geographic locations across the globe. This vast genomic diversity may be favored by the large variety of avian species susceptible to NDV infection and by the availability of highly mobile wild bird reservoirs (Miller et al., 2010).

Genome sequence analysis of a number of avirulent field isolates of Newcastle disease virus revealed the presence of viruses (within their quasispecies) that contained virulent F0 sequences. In the quasispecies model of RNA virus genomes, mutations occur through errors made in the process of copying existing RNA genome sequences. Due to the ongoing production of mutant sequences during the replication of the RNA virus genome, the virus population is made up of a ''mutational cloud'' of closely related sequences referred to as quasispecies (Domingo, 1996). Reverse-transcription real-time PCR (RRT-PCR) analysis of the quasispecies of a number of Newcastle disease virus field isolates, revealed variable ratios (approximately 1:4–1:4,000) of virulent to avirulent viral F0 sequences. The precursor F0 protein is only fusogenic after it is cleaved into F1 and F2 polypeptides. The different F protein-cleavage sequences of NDV strains are recognized by distinct

Reference	Type of Study	NDV Strain	Type of Cancer	No. of Patients: Enrolled; Treated; Control	Strongest Benefit Reported
Csatary, 1993	Phase II trial	MTH-68	Various advanced	59; 33; 26, placebo	Improved overall survival
Pecora, 2002	Phase I trial	PV701	Various advanced	79; 79; None	Partial tumor regression, 8 patients
Freeman, 2006	Phase I/II	HUJ	Glioblastoma multiforme, recurrent	14 (phase I–6; phase II–8); 11 (phase I–6, phase II–5); 0	1 transient (3 months) complete response, all other patients had progressive disease
Csatary, 1999	Case series	MTH-68	Various advanced	4; 4; None	Complete tumor regression, 2 patients
Csatary, 2004	Selected case series	MTH-68/H	Gliomas, high-grade	4; 4; 0	Radiographically documented responses and long survival with improved symptomatology
Laurie, 2006	Phase I	PV701	Various	16; 16; 0	Improved patient tolerability with two-step desensitization
Wagner, 2006	Case report	MTH-68/H	Anaplastic astrocytoma	1; 1; 0	Partial response
Cassel, 1965	Case report	73-T	Advanced cervical	1; 1; None	Partial tumor regression
Csatary, 1971	Anecdotal report	MTH-68	Various metastatic	3; 3; None	Tumor regression
Csatary, 1999	Case report	MTH-68	Glioblastoma multiforme	1; 1; None	Partial tumor regression
Wheelock, 1964	Case report	Hickman	Acute myeloid leukemia	1; 1; None	Partial response

Table 4: Summary on studies on the use of NDV for cancer treatment (Adapted from http://www.meb.uni-bonn.de/Cancernet/)

cellular proteases. The F0 proteins from lentogenic viruses are only cleaved by trypsin-like proteases found in the respiratory and intestinal tracts of birds, whereas the F proteins of velogenic strains can be cleaved by a broad range of proteases found in a wide range of tissue. Molecular analysis of NDV associated with outbreaks of virulent disease in chickens demonstrated that some field isolates were capable of harbouring virulent virus in their quasispecies without causing overt clinical signs of disease (Kattenbelt et al., 2006). The ratios of these sequences generally remained constant in the quasispecies population during replication, unless factors that could affect the balance of virulent to avirulent sequences during viral infection are present. It was shown both in vitro and in vivo that virulent virus present in the

quasispecies did not emerge from the ''avirulent background'' unless a direct selection pressure was placed on the quasispecies, either by growth conditions or by transient immunosuppression. For example, under selection pressure, in the form of prolonged passage in tissue culture (e.g 45 passages) virulent virus could emerge from an apparently avirulent inoculum. Furthermore, it has been demonstrated experimentally that the appearance of virulent NDVs can occur by the introduction of avirulent viruses into chickens followed by repeated passages in these bird populations (Shengqing et al., 2002).

Recent publication by Kattenbelt and colleagues (2010) strongly indicates that a prior infection of the host by infectious bursal disease virus (IBDV) has an effect on the subsequent emergence of virulent Newcastle disease virus. Experimentally, the pre-existence of IBDV significantly alter the apparent course of NDV infection in birds. It has been noted that if a prior infection with IBDV exists, this can cause transient immunosuppression in infected birds. After a delay of several days, respiratory clinical signs began to appear, followed by the appearance of classic neural signs caused by NDV. Virulent virus was isolated from these birds from both neural tissue and swabs and detected by RRT-PCR. This may explain the observation that after IBDV exposure (experimental inoculation), NDV infection occurred and immunosuppression allowed rampant NDV replication of the avirulent isolate, which then acquired a mutation at its F0 cleavage site. This virus could then reach neural tissue and replicate prior to elimination of the virus by the immune system, resulting in virulent virus dominating the quasispecies and with virulent clinical signs being seen in the infected bird (Kattenbelt et al., 2010). Use of reverse genetics to enhance the oncolytic properties of NDV has been described by Vigil and colleagues (2007).

NDV variants used in clinical trial(s)

PV701- In clinical trial study described by Laurie and colleagues (2006), the NDV variant designated as PV701 has been used. PV701 is a highly purified, naturally attenuated, replication competent isolate of NDV. This virus is a naturally attenuated, triple-plaque purified isolate from the MK107 mesogenic vaccine strain of NDV. The virus has been grown in specific pathogen-free embryonated chicken eggs and purified from the allantoic fluid. (Study performed in **Canada**)

MTH-68- The NDV variant MTH-68/H used in the study by Csatary and Bakács (1999) was generated by several passages in chicken embryos of the original Hertfordshire strain of NDV designated Herts'33, described in the early 1930s in England. MTH-68/H is a highly purified, attenuated NDV strain developed for human use by United Cancer Research Institute (Fort Lauderdale, FL). Lyophilized MTH-68/H vaccine (minimal titer for effective infective dose, $10^{8.8}$) was produced by Phylaxia-Sanofi (Budapest, Hungary). (Study performed in **Hungary**)

NDV-HUJ- NDV-HUJ is a highly purified isolate originally derived from the naturally attenuated B1 NDV vaccine strain (ATCC, 1971), passaged 50 times in 10- to 11-day-old embryonated chicken eggs, and cloned twice by limiting dilution in specific-pathogen-free (SPF) embryonated hen's eggs. The HUJ strain was classified as lentogenic and having no pathogenicity for its natural host, poultry, on the basis of its intracranial pathogenicity index of 0.0 determined by the Israel Ministry of Agriculture and a ^{112}G-R-Q-G-RL^{117} cleavage site sequence in its surface fusion protein, as well as having a mean death time of >100 hrs (Freeman et al., 2006). (Study performed in **Israel**).

Horizontal transmission and establishment in the human population

Transmission in natural host: Infected birds shed virus in exhaled air, respiratory discharges, and feces. Virus is shed during incubation, during the clinical stage, and for a varying but limited period during convalescence. Virus may also be present in eggs laid during clinical disease and in all parts of the carcass during acute virulent infections. Chickens are readily infected by aerosols and by ingesting contaminated water or food. Infected chickens are the primary source of virus, but other domestic and wild birds may be sources of NDV. Transfer of virus, especially in infective feces, by the movement of people and contaminated equipment is the main method of spread between poultry flocks. Under certain conditions, the virus can be wind-borne. Psittacine species can shed the virus intermittently for one year or more following recovery from clinical disease. Migratory birds only play a minor role in disease dissemination. Any animals, including flying insects that travel between infected and susceptible birds can spread the virus by mechanical means, although this form of transmission is a low priority.

*Zoonotic Risk***:** Newcastle disease viruses, whether virulent field viruses or live vaccine, can produce a transitory conjunctivitis in humans (the virus is present from 4-7 days in lacrimal fluid), but the condition has been limited primarily to laboratory workers and vaccination teams exposed to large quantities of virus. Before poultry vaccination was widely practiced, conjunctivitis from NDV infection occurred in crews eviscerating poultry in processing plants. Transmission from animals to humans occurs via aerosols from poultry or from workers rubbing their eyes with hands contaminated from contact with birds or the virus. The disease has not been reported in people who rear poultry or consume poultry products. Human to human transmission has not been reported.

Information available from a phase I clinical study of intravenous administration of NDV PV701 by Laurie and colleagues (2006): This trial was approved by Health Canada and the research ethics boards of the Ottawa Regional Cancer Centre and the Ottawa Hospital, and all patients provided written informed consent.

Although no human-to human transmission has been documented with Newcastle disease virus, as a precaution, patients were instructed to avoid infants ages <1 month, pregnant women, and those with immunodeficiency until 3 weeks following discontinuation of study therapy.

During the first two cycles of therapy before each infusion of PV701 and before dose 1 of each subsequent cycle, urine samples were collected and stored at below -65°C to test for viruria (the presence of viruses in the urine). The amount of virus in the samples was determined by establishing a relative potency for each sample compared with the standard. This viruria assay had a limit of detection of 70 pfu/mL and a lower limit of quantification of 600 pfu/mL. Patient urine output was not quantified, thus, the results described in the study only represent a semiquantitative assessment of viral shedding.

Side effects observed during clinical trials: The side effects associated with exposure to NDV have generally been described as mild to moderate. NDV has been reported to cause mild flu-like symptoms, conjunctivitis, and laryngitis in humans (Csatary et al. 1999 ; Csatary et al. 1993; Lorence et al. 1994; Lorence et al. 1994; Batliwalla et al. 1998; Reichard et al. 1992). The most commonly reported side effect after treatment of cancer patients with the virus alone is fever, which usually subsides within 24 hours (Csatary et al. 1993; Wheelock et al. 1964; Pecora et al. 2002). In one study of infectious virus, localized adverse effects, such as inflammation and edema, were observed in the vicinity of some tumors (Pecora et al. 2002). These adverse effects may have contributed to the death of one patient (Pecora et al. 2002). Other adverse effects reported in this study included fatigue, low blood pressure, shortness of breath, and hypoxia. Some of these adverse effects were serious enough to require hospitalization. Human-to-human transmission during clinical trials (patient-to-medical staff) and during post-treatment recovery (patient-to-family members) has not been reported.

Environmental risk assessment/ Biosafety

Vaccination for Newcastle disease is routinely practiced in countries where virulent strains of the NDV are endemic and in countries where virulent strains do not exist but ill-timed infection by a low virulent field strain may have significant economic consequences for the producer. The types of vaccines and vaccination schedules used vary depending on the potential threat, virulence of the field challenge virus, type of production, and production schedules. A combination of live and inactivated ND vaccine, administered simultaneously, is shown to provide better protection against virulent NDV and has been successfully used in control programs in areas of intense poultry production.

Strain	Description
F	Lentogenic. Usually used in young chickens but suitable for use as a vaccine in chickens of all ages.
B1	Lentogenic. Slightly more virulent than F, used as a vaccine in chickens of all ages.
La Sota	Lentogenic. Often causes post vaccination respiratory signs, used as a booster vaccine in flocks vaccinated with F or B1.
V4	Avirulent. Used in chickens of all ages.
V4-HR	Avirulent. Heat Resistant V4, thermostable, used in chickens of all ages.
I-2	Avirulent. Thermostable, used in chickens of all ages.
Mukteswar	Mesogenic. An invasive strain, used as a booster vaccine. Can cause adverse reactions (respiratory distress, loss of weight or drop in egg production and even death) if used in partially immune chickens. Usually administered by injection.
Komarov	Mesogenic. Less pathogenic than Mukteswar, used as booster vaccine. Usually administered by injection.

Table 5: Eight strains of Newcastle disease virus used in chickens (poultry) as live vaccines

Regarding clinical trials with NDV as an anti-cancer agent, the following safety information has been provided: PV701, a live attenuated virus, is safe to administer from an occupational health and a public health perspective. The virus is not a human pathogen, and no contacts of the patients enrolled to this trial developed any infectious illness potentially related to PV701. The levels of virus shed in the urine (viruria) diminished with repeated dosing, with no indication of persistent shedding (Laurie et al. 2006) This trial was approved by Health Canada and the research ethics boards of the Ottawa Regional Cancer Centre and the Ottawa Hospital, and all patients provided written informed consent. However, no information regarding visits/no-visits to local poultry farms, hatcheries, pet shops or any natural bird habitats was provided.

Interestingly, in all current clinical trials using NDV as anti-cancer agent, reverse-transcription real-time PCR (RRT-PCR) analysis of the virus present in the urine samples has not been performed. Thus there is no data to support that viruses shed in the urine are genetically identical to the master stock administered to the patients. Reverse transcription real-time PCR is used as a quantitative method to determine the presence of individual sequences within nucleic acid mixtures. By this method it is possible to determine the ratio of virulent to avirulent F cleavage sequences within a mixed population of nucleic acid sequences. This low level of virulent virus remains in the viral population without apparent affect in infected birds until a selection pressure is placed upon it. Thus, virulent sequences can remain undetected in the viral quasispecies until a selection pressure is placed upon the isolate so that the virulent virus becomes the dominant sequence present in the quasispecies and virulent clinical signs are then observed upon infection.

The relative environmental risk assigned to NDV is **'Medium'**. This is based on the evidence of zoonotic infections in human, which caused conjunctivitis and laryngitis. The virus replicates efficiently in human tumor cells and may be transmitted to others, e.g. via exhaled air.

Genus Respirovirus
Sendai virus

Pathogenesis/Disease association/Epidemiology / Host range / Tissue tropism
Sendai virus (SeV), also known as murine parainfluenza virus type 1 or hemagglutinating virus of Japan (HVJ) is responsible for a highly transmissible respiratory tract infection in mice, hamsters, guinea pigs, rats, and occasionally pigs. Rats, mice, and hamsters are the exclusive natural hosts of SeV. Sendai virus is the murine counterpart of human parainfluenza virus 1 (HPIV1), and these two viruses share high sequence homology and antigenic cross-reactivity. The virus can be detected in mouse colonies worldwide, generally in suckling to young adult mice. Epizootic infections of mice are usually associated with a high mortality rate, while enzootic disease patterns suggest that the virus is latent and can be cleared over the course of a year. Sub-lethal exposure to SeV can promote long-lasting immunity to further lethal doses of SeV.

The SeV is immunosuppressive and can have an immediate, as well as a long term effect, on the rat's immune system. SeV infection may cause high morbidity (illness) and mortality rates when combined with bacterial pathogens (e.g. bacterial inflammation of the trachea and lung).

Infection is most devastating to very young, elderly, and immunocompromised rats and mice who may develop a more severe pneumonia and in which the virus may persist for a longer amount of time. Following symptoms can be observed: sneezing, hunched posture, respiratory distress, porphyrin discharge from eyes and/or nose, lethargy, failure to thrive in surviving babies and young rats and anorexia.

SeV is a descending respiratory infection. It begins in the nasal passages, and moves through the trachea into the lungs. Viral replication occurs in the respiratory tract for approximately one week after initial exposure. It is rare for the virus to become a generalized infection in the blood. Virus infection causes necrosis of the respiratory epithelium. In the first few days of infection epithelium necrosis is mild. As the disease progresses the necrosis becomes severe and usually occurs around day 5. The virus titer peaks at 5 to 6 days. By day 9 the regeneration of respiratory tract surface cells occurs, and virus titer decreases to undetectable levels around day 14 post infection. Focal interstitial pneumonia occurs and inflammation and lesions of varying degrees can develop on the lungs. There is no carrier state and cessation of breeding in the colony will eventually naturally eliminate SeV infection.

SeV invades host cells by fusion (F) protein-mediated membrane fusion at the plasma membrane. The receptor binding protein for SeV, as well as the other parainfluenza viruses, is the hemagglutinin-neuraminidase (HN) protein. During viral entry the HN protein binds sialic acid-containing receptors on the surfaces of host cells and then triggers the F protein to refold and cause membrane fusion. Virus replication occurs in the cytoplasm of infected cells, where the viral nucleocapsid is formed by the encapsidation of the viral genome with the viral nucleoprotein (N), phosphoprotein (P), and the large RNA-dependent RNA-polymerase (L) protein. The assembly and budding of infectious parainfluenza virions from the plasma membrane are mediated largely by the matrix (M) protein, which interacts with the viral nucleocapsid and the cytoplasmic tails of the HN and F proteins. The paramyxovirus F protein mediates both virus-cell fusion and cell-cell fusion. In order to become activated for membrane fusion, uncleaved F0 precursor protein trimers must be cleaved into a fusion-capable complex formed by F1 and F2 subunits. Field isolates of Sendai virus that have a monobasic cleavage site are cleavage activated by tryptase Clara secreted from respiratory epithelial cells while the pantropic F1-R laboratory isolate of Sendai virus has a mutated cleavage site and is cleaved by more ubiquitously expressed proteases.

There is apparent correlation between the membrane fusion activity of the F protein and the virulence of SeV in mice. While wild-type SeV infection is an acute infection restricted to the respiratory tract in immunocompetent mice, the virus can persist longer and disseminate to the spleen, pancreas, kidney, liver, brain, and blood in cortisone- and cyclophophasmide-based immunosuppressed mice (Miyamae, 2005).

Current status and stage of the research activities

The stage of the research activities is currently at the level of *in vitro* studies, although a few *in vivo* (experimental animals) experiments have been performed. A recent review by Cattaneo (2010) describes current progress regarding the use of paramyxoviruses (including Sendai virus) as vectors for cancer therapy.

According to the current publications the following characteristics emphasize the use of SeV as a gene therapy vector: (a) an exclusively cytoplasmic replication cycle without any risk of integration into the genomic DNA, (b) transduction efficacy that is not dependent on the cell cycle of target cells, (c) no homologous recombination between different SeV genomes or to wild-type virus, (d) the remarkably brief contact time that is necessary for cellular uptake, (e) a high and adjustable expression of virally encoded genes in a broad range of host cells, and (f) the lack of association with any disease process in humans (Bitzer et al. 2003). The SeV vector has been shown to produce 2 to 3 logs higher transfection efficiency than the adenoviral vectors or lipofection, and high gene expressions have been noticed in a broad range of tissues, including the airway epithelial cells, vasculature tissues, skeletal muscle, activated T cells, stem cells, and neural tissues. In addition to these biological

features of the SeV vector, a nontransmissible recombinant SeV vector has been constructed by deleting the M and F genes from its genome to enhance its safety (Inoue et al. 2003; Inoue et al. 2004).

Available assessment information in humans or humanized systems

Currently there are no reports on use of Sendai virus as oncolytic agent in humans. Regarding reports describing *in vivo* (animal) experiments, Iwadate and colleagues have shown (Iwadate et al. 2005) that the nontransmissible recombinant SeV vector could transfer genes efficiently into the glioma cells *in vivo*, and this directly correlated with the therapeutic efficacy against the established brain tumors. The magnetic resonance imaging study showed that the intracerebral injection of hIL2-SeV/ΔMΔF brought about significant reduction of the tumor growth, including complete elimination of the established brain tumors. To minimize the side effects of oncolytic viruses, the anti-tumor activities of inactivated HVJ particles (HVJ-E) have been tested. It appears that HVJ-E itself mediates a powerful anti-tumor effect by enhancing cytokine production in dendritic cells (DCs), generating tumor-specific cytotoxic T cells (CTLs), and inhibiting regulatory T-cell activity (Kurooka and Kaneda, 2007).

Kawano and colleagues (Kawano et al. 2007) attempted to eradicate tumors derived from mouse colon cancer cells, CT26, by administering inactivated HVJ-E particles that incorporated bleomycin (BLM). This treatment was combined with cisplatin (CDDP) administration. HVJ-E/BLM was directly injected into the tumor mass with or without intraperitoneal administration of a drug cisplatin. The anti-tumor effect was evaluated by measuring tumor size and cytotoxic T cell activity against CT26. It is observed that three intratumoral injections of HVJ-E/BLM along with a single intraperitoneal administration of CDDP eradicated CT26 tumors with more than 75% efficiency. When tumor cells were intradermally re-injected on day 10 after the initial tumor inoculation, tumors on both sides disappeared in most of the mice that received the combination therapy of HVJ-E/BLM and CDDP. Eight months after the initial tumor eradication, surviving mice were re-challenged with CT26 cells and tumor formations were rejected in all of the surviving mice treated with the combination therapy. Surviving mice were able to generate cytotoxic T lymphocytes specific for CT26.

Furthermore, a few reports regarding anti-tumor effects of HVJ-E in murine tumor models have been published. Renal cancers transplanted to Balb/c mice intradermally were completely eradicated by three times injection of HVJ-E alone (Fujihara et al. 2008) and orthotopic bladder carcinoma was suppressed by intravesical injection of HVJ-E with doxorubicin (Kawano et al. 2008). Activation of anti-tumor immunity was detected in both cases after HVJ-E treatment.

Bio-selection and genetic modifications

In order to establish SeV vector system that is safe for future clinical application, a strategy has been applied to generate the gene(s)-deleted or modified SeV vectors. Kinoh and colleagues (Kinoh and Inoue, 2008) have succeeded in the recovery in high titers of fusion (F) gene-deleted (SeV/ΔF), matrix (M) gene-deleted (SeV/ΔM), hemagglutinin-neuraminidase (HN) gene-deleted (SeV/ΔHN), both M and F genes-deleted (SeV/ΔMΔF), all of the envelop-related genes-deleted (SeV/ΔMΔFΔHN) SeV vectors by using the packaging cell lines which express respective proteins of those deleted gene(s). Among them, SeV/ΔM vector was selected in generating an oncolytic SeV vector targeting a solid tumor tissue. Thus, redesigned SeV vector has lost the ability to form particle from transduced cells and gains cell-to-cell spreading in protease-dependent, namely controllable, manner. For the selective delivery to malignant tumor cells expressing matrix metalloproteinases (MMPs) or urokinase-type plasminogen activator (uPA), MMP-cleavage (PLGMTS) or uPA-cleavage (SGRS) sequences are introduced, respectively, immediately prior to the cleavage site for activation of fusion protein with remaining essential sequences for cell-fusion. The MMP-targeted SeV vector demonstrated syncytia formation in MMP expressing human sarcoma (HT1080) cell line in vitro, and growth inhibition of HT1080 subcutaneous xenografts in vivo. The uPA-targeted vector showed the same effects in uPA expressing human prostate cancer (PC-3) cell line. Severe apoptosis occurred in fused-cells. Thus, the vector selectively spreads to tumor cells in tumor-protease dependent manner and demonstrates the antitumor effects in solid tumors, indicating the value of selective targeting and killing of tumors by recombinant SeV technology.

Naturally occurring mechanism of Sendai virus attenuation is the presence of a nonconsensus gene start signal upstream of the F gene that decreases the reinitiation capacity of the viral polymerase, resulting in decreased expression of the downstream F, HN, and L genes. Mutation of the gene start sequence upstream of the F gene (AGGGATAAAG) to the more efficient sequence that is found upstream of the P, M, and HN genes (AGGG**TG**AAAG; mutated residues are in boldface) has been shown to increase the rate of Sendai virus replication in vitro and result in increased morbidity and mortality in mice (Kato et al. 1999).

Two other features of the SeV F gene have been previously discovered that prevent the virus from attaining maximum growth and virulence in mice. The SeV F protein has a monobasic cleavage site at residue R116 that requires the presence of extracellular protease for cleavage of the F0 precursor protein into its fusion-active F1-F2 form (Homma and Ouchi, 1973).

Tryptase Clara secreted from mouse respiratory epithelial cells cleaves wild-type SeV F protein in the lungs of mice (Tashiro et al. 1992). For the SeV variant F1-R that was

isolated from persistently infected tissue culture cells (Tashiro et al. 1988), F protein mutations that permit cleavage by more ubiquitously expressed proteases were found to support greater tissue tropism and increased pathogenicity in mice (Tashiro et al. 1999).

Horizontal transmission and establishment in the human population
SeV is highly contagious. Natural infection occurs by way of the respiratory tract. Transmission can be contact and airborne. Airborne transmission can occur over a distance of 1-2 meters as well as through air handling systems. Transmission by fomites can be reduced by strict hygienic practices.

Regarding the safety in clinical application, the biological features of SeV, such as the lack of integration into the cellular genome and the lack of homologous recombination between different SeV genomes, are desirable. Furthermore, by utilizing both M and F genes–deleted SeV (SeV/MF) it shows the ability to enhance vector's safety. These characteristics of SeV/ΔMΔF are considered suitable for the clinical application in gene therapy. In contrast, a theoretical obstacle for the clinical application could be the presence of antibodies against the human parainfluenza virus type I, which are known to cross-react with SeV HN proteins (Bitzer et al. 2003). However, it is currently not known whether a respiratory infection with human parainfluenza virus type I in the past will interfere with the transduction process of SeV vector at distant site, such as the central nervous system.

Environmental risk assessment/ Biosafety
Regarding the possibility of the environmental risk due to the clinical use of the SeV vectors (from human to animal) the F gene deletion made the SeV vector nontransmissible and M gene deletion worked well to make SeV incapable of forming particles from the infected cells. Although simultaneous deletions of these two genes in the same genome resulted in combining both advantages and contributed to increase the safety of the SeV vector, SeV/ΔMΔF still retains high levels of infectivity and gene expression in vitro and in vivo (i.e., similar to the wild-type SeV; Inoue et al. 2004).

There is no treatment for SeV infection of rat and mice. However, it is essential to treat immediately for secondary bacterial infections with broad-spectrum antibiotics as soon as a SeV infection is noticed. In the laboratory, where rats and mice are often free of specific pathogens, SeV is not as dangerous to rats and mice as it is in the pet population. SeV infection can devastate a pet or breeding colony. It moves quickly and the complications from secondary infections can virtually wipe out a group, thus, immediate antibiotic treatment is essential. During SeV outbreak a strict quarantine must be observed to ensure that the virus runs its course and is eliminated. The virus is inactivated by UV (ultraviolet) light, temperatures above 37°C, and lipid solvents (such as alcohol).

A formalin-killed SeV vaccine was developed in the 1980s. It involved repeated dosing to show any effectiveness at all. For the most part the research has not been continued. At present, Sendai virus infections are rarely an issue in many experimental animal facilities (laboratories).

The relative environmental risk category that replication-competent Sendai virus is assigned to is **'Medium'**. While the environmental hazards of Sendai virus are relatively small, there is little data on pathogenicity in human. The virus replicates efficiently in human tumor cells. Air borne transmission in humans can not be excluded.

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Family *Rhabdoviridae*

Rhabdoviridae are currently classified into seven genera. Rhabdoviruses carry their genetic material in the form of a single RNA strand (11 – 12 kb) of negative polarity (i.e. it cannot be directly translated) that is completely coated by the viral nucleoprotein. During infection, these viruses synthesize five subgenomic mRNAs that encode five distinct proteins; the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G), and the large polymerase protein (L). Replication characteristically occurs in an inclusion body (negri body) within the cytoplasm, from where they bud through various cytoplasmic membranes and the outer membrane of the cell. This process results in the acquisition of the M + G proteins, responsible for the characteristic bullet-shaped morphology of the virus. Rhabdoviruses that infect vertebrates are bullet-shaped.

The pathogen that humans should be most concerned with is the rabies virus (RaV) that belongs to genus Lyssavirus. This causative agent of disease Rabies, is capable of infecting all warm blooded animals.

Genus Vesiculovirus
Vesicular stomatitis virus

The prototypical and best studied Rhabdovirus is vesicular stomatitis virus. Since it is easy to grow in the laboratory, it is a preferred model system to study the biology of Rhabdoviruses.

Pathogenesis/Disease association/Epidemiology/Host range/Tissue tropism
Vesicular stomatitis virus is a causative agent of vesicular stomatitis (VS). In animals, vesicular stomatitis causes a mild fever, and the formation of blister-like lesions on the inside of the mouth, and on the lips, nose, hooves and udder. The blisters break, leaving raw, sore areas. Affected animals often salivate profusely, may become lame and are unwilling to eat or drink. The incubation period (the time between infection with the virus and clinical signs) may range from two to eight days, and animals generally recover completely in three to four days.

VS is mainly found in regions of North and South America. The Indiana (VSV-Ind) and New Jersey (VSV-NJ) virus strains are the two most prominent types in North America. Viruses from the same genus are present in Asia and Africa, and disease primarily affects people handling dairy cattle and secondarily beef cattle. While VS causes discomfort to affected animals, and may result in substantial loss in livestock trading and meat markets, it is most significant because it closely resembles early symptoms of foot and mouth disease (FMD), a devastating disease for producers.

VSV is an arthropod-borne virus with wide host range. It primarily affects rodents, cattle, swine and horses but can also infect humans and other species. The virus is transmitted from animal-to-animal by arthropod vectors such as sandflies, mosquitoes, and blackflies. Phlebotomine sandflies are confirmed biological vectors of VSV. The virus has also been found to replicate in *Culicoides* midges and *Simulium* blackflies. Interestingly, infected female insects can transmit the virus transovarially (Tesh et al 1972). Virus has been cultured from those vectors, and transmission has been demonstrated experimentally (Mead et al. 2000).

The glycoprotein of VSV (VSV-G) serves both to bind the surface of the host cell and to fuse viral and cellular membranes, enabling the release of the viral genome and replicase into the cytoplasm. The glycoprotein binds to phosphatidylserine, a near-universal component of cell-surface membranes, enabling VSV to infect virtually all animal cells. This extensive tissue tropism, therefore, enables VSV to be used as an anti-cancer agent in all types of tumors, although normal tissue can also be infected. VSV-G is also used for pseudotyping retroviruses and lentiviruses.

Current status and stage of the research activities

The lack of antibodies against VSV in the general population and the extremely rare incidence of adverse outcomes following human infection are important prerequisites for its potential as a candidate therapeutic virus. There is an ample of scientific publications describing the potential of VSV as anti-cancer agent (reviewed by Barber, 2004). At the level of in vitro studies, VSV induces preferential killing of infected tumor cells. In addition, VSV is capable to reduce tumor size and to spread in melanoma, lung cancer, colon cancer and certain brain tumors in laboratory in vivo models of cancer.

Mayo Clinic (Rochester, Minnesota, USA) researchers are working to bring several research projects in the Molecular Medicine Department to the stage of clinical trials in patients. Among planned clinical trials that will commence soon, a trial using vesicular stomatitis virus (VSV) as anti-cancer agent (oncolytic virus), is planned for patients with hepatocellular carcinoma. This clinical trial (phase I) is expected to open in 2010. This information has been obtained at the following site:
(http://mayoresearch.mayo.edu/molecular_medicine/)

Available assessment information in humans or humanized systems

Currently there are no reports on VSV in-use as a oncolytic agent in humans. Regarding in vivo experiments (humanized systems), in a recent study it was shown that i.v. administration of replication-competent VSV to immunocompetent rats with hepatocellular carcinoma significantly prolonged survival (Shinozaki et al. 2004). Furthermore, genetically engineered replication-competent VSV expressing either IL-4 or HSV-TK, killed syngeneic breast carcinoma and melanoma in immunocompetent mice more efficiently then wt virus (Frenandez et al. 2002). The

tumor specificity of VSV, as in many other RNA viruses, is largely determined by the inability of cancer cells to mount an effective anti-viral response due to defects in type I interferon signaling pathway (Stojdl et al. 2000). When a recombinant VSV virus termed VSV$^{\Delta M51}$ was recently used to target experimental human glioma xenografts in nude mice, the virus was lethal to nude mice when given i.c. However, when the virus was administered by i.v. procedure, the mutant virus was well tolerated and was able to prolong animal survival. These data indicate that other mechanisms then susceptibility to type I interferons govern the neurotoxicity of recombinant VSV (Lun et al. 2006).

Bio-selection and genetic modifications

Several naturally occurring or recombinant strains of VSV have been developed as potential therapeutic vectors (reviewed by Lichty et al. 2004) and they are currently used in experimental settings. However, it should be noted that members of Rhabdoviridae family display a high mutation rate, thus their tendency to change or revert could result in adaptation to human host. In addition, the laboratory adapted strains with demonstrated low virulence pose a lower risk of infection and pathogenicity (Novella et al. 2010; Jenkins et al. 2002; Drake, 1993).

Horizontal transmission and establishment in the human population

Naturally occurring human infections with VSV are rare. If vesicular stomatitis fever occurs in humans (incubation period up to 6 days, usually 3-4 days), it is manifested by influenza-like symptoms; headache, fever, retrobulbar pain on motion of eyes, malaise, nausea, pain in the limbs and back; possible vesicular lesions in mouth, lips and hands; leukopenia; In blood, the virus is infective for at least 24 hours before and after onset of fever. Recovery may be prolonged and death is unknown except for a case of encephalitis in a 3-year-old boy that has been reported, which is potentially associated with VSV-Ind infection (Roberts et al. 1999). At present, there are no published reports on human-to-human transmission of VSV.

Environmental risk assessment/ Biosafety

Control of outbreaks that occur in livestock is dependent upon rapid recognition of initial cases, quarantine and restriction of movement of infected and in-contact animals, and insect control. There are no commercially-available VSV vaccines in the US, but an autologous vaccine was made in 1995 to help control that outbreak. Several inactivated vaccines containing both the Indiana and New Jersey serotypes are used in Central and South America (veterinary use). Furthermore, in the US vaccination of pigs against VS is not allowed. Countries which are completely free from VS (e.g. countries in Europe) apply national preventative measures against the introduction of VS. The main feature is control over the importation of cattle and pigs from countries in which VS occurs.

The disease could be transmitted to humans who come into contact with infected animals (vesicular fluid, saliva) or by insect vector (via the bite of an infective sandfly, mosquito or blackfly). In experimental settings, it has been shown that after being bitten by black fly, viremia cannot be detected in infected mammalian host. However, non-infected black flies can acquire the virus if they feed on infected, non-viremic host (Mead et al 2000). Thus co-feeding is a mechanism of infection for an insect-transmitted virus. VSV can also be spread by direct contact among infected pigs. (Stallknecht et al. 1999).

The Vesicular Stomatitis virus is classified in the '**Medium'** category of the relative envronmental risk classification. Exposure to infectious aerosols has resulted in many laboratory-acquired infections, althoufh their pathogenicity is relatively mild. There is a documented hazard to personnel handling infected livestock, tissues and virulent isolates; between 40 and 46 laboratory-associated infections were reported before 1980 (CDC, USA). Primary hazard to humans are blood and throat secretion of infected individuals. Hence horizontal transfer and shedding in the environment may occur.

Maraba virus

Pathogenesis/Disease association/Epidemiology/Host range/Tissue tropism
Maraba virus belongs to the vesiculoviruses group. A single strain of Maraba virus (BeAr 411459) was isolated from a pool of 70 female phlebotomine sand flies (Lutzomyia spp.) captured from tree trunks in Serra Norte, municipality of Maraba, Para State, Brazil in 1983 (Travassos da Rosa et al. 1984). Animals and human sera collected at the time from the same region (the Amazon basin of Brazil) were tested for the presence of neutralizing antibodies. Interestingly, only one human sample appears positive for antibodies against Maraba virus. The pathogenicity of this virus in newborn mice is similar to that of VSV-Indiana and VSV-New Jersey. Maraba virus can kill newborn mice within 24 hours after intracerebral inoculation. It is also lethal to an adult mouse but not if administered intraperitoneally. The virus grows well on Vero cells. The virus is able to replicate in sand flies following experimental intrathoracic inoculation, and it can be transovarially transmitted in those flies.

At present it is not known if Maraba virus can cause disease in humans and/or animals. Serological tests indicate that humans are exposed to the source of the virus but rarely infected. However, it should be noted that the region from where the virus has been isolated is largely a forest with relatively few human inhabitants.

Nevertheless, Maraba virus is an interesting virus since it is antigenically closely related to VSV-Indiana, Cocal, and Alagoas viruses. These viruses are known to cause vesicular disease in cattle and swine. There are no records of Maraba virus being experimentally administered to domestic animals.

Current status and stage of the research activities

With the aim to expand current array of safe and potent oncolytic viruses, Brun and colleagues (Brun et al. 2010) preferentially screened a variety of wild-type rhabdoviruses against a panel of tumor cell lines. The results point to a number of viruses with varying degrees of cytolytic activity. Maraba virus was the most potent of all 20 rhabdoviruses tested. Maraba virus demonstrated good cytolytic activity against tumor cell lines (37 cell lines from the NCI 60 cell panel). Furthermore, Maraba virus replicated productively and killed the majority of cancer cell lines (e.g. breast, CNS, colon, melanoma, lung, ovarian, prostate, and renal cancer cell lines). Thus, Maraba virus appears to be a potent oncolytic agent.

Available assessment information in humans or humanized systems

Currently there are no reports on use of Maraba virus as oncolytic agent in humans.
Regarding in vivo experiments (humanized systems) in a recent study by Brun and colleagues (2010) it was shown that Maraba virus was well tolerated following intravenous injection of immunocompetent Balb/C mice. Maximum tolerable dose (MTDs) for Maraba WT and several attenuated strains was also determined. Animals that received a lethal dose of Maraba WT and two of most promising mutant strains (V221Y or Maraba MG1), display signs of central nervous system infection and had significant titers of virus in their brains. Interestingly, the MTD of Maraba MG1 mutant was 100-fold greater than the WT virus. At doses below MTD, mice generally showed transient weight loss and dehydration consistent with a virus infection. Observed symptoms resolved within 3-4 days post-infection and no virus could be detected in the brains of these mice euthanized 12 days later.

Importantly, it is shown that Maraba virus is efficacious in syngeneic and xenogeneic tumor models. Animals that received six systemic doses of Maraba MG1 (selected mutant, see below) responded to treatment with complete tumor regression (subcutaneous CT26; murine colon carcinoma) by day 35 and durable cures in 100% of the animals. Complementary to those studies, Brun and colleagues performed tests using immunocompetent animals bearing human ES2 ovarian xenografts. Even at very low dose (10^4 pfu) animals treated with Maraba MG1 had significant reduction in tumor burden. The results point to a general conclusion that Maraba MG1 is more effective at killing tumor cells then ΔM51 VSV or even WT Maraba virus.

Bio-selection and genetic modifications

Brun and colleagues (Brun et al. 2010) developed a system for genetic modification of Maraba virus by reverse genetics. Several recombinant strains of Maraba virus have now been developed as potential therapeutic vectors. Mutations have been introduced into recombinant Maraba backbone (rMaraba WT) that might improve the tumor-selective killing properties of WT Maraba virus. The authors explored two previously identified mutations in VSV that improved virus replication on BHK-21

cells (M protein L123W and L protein H242R). The corresponding mutations were identified to be L123W and Q242R in the Maraba virus sequence of the M and G proteins, respectively. The double mutant (referred to as Maraba MG1) showed no impairment in replication kinetics. Interestingly, Maraba MG1 appears to be attenuated on primary human skin fibroblasts but remained highly lytic when assayed on a panel of malignant cell lines. Furthermore, Maraba MG1 appears significantly more virulent in several cancer cells when compared to ΔM51 VSV (deletion of methionine 51 in the M protein of VSV).

Horizontal transmission and establishment in the human population
Naturally occurring human infections with Maraba virus appear to be rare and it is not known if virus infection is a threat to human health. However, it should be noted that Maraba virus is antigenically closely related to VSV-Indiana, Cocal and Alagoas viruses, which are known to infect cattle and pigs.

Environmental risk assessment/ Biosafety
It remains to be determined if Maraba virus could be transmitted to humans who come into contact with infected animals(note that the animal host is unknown) or by insect vector (via the bite of an infective sand fly, mosquito or blackfly). **Maraba virus** This virus is known to cause severe pathology in newborn mice. Despite the fact that the virus probably requires an insect host for transmission between susceptible mammals, we classify the relative environmental risk as '**High**' based on the scanty information on the biology and pathology of Maraba virus.

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Family *Coronaviridae*

Coronaviruses are species in the genera of animal viruses belonging to the subfamily *Coronavirinae* in the family *Coronaviridae*. Coronaviruses are enveloped viruses with a positive-strand RNA genome with size ranging from approximately 16 to 31 kilobases, thus coronaviruses are considered the largest of all RNA viruses. In electron microscopy, the virus envelope appears to be crowned by a characteristic ring of small bulbous structures. These structures are formed by the viral spike peplomers, proteins that populate the surface of the virus and determine host tropism. The name "coronavirus" is derived from the word “corona”, meaning crown.

Proteins that contribute to the overall structure of all coronaviruses are the spike (S), envelope (E), membrane (M) and nucleocapsid (N). In the specific case of SARS, a defined receptor-binding domain on S mediates the attachment of the virus to its cellular receptor, angiotensin-converting enzyme 2 (ACE2). Members of the group 2 coronaviruses also have a shorter spike-like protein called hemagglutinin esterase (HE) encoded in their genome.

At least five different coronaviruses are known to infect humans. The most publicized human coronavirus, SARS-CoV which causes SARS (Severe Acute Respiratory Syndrome), has a unique pathogenesis because it causes both upper and lower respiratory tract infections and can also cause gastroenteritis. Coronaviruses are believed to cause a significant percentage of all common colds in human adults, primarily in the winter and early spring seasons. Since human coronaviruses are difficult to grow in the laboratory, it is often difficult to assess the significance and economic impact of coronaviruses as causative agents of the common cold.

In 2003, following the outbreak of SARS, the World Health Organization issued a press release stating that a novel coronavirus identified by a number of laboratories was the causative agent for SARS. The virus was officially named the SARS coronavirus (SARS-CoV). Following the high-profile publicity of SARS outbreaks, there has been a renewed interest in coronaviruses in the field of virology. For many years, only two human coronaviruses were known HCoV-229E and HCoV-OC43. The discovery of SARS-CoV added another human coronavirus to the list, later followed by a fourth and in 2005, a fifth human coronavirus in two pneumonia patients.

Coronaviruses are common respiratory pathogens of mammals and birds. They primarily infect the upper respiratory and gastrointestinal tract. In the non-human hosts such as in farm animals and domesticated pets, coronaviruses cause a range of diseases, some of which can be serious and are a threat to the farming industry. Economically significant coronaviruses of farm animals include porcine coronavirus

(transmissible gastroenteritis coronavirus, TGE) and bovine coronavirus, which both result in diarrhea in young animals. Feline Coronavirus (e.g. Feline enteric coronavirus) is a pathogen of minor clinical significance, but spontaneous mutation this virus can result in feline infectious peritonitis (FIP), a disease associated with high mortality. There are two types of canine coronavirus (CCoV), one that causes mild gastrointestinal disease and one that has been found to cause respiratory disease. Mouse hepatitis virus (MHV) is a coronavirus that causes an epidemic murine illness with high mortality, especially among colonies of laboratory mice. Significant research efforts have been focused on elucidating the viral pathogenesis of these animal coronaviruses, especially by virologists interested in veterinary and zoonotic diseases.

Genus Coronavirus
Mouse Hepatitis virus

Pathogenesis/Disease association/Epidemiology/Host range/Tissue tropism
Prior to the discovery of SARS-CoV, mouse hepatitis virus (MHV) had been the best-studied coronavirus both *in vivo* and *in vitro* as well as at the molecular level. MHV causes a variety of diseases, such as enteritis, hepatitis and demyelinating encephalomyelitis, in susceptible rodents (Haring and Perlman 2001). *Mus musculus* (domestic "house" mouse) is the natural host and the virus infection in immunocompetent mice is usually asymptomatic. It is nearly ubiquitous among conventional mice, and not uncommon in "barrier maintained" mice. Enzootic (prevalent) subclinical infection is typical of conventional breeding colonies, in which adults are immune and suckling mice are passively protected by residual maternal antibody up to weaning. When inoculated experimentally, suckling rats had transient virus replication in nasal mucosa and seroconversion but no clinical disease is apparent. Also Deer mice (*Peromyscus sp.*) show seroconversion with no signs of clinical disease.

Manifestation and the outcome of the disease depend on the age and genotype of laboratory mice, in addition to the tropism and virulence of the virus strain. For example, in suckling BALB/c and ICR mice, enteric strains such as MHV-Y can cause encephalitis.

Infection with most strains follows the respiratory system. Viral antigen appears first in the nasal mucosa, then in the lungs, followed by lymphoid organs, bone marrow, brain, liver, and intestine. In most natural infections, gross lesions are not present or are transient, thus, not observed. Susceptible mice infected with virulent virus can have severe acute hepatitis, in which the liver is diffusely tan or yellow with an accentuated lobular pattern.

Mouse hepatitis virus is not a single virus, but a group of serologically and genetically related but distinct strains. To date, about 25 strains have been reported. The best studied strains are the "prototype strains" MHV-1, MHV-2, MHV-3, JHM, A59, and S, of which MHV-3 is regarded as the most virulent. Field isolates often differ more or less from laboratory used strains. MHV, like other coronaviruses, mutates rapidly, and strains readily form recombinants, so the genetic heterogeneity is very large. Strains can differ in tropisms, tendency to disseminate, and virulence.

Regarding virus tropism, enterotropic strains replicate initially in the intestinal epithelium. They exhibit a weak virulence and tend not to disseminate. Polytropic strains replicate initially in the respiratory tract, tend to disseminate to other organs (e.g. liver, brain, lymph nodes) and they are usually more virulent than enterotropic strains. Some strains of MHV can be maintained in culture, whereas others are very difficult. Enterotropic strains tend to be the most difficult to culture. The preferred cell line varies among strains; the most often used are NCTC-1469, 17Cl-1, DBT, BALB/c-3T3, and CMT-93.

In general, MHV has very narrow host range since viral tropism is determined solely on the basis of expression of the proper viral receptors on the cell surface.

Current status and stage of the research activities

The stage of the research activities is currently at the level of *in vitro* and *in vivo* animal model studies. There are few publications that provide interesting leads for further investigations on use of non-human coronaviruses as oncolytic agents. These viruses appear to possess a strong capacity to kill human cancer cells once they are able to enter those cells (Wurdinger et al. 2005; Verheije et al. 2006; Verheije et al. 2009). The results described in those papers demonstrate the feasibility of redirecting MHV to a non-native receptor on human cancer cells, extensive cell-cell fusion and efficient killing of the target cells.

Available assessment information in humans or humanized systems

Currently there are no reports on use of MHV as oncolytic agent in humans, and only few studies in animal models for human cancer. A recent study by Verheije et al. (2009) evaluated the oncolytic capacity of the redirected MHV (MHVsoR-EGF) in an established intracranial glioblastoma model (U87ΔEGFR) *in vivo*. Mice were injected intracranially at the position of a tumor with 10^5 infectious virus particles. Treatment with modified/redirected virus (MHVsoR-EGF) had a dramatic effect on survival resulting in six of seven animals becoming free of neoplasm. The oncolytic activity of this modified virus appeared to be fast (already observed at day 5 post-inoculation) and lasting with no recurrence of tumors. One major advantage of non-human coronaviruses as oncolytic agents is that they have a very short replication cycle, resulting in fast clearance of the tumor cells.

Bio-selection and genetic modifications

The utilization of a virus with specificity toward cancer cells allows to control and to treat various physiological responses of only these cells. Since MHV cannot establish an infection in either normal or cancerous human cells, its tropism can be modified either by substitution of the viral spike ectodomain (Kuo et al. 2000) or by the use of adapter proteins (Verheije et al. 2006; Wurdinger et al. 2005). These adapter proteins, composed of a virus-binding moiety coupled to a target cell-binding device, can redirect the virus to a specific receptor on the target cell. Once the host cell tropism barrier is alleviated, MHV is capable of establishing infection in nonmurine cells. The genetic information for the targeting device can be introduced into the viral genome to allow the virus to produce the adaptor itself in infected cells, thereby creating a self-targeted virus. It should be taken into account that coronaviruses exhibit high mutation rates and are prone to recombination. Of course, an obvious concern in such a treatment would be the occasional virus mutant that is more virulent to the host than is the original strain. Their application in adapter mediated targeting to tumors will thus raise questions about the safety of its application, particularly regarding the possibility of generating viruses that acquired the capacity to infect human cells independent of targeting devices (through cell-cell fusion and formation of large multinucleated syncytia). These questions will have to be addressed.

Horizontal transmission and establishment in the human population

Non-human coronaviruses do not normally infect human cells. Genetic modification of the viral envelope to alter tropism will result in infection of otherwise non-permissive host. Once inside of the cell, non-human coronaviruses are capable of initiating productive infection.

MHV transmission in its natural host occurs by several routes, including contact, aerosol, fomites, and airborne particles of feces, bedding, etc. Vertical transmission has been demonstrated in experimental infections, but it is not known whether this is important in natural transmission. MHV is a common contaminant of transplantable tumors and cell lines.

Environmental risk assessment/Biosafety

MHV is ubiquitous among general wild mice population and it is extremely contagious. It is regarded as the single most-important infectious agent of mice kept as research subjects. Difficulties may arise in multipurpose multi-user facilities. If infection occurs, it is best to eliminate affected population/stock. Alternatively, immunocompetent mice only shed virus for 2 or 3 weeks, and the virus is not environmentally resistant, so infection can be eliminated by not introducing new susceptible mice for several weeks.

In view of biosafety issues, it should be noted that coronaviruses exhibit strict species specificity and strongly depend on the receptor-specificity of the viral envelope, thus,

zoonosis and reverse zoonosis should not occur unless the tropism barrier is moderated.

Mouse hepatitis virus is rated '**Medium**' in the relative environmental risk score. This is based on its association with severe pathology in rodents and its capacity to be transmitted via aerosols. For therapeutic applications MHV will require artificial expansion its host-range. The biological consequences of such modifications on their biology (pathology, tissue tropism, shedding) in humans are unpredictable.

Feline Coronavirus
Feline enteric coronavirus (FECV)
Feline infectious peritonitis virus (FIPV)

Pathogenesis/Disease association/Epidemiology/Host range/Tissue tropism
Feline enteric coronavirus is highly contagious among cats in close contact. Although the feline enteric coronavirus is antigenically similar to the virus of feline infectious peritonitis (FIP, Feline Infectious Peritonitis and Pleuritis), the pathogenesis of each differs. The enteric form of infection is limited to the gastrointestinal tract. In catteries, the virus may be a cause of unapparent to mildly severe enteritis in kittens 6-12 weeks old. Adult cats often have subclinical infection. Death from the enteric form of disease is uncommon.

Although a large number of cats may be infected with the feline coronavirus, only a few develop clinical FIP. Positive coronavirus antibody titers are seen in ~10-40% of cats in the general cat population and in 80-90% of cats in catteries, but only 8% develop FIP. This disease is progressive and may manifest clinically as a continuum between the effusive (serositis or wet) and noneffusive (granulomatous or dry) forms. Mortality, even with therapy, approaches 100%.

The acute or primary infection often is asymptomatic, but in some cases, fever of unknown origin, conjunctivitis, and other upper respiratory signs and diarrhea may occur. This stage may last several days or weeks or longer before signs of effusive or noneffusive FIP develop. The development of FIP, and the particular clinical form of disease (ie, effusive or noneffusive) depends on the intrinsic immune responses of the cat. Cats with a strong humoral immunity and a weak or absent cell-mediated immune response against FIPV develop a persistent viremia and effusive FIP. Cats with partial cell-mediated immune responses along with humoral immunity develop the more chronic noneffusive FIP. Cats with strong cell-mediated immune responses with or without humoral responses can either completely recover or become persistently infected asymptomatic carriers. The latter may infect contact cats and may themselves later develop FIP, usually after periods of stress or co-infection with feline leukemia virus (FeLV). Some asymptomatic, seropositive carrier cats subsequently may become seronegative and stop excreting virus.

Field strains of feline coronavirus vary in their ability to induce FIP. Some isolates cause FIP (feline infectious peritonitis virus [FIPV]); others cause more localized gastrointestinal disease (Feline Enteric Coronavirus). The exact relationship between low virulence FIPV strains and feline enteric coronavirus, which is relatively nonpathogenic, is not clear. The close antigenic relationship of the enteric form of the virus and that causing clinical signs of FIP has led to speculation that FIP virus may be a mutated form of enteric coronavirus. Cross-protection is not induced by either virus to the other, and it is not clear if preexisting infection with the enteric form of disease accelerates or enhances the severity of disease associated with FIP. There is no known treatment that can cure FIP once clinical signs arise. Although spontaneous remission in treated cats has been reported, it is uncommon.

The prevalence of FIPV infection in the general cat population is difficult to determine because current serologic tests for detecting FIPV antibodies cannot discriminate between FIPV and other feline coronaviruses that do not produce disease and that may be more prevalent. Furthermore, FIPV is antigenically related to and serologically cross-reacts with a subgroup of mammalian coronaviruses, including transmissible gastroenteritis virus of swine, human coronavirus 229-E, canine coronavirus, and feline enteric coronavirus. FIPV and canine coronavirus are very closely related antigenically and may have crossed between hosts.

Although primarily a disease of domestic cats, FIP has been recognized in exotic *Felidae*, including the large and small wild cats. Among larger cats, FIP is seen in lions, leopards, jaguars, mountain lions, and especially cheetahs. Smaller cats susceptible to FIP include the sand cat, lynx, caracal, and Pallas' cat.

Feline coronavirus infects the apical columnar epithelium of intestinal villi of the duodenum, jejunum, and ileum, and causes the tips of villi to slough, fuse with adjacent villi, and atrophy. After ingestion of virus or aerosol exposure, FIPV initially replicates in tonsil or intestinal epithelium and then is transported via macrophages and monocytes to primary target organs such as liver, spleen, and visceral lymph nodes.

Current status and stage of the research activities

The current stage of research activities is at the level of *in vitro* studies. To explore the potential of non-human coronaviruses for oncolytic virotherapy Würdinger et al. (2005) investigated whether the feline coronavirus (feline infectious peritonitis virus; FIPV strain 79-1146; type II feline coronavirus (Horzinek, 2006.; Herrewegh et al. 1998) could be targeted to human cancer cells. FIPV normally infects feline cells via specific cellular receptor feline aminopeptidase N (fAPN). Interestingly, when fAPN was expressed on human cancer cells FIPV could efficiently enter, replicate and rapidly destroy those cells (tumor cells as monolayers and tridimensional multilayer

spheroids) via syncytia formation, a process typical of productive coronavirus replication. Furthermore, the group also describes the use of a bispecific single-chain (scFv) antibody to target FIPV to a relevant tumor-associated cellular receptor thereby alleviating the coronavirus entry barrier. This "target adaptor" antibody binds on one side to the feline coronavirus spike protein and on the other side will interact with the human epidermal growth factor (EGF) receptor. The data presented show that the use of the target-adaptor antibody mediated specific coronavirus infection of EGF receptor-expressing cancer cells. Feline coronavirus infection exhibited potent cytotoxicity by rapid and effective killing of human cancer cells.

According to the data available, the selective targeting of non-human coronaviruses to human cancer cells provides a rationale for further investigations into the use of these viruses as anti-cancer agents

Available assessment information in humans or humanized systems
Currently there are no reports on use of feline coronavirus as oncolytic agent in humans.

Bio-selection and genetic modifications
At present there is no information available on use of genetically modified replication-competent feline coronavirus for the purpose of developing anti-cancer viral agent.

Horizontal transmission and establishment in the human population
Most feline coronavirus infections probably result from ingestion of the virus; however, aerosol transmission is also possible. The virus is shed in the feces of seropositive cats thus close contact with an infected cat or its excreta are required for virus transmission. Transplacental (vertical) transmission is suggested by the occasional observation of FIP in stillborn kittens, but the frequency with which this occurs is unknown. Non-human coronaviruses do not normally infect human cells. Genetic modification of the viral envelope to alter tropism will result in infection of otherwise non-permissive host. Once inside of the cell, non-human coronaviruses are capable of initiating productive infection.

Environmental risk assessment/Biosafety
The virus is ubiquitous in cats, and many cats that recover from the infection remain carriers. Enteric coronavirus infection can be prevented only by minimizing exposure to infected cats and their feces. Feline coronaviruses are fairly stable in the environment and, once dry, can survive for 4-6 wk. Cats with the enteric disease do not progress to develop clinical signs of FIP. Most cats develop an effective immune response on exposure and recover from infection. However, once clinical signs of disease develop in cats with FIP, the disease is invariably fatal. Cats living in multiple-cat households are at greater risk of contracting feline coronavirus and

developing FIP because of sharing multiple strains of the virus and stress-associated immunosuppression. In the past, up to 50% of cats with FIP were co-infected with FeLV; FeLV potently suppresses cell-mediated immunity, which is required for resistance to FIP. Currently, the co-infection rate is only 5%, due to FeLV testing and vaccination.

An intranasal, modified live virus vaccine to help prevent FIP is available. It protects 60-90% of the cats vaccinated as determined by experimental challenge several weeks after vaccination. The duration of significant protection is unknown but is thought to be limited. Because FIP in the general cat population is relatively rare, vaccination of individual pet cats that live mostly or entirely indoors appears to be unwarranted.

Feline coronavirus is rated '**Medium**' in the relative environmental risk score. This is based on its association with severe pathology in cats and its capacity to be transmitted via aerosols. Feline coronaviruses for therapeutic applications will require carrying modifications that expand the virus's host-range. The biological consequences of such modifications on their biology (pathology, tissue tropism, shedding) in humans are unpredictable.

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http://www.newmanveterinary.com/
http://www.cat-health-guide.org/feline-corona-virus.html

Family *Picornaviridae*

Members of family *Picornaviridae* are non-enveloped viruses with an icosahedral capsid containing a single stranded, positive sense RNA genome of between 7.2 and 9.0 kb (kilobases) in length. Their RNA genome is unusual because it has a protein on the 5' end that is used as a primer for transcription by RNA polymerase. Both RNA dependent RNA polymerase and polyprotein synthesis were discovered by studying virus infected cells. The whole replication cycle occurs within the host cell cytoplasm. Like most positive sense RNA genomes, the genetic material alone is infectious. Although substantially less virulent than if contained within the viral particle, the RNA can have increased infectivity when transfected into cells. The name is derived from pico meaning small, and RNA referring to the ribonucleic acid genome, so "pico-rna-virus" literally means small RNA virus.

Picornaviruses are among the most diverse (more than 200 serotypes) viruses and they are separated into 12 distinct genera. Members of this family of viruses include many important pathogens of humans and animals. The diseases they cause are various, ranging from acute "common-cold"-like illnesses, to poliomyelitis, to chronic infections in livestock.

Foot-and-mouth disease virus (FMDV) is the prototypic member of the Aphthovirus genus and one of the first viruses to be recognized (Loeffler and Frosch in 1898). It is a causative agent of Foot-and-mouth disease (FMD). This is a highly contagious and sometimes fatal viral disease of cloven-hoofed animals, including domestic animals such as cattle, water buffalo, sheep, goats and pigs, as well as antelope, bison and other wild bovids, and deer.

Humans can be infected with foot-and-mouth disease through contact with infected animals, but this is extremely rare. Some cases were caused by laboratory accidents. Symptoms of FMD in humans include malaise, fever, vomiting, red ulcerative lesions (surface-eroding damaged spots) of the oral tissues, and sometimes vesicular lesions (small blisters) of the skin. Because FMDV rarely infects humans but spreads rapidly among animals, it is a much greater threat to the agriculture industry than to human health.

There is another viral disease with similar symptoms, commonly referred to as "hand, foot and mouth disease", that occurs more frequently in humans, especially in young children; the cause, Coxsackie A virus, is different from FMDV. Coxsackie A also belongs to the Picornaviridae family, the genus Enterovirus.

Poliovirus (PV, also Wild Polio Virus WPV) is a member of the genus Enterovirus, species Human enterovirus C. This group of RNA viruses prefers to inhabit the gastrointestinal tract. Poliovirus infects and causes disease in humans alone

(poliomyelitis). Poliomyelitis as a viral disease was first recognized by Landsteiner and Popper, 1909 although the virus was not isolated until the 1930's. Three serotypes of poliovirus have been identified: poliovirus type 1 (PV1), type 2 (PV2), and type 3 (PV3), each with a slightly different capsid protein. All three are extremely virulent and produce the same disease symptoms. PV1 is the most commonly encountered form, and the one most closely associated with paralysis. Individuals who are exposed to the virus, either through infection or by immunization with polio vaccine, develop immunity. Infection or vaccination with one serotype of poliovirus does not provide immunity against the other serotypes, and full immunity requires exposure to each serotype. Poliovirus is one of the most, well-characterized viruses, and has become a useful model system for understanding the biology of RNA viruses.

Several members of Picornaviridae family of viruses are being developed into tools for virotherapy. During late 50's, oncolytic potential of Coxsackie B viruses was already recognized (Suskind at al. 1957). At present, oncolytic vectors based on coxsackievirus A21 are available (Au et al. 2005; Shafren et al. 2004; Parato et al. 2005) and phase I clinical trial has commenced at two locations in Australia: http://clinicaltrials.gov/ct2/show/NCT00636558; trial under title: Coxsackie Virus A21 Administered Intravenously (IV) for Solid Tumor Cancers; sponsored by Viralytics Ltd.). Another member of genus Enterovirus, a Poliovirus, infects a wide variety of human cancer cells and primary explants (Ansardi et al. 2001). Furthermore, a recombinant (attenuated) poliovirus named PV1(PVS)-RIPO displays significant tumor tropism and oncolytic potential (Dobrikova et al. 2008). This vector is currently in investigational new drug-directed toxicology studies in preparation for Phase I clinical trials against recurrent glioblastoma multiforme. In addition, a live attenuated strain PV-1 has been shown to hold great potential as an oncolytic agent (Toyoda et al. 2004).

Another oncolytic picornavirus tested in the 1970's (Casto and Hammon, 1970; Taylor et al. 1971), bovine enterovirus, has gained renewed attention as a potential viral anti-cancer agent (Smyth et al. 2002). Although just recently discovered in 2002, Seneca Valley virus (SVV)-001 (newly assigned genus Senecavirus) has entered phase I clinical studies as live attenuated virus to treat carcinoma of neuroendocrine origin (NeoTropiX, Inc. USA).

Genus Enterovirus
Bovine enterovirus

Pathogenesis/Disease association /Epidemiology /Host range /Tissue tropism
Bovine enteroviruses (BEVs) are picornaviruses of the genus Enterovirus. The virus (BEV- 0) was originally isolated by Moll & Davis (1959). BEVs were firstly classified into seven serotypes but are now accepted as falling into two serotypes, 1 and 2,

which are further classified into subtypes. BEV strain VG-5-27 of serotype 1, subtype 1 is the most extensively studied. BEV is endemic in cattle populations worldwide (Andersen, 1978; McCarthy et al. 1999; Zhang et al. 1990). Infections are typically asymptomatic, with healthy animals as carriers. Difficulties in reproducing clinical symptoms, following experimental infection of animals, led to the conclusion that bovine enteroviruses were of only minor veterinary medical importance. However, infections can also been associated with diarrhea and abortions. Enteroviruses are shed in large numbers in the feces and transmitted via the fecal-oral route (Taylor et al. 1974). As bovine enteroviruses (BEV) are excreted in the feces of infected animals in large numbers, it seemed reasonable to hypothesize that BEV could be found in the environment, due to the presence of feces originating from cattle farms.

The tissue tropism of BEV is extremely broad, including human, sheep, horse, dog, camel and other mammalian hosts. BEV-like sequences have even been reported in shellfish from water contaminated with bovine feces. The host cell surface receptor for BEV has yet to be identified but is sensitive to neuraminidase (Stoner et al. 1973). BEV is routinely cultured in baby hamster kidney (BHK-21) cells, yet is commonly known to be readily adaptable to grow in the HeLa, human cervical carcinoma cell line, to equivalent titer.

Current status and stage of the research activities

The stage of the research activities is currently at the level of in vitro studies, although a few in vivo (experimental animals) experiments have been performed in the early 1970's (Taylor et al. 1971; Sedmak et al. 1972; Hodes et al. 1973). It was observed that BEV was successful in infecting and destroying a number of tumors in mice. The virus produced a cytopathic effect in an astrocytoma and several glioblastomas derived from humans but did not affect either a normal brain or a mengioma cell line.

In 2002, the publication by Smyth et al. (2002) describes the comparison of freshly isolated human monocytes, monocyte-derived macrophages, and monocytes-like U937 tumor cell line susceptibility to BEV infection. The experimental data demonstrates the ability of BEV to induce cytolysis of human U937 cells whereas the virus-induced cytopathic effect was marginal in the human monocytes and human monocyte-derived macrophages.

Available assessment information in humans or humanized systems

Currently there are no reports on use of bovine enterovirus as oncolytic agent in humans. Regarding reports describing in vivo experiments, it has been shown that the treatment of ascites and solid tumors in mice (Sarcoma-1 and Ehrlich ascites carcinoma) with bovine enterovirus resulted in regression of the tumors without any pathological effect on the animals (Taylor et al. 1971). Approximately 10^8 PFU of BEV were inoculated into the tumor. Ehrlich ascites tumor and Sarcoma-1 grown in the

peritoneal cavity of adult mice regressed rapidly after treatment with BEV. No tumor cells were detectable by microscopic examination in such mice 48 hr after treatment. In control animals (no tumors, treated with BEV-1) no adverse effect on the animals was noted; such animals appear to be healthy, with sleek fur and normal weight gain. "Cured" mice have been retained as long as 1 year without recurrence of the tumor. Furthermore, treatment of normal mice, rabbits, and dogs with BEV by intracranial injection led to no clinical abnormalities, nor could virus be recovered from the brain (Hodes et al. 1973). The specificity of killing extends to cells in culture, since viral-transformed cells and oncogenic cells are susceptible to the virus, in contrast to cells of untransformed lines and cells of primary cultures, which are resistant. BEV infection of mouse cells derived from Sarcoma 180, Ehrlich ascites, A755, chemically-induced epithelial carcinoma, and L-cell lines causes highly significant decreases (18 to 98%) in viability, whereas cells derived from spleen, lung, thymus, liver, and kidney of normal mice showed no significant change after exposure to virus.

Indeed, when primary mouse embryo cell cultures or primary mouse kidney cell cultures were infected with BEV, no cytopathic effect was noted. When virus adsorption was measured on these cells, after 1 hr at 37°C, less than 1% of the added virus was on the cells. On the other hand, adsorption to Ehrlich ascites tumor cells was very efficient. Furthermore, the "normal" cells of the continuous lines Vero, BSC-1 and LLC-RK, are also susceptible to the virus.

Bio-selection and genetic modifications

The utilization of a virus with specificity toward cancer cells allows to control and to treat various physiological responses of only these cells. It should be possible to screen non-virulent viruses for their tissue (or tumor) specificity and to use these viruses against early stages of tumor development. In principle, it should be possible and practical to select or adapt viruses to different tumors for therapeutic usage. Of course, an obvious concern in such a treatment would be the occasional virus mutant that is more virulent to the host than is the original strain. This concern is more pronounced for RNA viruses, due to the inherent high error rates of RNA-dependent RNA polymerases and resulting genetic variability (Domingo and Holland, 1992). Furthermore, genetic variability of RNA viruses has been implicated in efficient viral propagation and invasion in vivo (Vignuzzi et al. 2006) and, hence, may contribute to anticancer activity of oncolytic viruses.

Horizontal transmission and establishment in the human population

Enteroviruses are the most common viruses infecting a wide range of mammals. Eighty-nine serotypes have been identified: 62 associated with human infections and 27 associated with animal infections. It is generally accepted that only a small proportion of enteroviruses are known, and every year, several new isolates are identified and the classification is revised. Most virus identification and typing of

new enterovirus isolates are for those infecting humans. Like other RNA viruses, their genetic variability is very high, and it is likely that new virus derivatives are frequently generated from the current population (Domingo et al. 2001). BEV is endemic in cattle population worldwide, but it is unknown in humans. Given the fact that, a large numbers of progeny virus are produced that are shed in the feces of cattle, it seems likely that human population (especially with agricultural background) has already been well exposed to this virus.

Environmental risk assessment/ Biosafety

Bovine enterovirus infections are typically asymptomatic, with healthy animals as carriers. However, the process of selection or adaptation of viruses to different tumors for therapeutic usage in humans is of concern since such treatment might result with the occasional virus mutant that is more virulent to the host than is the original strain. In nature, enteroviruses have many advantages for transmission compared with other viruses. They are easily introduced in the environment due to a large numbers of progeny produced that are shed in the feces (Ley et al. 2002). Importantly, the virus particles are very stable under a wide range of environmental conditions, such as pH, temperature, and salinity. Thus, enteroviruses can remain infective for long periods in soils, biological specimens, and aquatic environments, including marine environments.

Bovine enterovirus is rated '**Medium**' in the relative environmental risk score. While not associated with significant pathology in cattle, the virus family is notorious for its capacity to adapt. It is conceivable that clinical application leads to amplification of new quasi species. Also the virus can be shed in relatively large amounts and is physically very stable. This increases the likelihood shedding and transmission.

Genus Senecavirus
Seneca Valley Virus

Pathogenesis/Disease association /Epidemiology /Host range /Tissue tropism

Seneca Valley virus or SVV-001 is the first member of a new genus called Senecavirus in the family of Picornaviridae that is proposed to include other porcine picornaviruses that share similarity in sequence and biochemical properties with SVV-001 (Hales et al. 2008; Reddy et al. 2007; Venkataraman et al. 2008). SVV-001 was isolated at Genetic Therapy Inc. (Gaithersburg, MD) in 2002 from cell culture media as a contaminant during cultivation of PER.C6 cells (transformed fetal retinoblast cells; Fallaux et al., 1998; Reddy et al., 2007). It is presumed to be introduced via bovine serum or porcine trypsin source. SVV-001 shows closest sequence similarity to cardioviruses among other known picornaviruses and has an infectious RNA genome (Genbank accession no. DQ641257; Knowles and Hallenbeck, EUROPIC 2005, Abstract A14) of 7310 bases that codes for four capsid proteins and seven other

non-structural proteins. There are long and short UTRs at the 5' and 3' ends of the genome, respectively (Hales et al., 2008).

Between 1988 and 2005, 12 picorna-like viruses were isolated from pigs showing a variety of clinical symptoms in various locations across the United States. Virus neutralization tests using a specific antiserum raised against one of the isolates showed them to be antigenically related. Six of these isolates were subjected to a pan-picornavirus RT-PCR which employed primer sets targeted to the 3′ end of the genome. Sequence analysis of the resulting amplicons revealed all the virus isolates to be closely related to each other and to a newly described picornavirus, Seneca Valley virus (SVV).

In study by Knowles and colleagues (abstract), serum samples obtained from farm animals including pigs, cows and wild mice were tested individually for the presence of neutralizing antibodies to SVV-001 in a neutralization assay. The results showed that the highest percentage of serum samples that contained neutralizing antibodies came from pigs and cows. This indicated that pigs and other animals are exposed to SVV at levels and in a manner sufficient to induce a measurable immunological response. However, attempts to infect pigs with two of the isolates failed to demonstrate any specific disease.

Additionally, 52 serum samples from four different species of primates were obtained and tested in neutralization assays. None of the samples contained neutralization antibodies to SVV-001. This information, coupled with the isolation of members of SVV in pigs, supports hypothesis that pigs and possibly other farm animals are natural hosts for SVV.

Furthermore, the exposure of the human population to SVV-001 has been tested. For that purpose, serum samples were obtained from general population (60 volunteers), as well as from farmers. Only one sample contained neutralizing antibodies to SVV-001 and had a neutralization titer of 1:8. This data indicates that human exposure to SVV-001 and SVV-001-like viruses is not prevalent in the human population.

SVV-001 does not infect humans but propagates in tumor cells showing neuroendocrine features. The cytolytic potential and selectivity of SVV-001 was determined in neuroendocrine and pediatric tumor cell lines and normal cells. SVV-001 was more cytotoxic to small-cell lung cancer cell lines and solid pediatric cancer cell lines than other tumor cell lines and normal cell lines. Suitability of the virus for intravenous delivery in humans was assessed by blood inactivation assays. The virus activity was not inhibited by components in human blood (Reddy PS et al. JNCI 99, 2007).

This property is being exploited for developing SVV-001 as an oncolytic agent against tumors such as small cell lung cancer (Hallenbeck et al. 2005; Reddy et al., 2007). The virus is presently being studied extensively and characterized by Neotropix Inc. under the trade name NTX-010.

Current status and stage of the research activities
Information obtained from http://www.neotropix.com/index.htm regarding clinical trials: A recently completed Phase I trial evaluated safety of NTX-010 (Seneca Valley virus) in a dose escalation study at log intervals from $1x10^7$ to $1x10^{11}$ viral particles per kg administered intravenously to patients with advanced cancers expressing at least one neuroendocrine marker (synaptophysin, chromogranin A, CD56) suggesting the possibility of susceptibility to the virus. Thirty patients with advanced cancers were enrolled. No dose limiting toxicity was encountered. Currently, there are three clinical studies evaluating the safety and activity of NTX-010.

Information obtained from http://clinicaltrials.gov
Current clinical trials:

Study: "Safety study of Seneca Valley virus in patients with solid tumors with neuroendocrine features"
Status: **Active, not recruiting**/ Last update date: February 23, 2010
Conditions for treatment: Carcinoid; Neuroendocrine
Interventions: Drug: Seneca Valley Virus (biological agent)
Sponsors: Neotropix, Inc.
Phase: **Phase I**
Funded by: Neotropix, Inc.(Industry)

Study: "Seneca Valley Virus-001 after chemotherapy in treating patients with extensive-stage small cell lung cancer"
Status: **Recruiting**/ Last Update Date: May 27, 2010
Conditions for treatment: Lung Cancer
Interventions: Drug: Seneca Valley virus-001(biological agent); Other: placebo
Sponsors: North Central Cancer Treatment Group; National Cancer Institute (NCI)
Phase: **Phase II**
Funded by: NIH

Study: "Seneca Valley Virus-001 in treating young patients with relapsed or refractory neuroblastoma, rhabdomyosarcoma, or rare tumors with neuroendocrine features";
Status: **Suspended**/ Last Updated Date: **May 19, 2010**
Status: **Recruiting**/ Last Update Date: **November 22, 2010**
Conditions for treatment: Adrenocortical Carcinoma; Gastrointestinal Carcinoid Tumor; Kidney Cancer; Neuroblastoma; Retinoblastoma; Sarcoma

Interventions: Drug: Seneca Valley virus-001 (biological agent); Other: laboratory biomarker analysis; Other: pharmacological study
Sponsors: Children's Oncology Group; National Cancer Institute (NCI)
Phase: **Phase I**
Funded By: NIH

Available assessment information in humans or humanized systems
Information obtained from http://www.neotropix.com/index.htm The Therapeutic Index helps to predict whether an efficacious dose can be safely administered in humans and is defined as the maximum amount of drug delivered without toxicity vs. the effective dose. Efficacy is observed in several mouse models at $1x10^7$ viral particles/kg, while no toxicity was observed even at $1x10^{14}$ viral particles/kg (Wadhwa et al. 2007). This translates to a therapeutic index of over 1 million. This is significantly higher (by orders of magnitude) then most if not all cancer therapeutics used to treat metastatic disease, and is a key feature of NTX-010.

The data and conclusions discussed above have been reproduced and extended in part by investigators at the National Cancer Institute, Johns Hopkins University, St. Jude Children's Research Hospital, and Baylor College of Medicine (all in the USA). As an example, a collaborator at Baylor College of Medicine has demonstrated that after establishment of an orthotopic metastatic retinoblastoma model in the eye (Hales et al. 2006; these metastases normally spread to neighboring brain cells), systemic administration of NTX-010 killed metastasized cancer cells. Collaborators at St. Jude Children's Research Hospital have also demonstrated significant efficacy following systemic administration in 3 of 3 pre-established pediatric cancer models that are believed to be highly predictive of a response in the clinic.

45th annual meeting of the American Society of Clinical Oncology
May 29-June 2 2009. (General Poster Session; abstract number: 4629) No publication available.
Phase I study of intravenous Seneca Valley virus (NTX-010), a replication competent oncolytic virus, in patients with neuroendocrine (NE) cancers.
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Background: NTX-010 is a naturally occurring replication competent picornavirus with potent and selective tropism for human NE tumors, including small cell cancers and carcinoid. NTX-010 elicits rapid cytolysis in vitro and durable responses following IV dosing in multiple xenografts models.
Methods: A first-in-human phase I study of IV NTX-010 was conducted across 5 log-increment dose cohorts from 10^7 vp /kg to 10^{11}vp/kg, in patients with NE cancers. Study endpoints included toxicity assessment, response assessment, evaluation of viral titers and

clearance in blood, sputum, nasal swabs, urine, and stool, and assessment of neutralizing antibody (Ab) development.
Results: 30 patients were treated (6 small cell, 24 carcinoid-type). All small cell patients were heavily pretreated (third line) and received 10^7 vp/kg. In these patients, median PFS was 1.2 months and median OS was 4.1 months, including 1 long term (16 months) survivor with prolonged SD after progressing through prior therapies. Carcinoid patients in cohorts 1–4 have 70% SD rate and median PFS of 5.4 months (95% CI 3.6 to NE); median OS has not been reached. Cohort 5, a 12 patient expansion cohort at 10^{11} vp/kg restricted to carcinoid, is still being monitored and shows promising antitumor activity including improvement in carcinoid syndrome symptoms, decline in 5HIAA and other serum markers, minor responses by CT scan, and an objective PET response (50% decrease in SUV). There were no DLTs in any cohort. Evidence of intratumoral viral replication includes delayed kinetics in serum viral titer, post-infusion serum titers greater then the dose administered and positive immunohistochemistry and/or RT-PCR signal for viral antigens in tumor mass despite Ab production. Viral clearance was documented in all subjects and correlated temporally with development of antiviral Ab.
Conclusions: NTX-010 is the first picornavirus to be evaluated as an anticancer therapeutic. A single IV dose of 10^{11} vp/kg of NTX-010 is safe, has predictable viral kinetics, and shows promising activity against NE tumors. Phase II testing of this novel agent either as a single agent or in combination with standard cytotoxic therapies is warranted.

Safety was evaluated in vivo using an immune-competent mouse model, and efficacy was evaluated in vivo in athymic mice bearing tumors derived from human small-cell lung cancer and retinoblastoma cell lines (Reddy et al. 2007).

Single intravenous treatment with SVV-001 in mouse models led to complete responses of all mice carrying small-cell lung cancer xenograft tumors and a majority of mice carrying retinoblastoma xenograft tumors. A single intravenous dose of 1×10^8 vp per kg into athymic mice bearing pre-established small-cell lung or retinoblastoma tumors resulted in complete, durable responses in ten of ten and five of eight mice, respectively. The virus was very effective, with 100% of large pre-established H446 neuroendocrine tumors being eradicated. Such high efficacy following a single intravenous administration may be due to the efficient entry of the virus into the tumor mass and rapid replication cycle of picornaviruses. No deaths or toxic effects were noted in mice that received up to 1×10^{14} vp per kg, that is, a therapeutic index of greater than 1,000,000. Thus, SVV-001 was well tolerated in mice, and no dose-limiting toxicity was observed in immune-competent mice.

Recent findings by Yu and colleagues (2010) support the use of Seneca Valley virus (SVV-00) for treatment of recurrent brain tumors (e.g. medulloblastoma, MB). SVV-001 exhibits efficient replication and killing tumor cells expressing cancer stem cells expressing surface marker CD133. In addition, the virus eliminated tumor cells capable of forming neurospheres in vitro. We confirmed that SVV-001 could pass through BBB in vivo. A single i.v. injection of SVV-001 in 2 anaplastic MB mouse models led to widespread infection of the preformed intracerebellar (ICb) xenografts,

resulting in significant increase in survival (2.2-5.9-fold) in both models and complete elimination of ICb xenografts in 8 of the 10 long-term survivors.

Bio-selection and genetic modifications

Information obtained from http://www.neotropix.com/index.htm. NTX-010 (SVV-001) is naturally occurring and does not require re-engineering or genetic modification to demonstrate the desired therapeutic effect. As a result, NTX-010 (SVV-001) can be produced to extremely high levels in multiple production systems, has a rapid life cycle, and is easily manipulated. The FDA has extensive experience with picornaviruses, the class of virus to which NTX-010 belongs. The Picornavirus class is used in polio vaccines that have been in production for nearly 50 years. These properties confer significant advantages in manufacturing.

To evaluate the ability of SVV-001 to adapt to replicate in nonpermissive cells, Reddy et al. (Reddy et al. 2007) performed experiments where the virus was passaged intentionally three times in nonpermissive cell lines A549, H460, and Hep3B. The authors declare that no virus was produced, suggesting that SVV-001 did not change its tropism. In addition, no antibody escape mutants of SVV-001 were produced in PER.C6 cells when SVV-001 was grown with media containing anti-SVV mouse hyper immune serum (Reddy et al. 2007). These data suggest that the genome of SVV-001 is stable. However, it should be noted that like other RNA viruses, their genetic variability is very high, and it is likely that new virus derivatives are frequently generated from the current population (Hellen and de Breyne, 2007).

Horizontal transmission and establishment in the human population

Information obtained from http://www.neotropix.com/index.htm. To elucidate whether NTX-010 could be transmitted by the host following administration, Neotropix performed a study whereby mice were injected with NTX-010 and then co-mingled with naïve mice. After 30 days no naïve mice were found to have neutralizing antibodies, demonstrating a lack of viral transmission. In contrast, all control injected mice developed neutralizing antibodies. In other tests, the Company did not observe any effects on fetal development, birth or early development when injected mice were commingled with naïve pregnant mice.

Furthermore, the exposure of the human population to SVV-001 has been tested and the data indicated that human exposure to SVV-001 and SVV-001-like viruses is not prevalent in the human population.

Interestingly, the virus sequences from isolates described by Knowles (poster) appeared to fall on an evolutionary time-line. Regression analysis confirmed this and suggested that all the viruses had a recent common ancestor, possibly sometime in the early 1980's. Since the viruses were isolated from samples collected in diverse geographic areas of the United States, the results are suggestive of a recent

introduction of this virus into the US pig population (Pasma et al. 2008). Sequence analysis of further isolates may help to confirm this hypothesis. Based on these observations, the introduction of SVV-001 in human population through medical treatments has to be carefully evaluated. According to the information obtained from Neotropix, Inc. official web page, to date there has been no evidence of transmission of the virus among regularly tested employees or among a limited number of caregivers tested for the NTX-010 (SVV-001) antibody.

Environmental risk assessment/ Biosafety

It is clear that SVV is found in pigs (but also in cattle) and infection may be common in the United States, however, attempts to infect pigs with two of the isolates failed to demonstrate any specific disease. Importantly, phylogenetic studies suggest that the virus may have only recently been introduced into pig population. It is possible that SVV exists in pigs elsewhere in the world, or perhaps the virus has crossed from another species. It is tempting to speculate a rodent origin, since SVV's closest relatives, the cardioviruses, are viruses of rodents. Since SVV-001 is not associated with any specific disease it is difficult to foresee possible impact on the environment.

The relative environmental risk of Seneca Valley virus. In 2002 SVV was isolated as a contaminant in cell culture medium of cultured cells. While there is some evidence that it was derived from infected pigs, the information of its natural host and disease associations is limited. The virus was found to be strongly cytolytic in a range of human tumor cells, while barely affecting non-transformed cells. Based on these findings the virus was developed as clinically applicable oncolytic agent in record time, and so far 3 clinical trials have been initiated, including one in children (clinical trial ID: NCT01048892). Nevertheless, the limited information on the susceptible of other mammalian species, the uncertainties on its natural host, and the absence of shedding data in published literature led us to classify the Seneca Valley virus '**High**' in the relative environmental risk score.

Genus Cardiovirus

Encephalomyocarditis virus

Pathogenesis/Disease association /Epidemiology /Host range /Tissue tropism

Encephalomyocarditis virus (EMCV; family *Picornaviridae*, genus Cardiovirus) is a group of closely related virus strains belonging to one serotype with a wide host range. These picornaviruses infect many animal species including pigs, rodents, cattle, elephants, raccoons, marsupials, and primates such as baboons, monkeys, chimpanzees, and humans. However, only a small number of animal species appear to be adversely affected, including swine, non-human primates and mice. Importantly, a causal relationship between EMCV infection of humans and illness has never been established (Brewer et al, 2001; Moran et al, 2005). Mice can be infected by the oral route, but do not shed large quantities of virus. Infection with

EMCV is associated with sporadic cases and outbreaks of myocarditis and encephalitis in domestic pigs, in numerous species of nonhuman primates, and in other mammalian species. The disease is often fatal—frequently, sudden death is the first indication of infection—and most outbreaks have been associated with captive animals, such as those found in piggeries, primate research centers, and zoos. Virus isolation and serologic studies indicate EMCV is distributed worldwide, but clinical disease in humans or domestic animals is relatively infrequent. Although disease transmission is poorly understood, rodents appear to be the natural reservoirs. Rodent infestation has been implicated in the genesis of several epizootics; disease transmission apparently results from close contact between rodents or their excreta and individuals of susceptible mammalian species. In several instances, rodent control measures have interrupted disease transmission and halted institutional epizootics (Oberste et al. 2009).

Current status and stage of the research activities

Very recently, ECMV has been evaluated as oncolytic virus for treatment of clear-cell renal cell carcinoma, neuroblastoma and retinoblastoma. In animal murine models there was clear evidence of antitumor efficacy (Roos et al., 2010; Adachi et al., 2006; AdacHi et al., 2009).

Available assessment information in humans or humanized systems

Currently there are no reports on use of EMCV as oncolytic agent in humans. There is no evidence for ECMV induced encephalitis in humans. Human EMCV infection and disease have been documented by virus isolation from several specimen types, including serum, stool samples, cerebral spinal fluid, and throat washings. However, because this disease is so infrequent in humans, positive association with EMCV is difficult to establish. In addition, results of several early studies were questionable because EMCV was isolated by using laboratory mice; researchers could not unequivocally establish that the virus did not originate from the mice used for isolation and passage rather than from human clinical specimens. Serologic studies also indicate that humans have been infected by EMCV or immunologically related viruses. Antibody prevalence varied somewhat from study to study, but seroconversion rates tended to increase in persons of advancing age, consistent with a continuous risk for infection throughout life. (Oberste et al. 2009, and refs therein).

Environmental risk assessment/ Biosafety

ECMV pathogenicity is strain and species dependent. Healthy animals function as carriers. However, the process of selection or adaptation of viruses to different tumors for therapeutic usage in humans is of concern since such treatment might result with the occasional virus mutant that is more virulent to the host than is the original strain. It is unclear if the virus is shed in significant quantities by infected human hosts.

Encephalomyocarditis virus is rated '**Medium**' in the relative environmental risk score. While not directly associated with significant pathology in most host species (including humans), the family is notorious for its capacity to adapt. It is conceivable that clinical application leads to amplification of new quasi species. While the virus may not be shed in large amounts the virus particles are physically very stable. This increases the likelihood shedding and transmission.

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Family *Togaviridae*

The Togaviridae are a family of viruses with linear, single-stranded, positive sense RNA genome that is 10-12 kb in length. The 5'-terminus carries a methylated nucleotide cap and the 3'-terminus has a polyadenylated tail, therefore resembling cellular mRNA. The genome is contained within icosahedral capsid. The virus is enveloped and forms spherical particles (65-70 nm diameter). The envelope consists of two or three major proteins forming heterologous spikes. The receptors for binding are unknown, however the tropism is varied and it is known that the glycoprotein spikes act as attachment proteins. After virus attachment and entry into the cell, gene expression and replication takes place within the cytoplasm.

The Togaviridae family includes the following genera:
Genus Alphavirus: type species: **Sindbis virus**, **Semliki Forest virus**, Eastern equine encephalitis virus, Western equine encephalitis virus, Venezuelan equine encephalitis virus, Ross River virus, O'nyong'nyong virus, Chikungunya virus.
Genus Rubivirus : type species: Rubella virus

Genus Alphavirus
There are 27 alphaviruses, able to infect various vertebrates such as humans, rodents, fish, birds, and larger mammals such as horses as well as invertebrates. Transmission between species and individuals occurs mainly via mosquitoes making the alphaviruses a contributor to the collection of Arboviruses or Arthropod Borne Viruses. There are many alphaviruses distributed around the world with the ability to cause human disease. Infectious arthritis, encephalitis, rashes and fever being the most commonly observed. Larger mammals such as humans and horses are usually dead-end hosts or play a minor role in viral transmission, however in the case of Venezuelan equine encephalitis the virus is mainly amplified in horses. In most other cases the virus is maintained in nature in mosquitoes, rodents and birds.

Alphavirus infections are spread by insect vectors such as mosquitoes. Once a human is bitten by the infected mosquito, the virus can gain entry into the bloodstream, causing viremia. Some alphaviruses can also get into the CNS and multiply within the neurons, leading to encephalitis that could have fatal outcome. When an individual is infected, its immune system can play a role in clearing away the virus particles (antibodies and T cells). Once seroconverted, the neutralizing antibodies play an important role in preventing further infection and spread.

Among the many alphavirus members, Semliki Forest virus (SFV), Sindbis virus (SINV) and Venezuelan Equine Encephalitis (VEE) virus have been engineered as efficient delivery and expression vectors. The following types of vectors have been constructed:

1. Replication-deficient vector systems, which usually comprises of two vectors, expression and helper vectors. Co-transfection with a helper vector containing the structural genes will provide the means for generating replication-deficient virus particles.

2. Replication-proficient vector systems, which contains the full-length alphavirus genome and a foreign gene of interest. These vectors provide heterologous gene expression as well as generation of new virus progeny. The advantage of replication-proficient vectors is their prolonged expression profile and possibility to extend the infection to neighboring cells in vivo. On the other hand, these vectors pose a greater safety risk. Replication-competent alphaviruses have been engineered (oncolytic vectors) in attempts to prolong the effect of transgene expression and to enhance distribution in vivo.

A number of clinical trials that used alphaviruses as a treatment agent have been completed or initiated. Most of these trials are phase-I safety trials in healthy volunteers which employ alphavirus replicons for vaccination purposes.
The information can be found at http://clinicaltrials.gov/ct2/results?term=alphavirus (May, 2010).

The cytotoxic effect of alphaviruses must also be considered as a limitation, although it can be turned into an advantage in cancer therapy. The broad host range and especially the strong neurotropism certainly raise questions of concern about safety.

Genus Alphavirus
Sindbis virus

Pathogenesis/Disease association/Epidemiology/ Host range /Tissue tropism
Sindbis Virus (SINV) is a member of the Alphavirus genus. The virus was first isolated in 1952 in Cairo, Egypt. The virus is transmitted by mosquitoes (*Culex spp*.). SINV causes sindbis fever in humans that is considered as self-limiting febrile viral disease but can cause fatal encephalomyelitis in mice. The symptoms include arthralgia, rash and malaise. Rash may precede or follow joint pain for 1-2 days. The exanthema is observed on trunk and progressing to face, legs, palm, soles, lasting on average 10 days. In addition, signs of jaundice and myocardial damage are reported but those are rare.

Sindbis fever is most common in South and East Africa, Egypt, Israel, Philippines and parts of Australia. It is also present in Eastern Europe and Scandinavia. Sindbis virus is an "arbovirus" (arthropod-borne) and is maintained in nature by transmission between vertebrate (bird) hosts and invertebrate (mosquito) vectors. Humans are infected with Sindbis virus when bitten by an infected mosquito. The reservoir of infection is within birds.

Recently SINV has been linked to Pogosta disease in Finland. Pogosta disease is a viral disease, established to be identical with other diseases, Karelian fever and Ockelbo disease. It has long been suspected that the disease is caused by a Sindbis-like virus, a positive-stranded RNA virus belonging to the Alphavirus genus. In 2002 a strain of Sindbis was isolated from patients during an outbreak of the Pogosta disease in Finland, confirming the hypothesis. This disease is mainly found in the Eastern parts of Finland; a typical Pogosta disease patient is a middle-aged person who has been infected through a mosquito bite while picking berries in the autumn. The prevalence of the disease is about 100 diagnosed cases every year, with larger outbreaks occurring in 7-year intervals. The symptoms of the disease include usually rash, as well as mild fever and other flu-like symptoms; in most cases the symptoms last less than 5 days. However, in some cases, the patients develop a painful arthritis. There are no known chemical agents available to treat the disease.

SINV has a wide host range from vertebrates (including humans) to insects. The wide host range suggests that SINV might have promiscuous receptor interactions, which would not be in keeping with the remarkable tumor cell specificity seen in some animal studies. However, there are considerable differences between the susceptibly of various cell lines to infection. For instance, BHK cells, one of the cell lines used in animal tumor model, are particularly susceptible to infection (Wahlfors et al. 2000). The 293 human embryonic kidney cancer cells are also highly susceptible, whereas A549 human lung cancer cells are poorly infected. Two cellular targets seem to be important for infection by Sindbis virus: heparan sulphate proteoglycans and the laminin receptor (Fulop and Larbi 2002). Furthermore, normal human keratinocytes, two human cervical, and three ovarian cancer cell lines were tested for their susceptibility to SINV infection in vitro. SINV infection was able to induce cytopathic effects and apoptosis in two cervical cancer cells (HeLaS3 and C33A) and three ovarian cancer cells (HOC-1, HAC-2, and OMC-3) but not in normal human keratinocytes (Unno et al. 2005). Recently, the cytotoxicity and growth of SINV were tested in 13 oral squamous cell carcinoma (OSCC) cell lines and on normal human oral keratinocytes (NHOKs). High SIN growth was observed in all OSCC cell lines but not on NHOKs (Saito et al. 2009).

Current status and stage of the research activities

The stage of the research activities is currently at the level of in vitro and in vivo animal model studies. Replication-competent Sindbis virus is shown to have the ability to infect tumor cells without infecting normal cells as judged by the histological evidence of remarkable targeting of tumor cells distinct from normal cells. Dramatic tumor responses are also shown after single and repeat dosing in several tumor models, in both immune deficient and immune competent mice. Sindbis has also been shown to be effective after intravenous and intraperitoneal administration. Specifically, SINV is effective in vitro and in vivo for all of cervical and ovarian cancers tested (Unno et al. 2005). Therefore, SINV has a possibility as a

novel agent for the treatment of primary and metastatic cancers in humans, specifically for cervical and ovarian cancer therapy (Unno et al. 2005) and for oral squamous carcinomas (Saito et al. 2009). The data presented so far are exciting and suggest that Sindbis virus has the potential to evolve into an important gene therapy vector for cancer therapy.

Available assessment information in humans or humanized systems

Currently there are no reports on use of Sindbis virus as oncolytic agent in humans. In 2005, Unno et al. evaluated the feasibility of the attenuated replication-competent SINV AR339 strain as an agent for cervical and ovarian cancer therapy. The AR339 strain of SINV has not been reported to induce any serious disease to humans. In nude mice, intra-tumoral ($1x10^6$ pfu) and intra-venous inoculation of SINV resulted in significant regression of established cervical tumors implanted at their backs. Observation of tumor histology revealed that systemic treatment with the single injection of SINV ($1x10^6$ pfu) induces necrosis within tumors at a remote site. In the metastasis model of ovarian cancer, suppression of ascites formation was observed in nude mice with intra peritoneal ($2x10^6$ pfu injected) SINV treatment. By using an *in vivo* green fluorescent protein imaging system, it has been shown that systemic treatment with SIN targeted tumors specifically (Unno et al. 2005). In these experiments, the wellbeing and the body weight of mice was checked and measured every week. In addition, all mice in which tumors had disappeared after intra-tumoral treatment with SINV have been in very good health and living more than 6 months.

Bio-selection and genetic modifications

At present there are no available reports on use of genetically modified replication-competent Sindbid virus for the purpose of developing anti-cancer viral agent. Published results regarding the exploitation of this virus as oncolytic agent are restricted to attenuated SINV AR339 strain (Unno et al. 2005; Saito et al. 2009). To examine if the virus has significant effect on human cancer cell lines as well as for in vivo imagining, the recombinant SINV virus was constructed by inserting a GFP or the firefly luciferase gene for visualization purposes (Unno et al. 2005).

In the context of bio-selection, it should be noted that during infection of mice, the development of large-plaque viral variants (virus mutants) has been frequently observed. These mutants have a reduced ability to bind to heparan sulfate. Sequence analysis of these mutants revealed changes of positively charged amino acids in putative heparin-binding domains of the E2 glycoprotein. Recombinant viruses were constructed with these changes as single amino acid substitutions in a strain Toto 1101 background (Byrnes and Griffin, 2000). All mutants exhibited decreased binding to heparan sulfate and had larger plaques than parental laboratory strain (Toto 1101). When injected subcutaneously into neonatal mice, large-plaque viruses produced higher-titer viremia and often caused higher mortality (Byrnes and Griffin, 2000).

Horizontal transmission and establishment in the human population

In humans, Sindbis infection is common in Africa, Asia, Australia and Eastern Europe. Sindbis is transmitted to humans by mosquito bites, and the reservoir of infection is within birds. Sindbis infection typically is a self-limiting viral disease with so far no evidence of viral persistence in humans. Importantly, no evidence of person-to-person transmission of SINV has been documented.

Environmental risk assessment/ Biosafety

For vector-borne infections in general, the vector's choice of feeding host will ultimately determine which species, and individuals within a species, will be infected. Studies of the prevalence of antibodies to Sindbis virus in birds have shown that the virus infects many species of several taxonomic orders. Sindbis virus has a wide distribution and the virus has been isolated from several mosquito species, frogs, reed warblers, bats, ticks, and humans. For virus to be transmitted from human to animal, infected individual should generate enough viremia for mosquitoes to pick up Sindbis virus during blood-feeding. Sindbis virus infection does not pose a threat to bird population since it is not lethal. However, Sindbis virus infection can cause fatal encephalomyelitis in mice.

Sindbis virus has been assigned to the '**Low**' category in the relative environmental risk table. While Sindbiss can be pathogenic in mice, and some strains have been associated with diseases in humans, apathogenic strains are available. In a clinical application the shedding of Sindbis from the patients is strongly limited by the requirement of an insect vector for transmission. Clinical application may give rise to more virulent viruses, but the limitations to shedding reduce the environmental risks.

Semliki Forest virus

Pathogenesis/Disease association/Epidemiology /Host range/Tissue tropism

The Semliki Forest virus (SFV) was first isolated from mosquitoes in the Semliki Forest, Uganda by the Uganda Virus Research Institute in 1942. It is known to cause disease in both animals and humans. It is found in central, eastern, and southern Africa.

Semliki Forest virus is spread mainly by mosquito bites. It is not able to infect mammals through inhalation or gastrointestinal exposure although rodents in the laboratory can be infected by intranasal instillation. The virus is able to cause lethal encephalitis in rodents. Although SFV is one of the most extensively studied of the alphaviruses and serosurveys indicate that human infection is relatively common, SFV has been linked to human disease on only two occasions, but only one lethal human infection has been reported in 1979. Even in this one case, the patient was immunodeficient (immunodeficiency disorder involving antibody production) and had been exposed to large amounts of virus in the laboratory. In 1987, SFV was

isolated from serum samples of individual in the Central African Republic with fever, persistent headache, myalgias, and arthralgias.

SFV virus can cause encephalitis in horses, mice, rats, hamsters, rabbits and guinea pigs. Severity and type of disease depend on the age of an animal, route of infection and the strain used. Experimental evidence from studies in mice suggests that SFV enters the brain across cerebrovascular endothelial cells. Once within the CNS, the virus replicates primary in neurons and spreads rapidly along neural pathways, producing neuronal cell death. The primary target cells in the brain are neurons, but oligodendrocytes are also infected. Mice that survive infection develop demyelination, accompanied by mild paralysis, 2 to 4 weeks after infection. Antibody-mediated clearance of infectious virus is completed around 7 to 10 days with viral RNA and protein persisting for months.

Regarding in vitro tissue culture conditions, most cancer cell lines studied were susceptible to Semliki Forest virus infection and virally mediated cell death (e.g. human glioma cells U87, U251, and A172; cell lines such as BHK-21, A2058, A549, LS174T, SK-MEL-5, and SW620).

Current status and stage of the research activities

The stage of the research activities is currently at the level of in vitro and in vivo animal model studies. Semliki Forest virus has been used extensively in biological research as a model of the viral life cycle and of viral neuropathy. Due to its broad host range and efficient replication, it has also been developed as a vector for genes encoding vaccines and anti-cancer agents, and as a tool in gene therapy. Importantly, their inherent neurotropism and establishment of persistent infection in the brains of mice may serve as an asset in targeting CNS tumors which are notoriously difficult to reach by surgery. Currently (2010) there are no planned or scheduled clinical trials using replication-competent Semliki Forest virus as oncolytic agent in cancer patients.

Available assessment information in humans or humanized systems

At present, the information regarding the use of Semliki Forest virus as therapeutic agent in humans is limited to replication-incompetent SFV vectors expressing transgenes. A phase-I clinical trial in melanoma and kidney carcinoma patients was conducted with encapsulated SFV particles (replication-incompetent) expressing the p40 and p35 subunits of IL-12 (Lundstrom and Boulikas, 2003). The study showed no liposome- or SFV-related toxicity. The maximum tolerated dose was determined to 3 x 10^9 encapsulated particles per m^2, which was strongly influenced by the high transient expression of recombinant IL-12 resulting in a fever response. Due to the liposome-encapsulation, SFV particles were not recognized by the host immune defense system and allowed therefore repeated systemic administration. The phase I study clearly demonstrated that encapsulated SFV vectors can be safely administered

systemically to cancer patients. However, despite the protocol for a phase I/II trial for the treatment of recurrent glioblastoma multiforme with encapsulated SFV vectors published in 2003 (Ren et al. 2003) the study has not been conducted.

In the field of oncolytic virotherapy the attention has been directed toward replication-competent and conditionally replicating viruses that have retained the capacity to produce new virions and spread from cell to cell, but they are either intrinsically attenuated or have been rendered less pathogenic for safety reasons.

The study by Vähä-Koskela and colleagues (2006), describes the assessment of the replication-competent avirulent Semliki Forest virus (SFV) strain A7 (VA7-EGFP) of its ability to target subcutaneous human melanoma xenografts in severe combined immunodeficient (SCID) mice. The oncolytic potential of this virus was exhibited through marked regression of the xenografts was observed following a single injection of 10^6 plaque-forming units of virus given either intraperitoneally, intravenously, or intratumorally (Vähä -Koskela et al. 2006). Despite initial tumor destruction, however, small isolated groups of dividing tumor cells were detected within strands of connective tissue in association with the tumor capsule and those cells were resistant to the virus. Furthermore, tissue analysis revealed the presence of virus not only in all treated tumors but also in the brains of the treated mice, causing progressing neuropathology beginning at day 16 after infection. Lacking the adaptive immune system, SCID mice are unable to control the infection and develop slow-progressing neurologic symptoms due to persisting virus. Importantly, no symptoms appear in nude mice or immunocompetent mice, in which antibodies are critical in controlling and clearing the infection. Thus, to be eligible for use in virotherapy, the ability of avirulent SFV to spread within tumor tissue may have to be improved and the biological safety of the virus may have to be addressed thoroughly in higher animals.

Given the oncolytic potential and CNS tropism of virus strain VA7 upon peripheral administration, the same group further investigated whether VA7 would be able to inhibit the growth of human glioma xenografts implanted either subcutaneously or orthotopically in nude mice. In the report by Heikkilä and colleagues (2010), it is show that intravenously administered VA7 virus effectively eradicated both subcutaneous and orthotopic U87MG tumor xenografts in nude Balb/c mice. A single intravenous injection of VA7 into mice bearing orthotopic U87MG tumors resulted in long-term survival in total 16 of 17 animals. Intravenously administered VA7-EGFP completely eradicated 100% of small and 50% of large subcutaneous U87MG tumors. The initial results that small subcutaneous tumors were completely eradicated by a single injection of VA7-EGFP were promising, but in case of large tumors, after initial regression some regrowth was detected thus suggesting emerging resistance, as seen in a human melanoma model (Vähä-Koskela et al. 2006).

Besides initial transient viremia none of the mice displayed any neurological symptoms or signs of pathology. Virus was well tolerated and no damage to heart, liver, spleen, or brain was observed upon pathological assessment at three and ninety days post injection, despite detectable virus titers in these organs during the earlier time point (Heikkilä et al. 2010).

Another study describes the assessment of an oncolytic Semliki Forest virus (SFV) vector, VA7(74), carrying the enhanced green fluorescent protein gene (EGFP), as a novel virotherapy candidate against unresectable osteosarcoma (Ketola et al. 2008). In subcutaneous human osteosarcoma xenografts in nude mice treatment with the vector reduced tumor size, whereas tumors in control mice expanded quickly. The VA7-EGFP–treated tumors were either completely abolished or regressed to pinpoint size. The efficacy of VA7-EGFP vector was studied also in an orthotopic osteosarcoma nude mouse model characterized by highly aggressive tumor growth. Treatment with oncolytic SFV extended survival of the animals significantly ($P < 0.01$), yet none of the animals were finally cured. Sera from SFV-treated mice contained neutralizing antibodies, and as nude mice are not able to establish IgG response, the result points out the role of IgM class antibodies in clearance of virus from peripheral tumors (Ketola et al. 2008).

Bio-selection and genetic modifications

Several strains of SFV exist, which differ markedly in virulence. The most frequently used SFV strain to study oncolytic potential in cultured cells and in experimental animals is the avirulent SFV strain A7(74). The A7(74) strain is a naturally attenuated strain (Bradish et al. 1971) which, like the virulent wild type strains L10 and SFV4, is neurotropic, infecting both neurons and glial cells, but unlike the these strains which induce fatal encephalitis in mice irrespective of their age, A7(74) is lethal only for newborn mice. Avirulent SFV has the capacity to replicate in immature, actively growing neural cells while being severely restricted and controlled in adult, mature CNS tissue. In pregnant mice, avirulent SFV can cross the blood-placental barrier and cause early abortion while leaving the mothers unaffected.

For visualization purposes, an expression vector based on SFV A7(74) carrying the gene for enhanced green fluorescent protein (EGFP) has been constructed (Vähä-Koskela et al. 2003). This vector, termed VA7-EGFP, replicates similarly to the parental SFV A7(74) and wild-type SFV in culture, indicating that, despite avirulence in mice, it had not lost its capacity to infect and kill transformed cells.

However, it should be noted that RNA viruses by default display a high mutation rate, thus their tendency to change or revert could result in adaptation to a new host. In addition, the laboratory adapted strains with demonstrated low virulence pose a lower risk of infection and pathogenicity.

Horizontal transmission and establishment in the human population

Semliki Forest virus infection does not associate with any disease in humans but it may cause mild symptoms such as headache, fever and rash (Mathiot et al. 1990). Human infections are common and in certain parts of Africa where up to 40% of the population has been shown to be seropositive, the evidence of human-to-human virus transmission has not been documented. This Old World Alphavirus is common in Angola and Nigeria where up to 37% seroprevalence rate has been found (Kokernot et al. 1965; Adekolu-John and Fagbami 1983).The available data suggests that vast majority of European population does not have pre-existing antibodies to SFV (Lundström, 1999).

Environmental risk assessment/ Biosafety

The natural hosts of Semliki Forest virus are small rodents and birds while mosquitoes are the usual vector. Several different strains of SFV exist, some of which are highly virulent for laboratory mice, while others are virulent only for neonatal mice. As mentioned before, for vector-borne infections in general, the vector's choice of feeding host will ultimately determine which species, and individuals within a species, will be infected.

Semliki Forest virus has been assigned to the '**Low**' category in the relative environmental risk table. While SFV can be pathogenic in mice, hardly any pathology has been associated with this virus in those regions with a high prevalence in the population (e.g. Africa). In a clinical application the shedding of Sindbis from the patients is strongly limited by the requirement for an insect vector in transmission. The insect-mediated transmission reduces the relative environmental risks.

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Family *Retroviridae*

Retroviruses comprise a diverse family of enveloped RNA viruses with genomes sizes of 7000-11000 nucleotides. The virions carry two copies of the single-strand + strand mRNA including the 5'cap and 3' polyA tail inside the enveloped virion. Retroviral genome is converted into a DNA form in the cytoplasm of a host cell by the enzyme reverse transcriptase (virus-encapsulated enzyme). The retroviral DNA then integrates into the chromosomal DNA of the host cell; a process mediated the viral integrase. The integrated virus, called provirus, is transcribed by the cellular transcriptional machinery. The enzyme reverse transcriptase as well as the cellular polymerases transcribing the provirus lack proofreading activity, resulting in a high mutation rate. Genetic variation provides a mechanism for retroviruses to escape host immune responses and develop resistance to antiretroviral drugs (Svarovskaia et al. 2003).

Retrovirus genomes contain at least three open reading frames that encode for proteins that can be found in the mature virus: the group-specific antigen (gag) codes for core and structural proteins of the virus; polymerase (pol) codes for reverse transcriptase, protease and integrase; and, envelope (env) codes for the retroviral coat proteins.

Members of this family include important pathogens such as HIV-1, feline leukemia, and several cancer-causing viruses. However interest in these viruses extends beyond their pathogenicity. For example, their research led to the discovery of proto-oncogenes. Because retrovirus DNA is inserted randomly into the host genome, the insertion can occur near proto-oncogenes, perturbing their regulation. In this way retroviruses can convert normal cells into cancer cells. Many retroviruses that cause cancer in their hosts, notorious examples are Rous sarcoma virus, mouse mammary tumor virus and Moloney murine leukemia virus.

Retroviruses are currently classified into 7 genera (Table 6)

Family	Genus	Type species
Retroviridae	Alpharetrovirus	Avian leukosis virus
	Betaretrovirus	Mouse mammary tumor virus
	Gammaretrovirus	Murine leukemia virus
	Deltaretrovirus	Bovine leukemia virus
	Epsilonretrovirus	Walley dermal sarcoma virus
	Lentivirus	Human immunodeficiency virus 1
	Spumavirus	Chimpanzee foamy virus

Table 6: Retrovirus genera

Lentiviruses affect both non-primates and primates. They are naturally present in Africa but not in Asia, North and South America. In 2006, HIV-1 and HIV-2 infection was estimated by the United Nations Programme on HIV/AIDS to have affected 39.5 million people. The worldwide prevalence among adults was estimated to be 1%. Most affected by the AIDS epidemic is the population living in sub-Saharan Africa. SIV and HIV are transmitted through sexual contact and by contaminated needles and blood. Scientific evidence strongly indicates that the HIV epidemic is the result of the zoonotic transmissions of SIV from chimpanzees. The Chimpanzee strain (SIVcpz) came from endangered *Pan troglodytes* Chimpanzees living in the forests of Cameroon. It is this SIV strain that most likely crossed species to infect humans.

In addition retroviruses are proving to be valuable research tools in molecular biology and have been used successfully in gene therapy. Gammaretroviral and lentiviral vectors for gene therapy have been developed that mediate stable genetic modification of treated cells by chromosomal integration of the transferred vector genomes. This technology is of use, not only for research purposes, but also for clinical gene therapy aiming at the long-term correction of genetic defects, e.g., in stem and progenitor cells. Retroviral vector particles with tropism for various target cells have been designed. Gammaretroviral and lentiviral vectors have so far been used in more than 300 clinical trials, addressing treatment options for various diseases (Kurth and Bannert, 2010).

Furthermore, the members of retrovirus family have been extensively studied and tested for cancer gene therapy applications. Perhaps the most frequently used retrovirus to target cancer is the Moloney murine leukemia virus (MoMuLV). Due to the lack of active nuclear transport of the viral genome, all gammaretroviruses, including MoMuLV, are unable to transduce nondividing cells, which can be considered an important safety aspect. Besides the gammaretroviruses, vectors based on lentiviruses have also been used to target cancer in pre-clinical experiments (Pellinen et al. 2004). Unlike their retroviral counterparts, lentiviruses are not oncogenic. They do have the capacity to transduce both dividing and non-dividing cells, which may be advantageous under certain circumstances. In contrast to both gammaretroviruses and lentiviruses, the spumavirus 'foamy virus' shows inherent oncolytic capacity. In this chapter, the specific focus will be on use of non-human replication-competent retroviruses as anti-cancer agents.

Replication-defective retroviruses have been used extensively in clinical trials in cancer. Data from these studies suggest that the distribution of the vectors in solid tumors needs to be improved to reach clinical efficacy (Ram et al. 1997; Pulkkanen and Ylä-Herttuala, 2005). Only few of the *Retroviridae* have been developed for use as replication-competent virus for therapeutic intervention in humans. Those include murine leukemia viruses (MuLV) and the chimpansee Foamy virus.

Genus Gammaretrovirus
Murine Leukemia virus

Pathogenicity/Disease association/Host range/Tissue tropism
The murine leukemia viruses (MuLVs) belong to the genus Gammaretrovirus. They were originally identified by breeding of high-leukemic incidence mouse strains in the 1920s and 1930s by Furth (Furth et al 1933). Attempts to passage a cell-free leukemogenic agent were ultimately successful, leading to independent MuLV isolates by Gross, Friend, Moloney, Rauscher and others. The Moloney, Rauscher, Abelson and Friend MuLVs strains, named for their discoverers, are used in cancer research. The MuLVs include both exogenous and endogenous viruses. Exogenous forms are transmitted as new infections from one host to another. Endogenous MuLVs are integrated into the host's germ line and by cell division conveyed to the next generation. Stoye and Coffin have classified MuLVs into four categories (Stoye and Coffin, 1987) by host specificity, determined by the genomic sequence of their envelope region.

Different strains of mice may have different numbers of endogenous retroviruses, and new viruses may arise as the result of recombination of endogenous sequences. Different MuLVs induce T lymphoma (Gross and Moloney MuLVs), while Fried and Rauscher MuLVs induce erythroleukemia. Beginning in the 1970s, MuLVs have been among the most intensively studied retroviruses. Much of basic retroviral replication was worked out for MuLVs, and they have also been important subject for the study of retroviral leukemogenesis and carcinogenesis in general. In early experiments, passage of MuLV or MuLV-induced tumor cells occasionally gave a rise to variants with increase pathogenicity *in vivo* (e.g. the appearance of solid tumors instead of leukemias). Follow-up studies indicated that these variants were classical acute transforming retroviruses that carry transduced cellular oncogenes with examples of Moloney murine sarcoma virus (M-MuSV, the *mos* oncogene), Kirsten MSV (K-*ras*), and FBJ-MSV (v-*fos*).

MuLVs are widely distributed in domestic and feral mice. While the original isolations were from laboratory mouse strains, related MuLVs have been important because they have shown new receptor specificities and pathologies. All mouse strains carry genetic information for MuLVs-related viruses (endogenous viruses) in their chromosomes. Endogenous viruses represent rare germline infections which result in mendelian transmission of the integrated proviruses to all progeny. Most endogenous MuLV proviruses are replication-defective, although some inbreed mouse strains (e.g. ARK) carry and spontaneously activate replication-competent endogenous viruses. There are approximately 50-80 endogenous MuLV proviruses in most mouse strains.

The host range of MuLV is controlled by two systems. The first system is the interaction of envelope glycoprotein with the cell surface receptor. On the basis of cell surface receptor specificities, different classes of MuLVs have been identified so far: ecotropic, xenotropic, amphotropic, and polytropic. The ecotropic MuLVs (from eco, "house") are capable of infecting mouse and rat cells in culture. Non-ecotropic MuLVs may be xenotropic (from xeno, "foreign", infecting non-mouse species), amphotropic or modified polytropic (infecting a range of hosts including mice).

MuLVs induce leukemias in mice with latencies ranging from 2 to 18 months, depending on the strain of virus and strain of mouse. Neonatal infection is by far the most efficient mean of leukemogenesis, whereas infection of adults is not leukemogenic for most viruses. Prior to development of leukemia, the predominant sites of infection are hematopoietic tissues (bone marrow, spleen, thymus), although the tissue specificity of the enhancers in the LTR can modulate this. Oncogenesis is a multistep process and LTR activation of proto-oncogenes probably supplies only one step in the process. For example, MoMuLV induces T lymphoma in mice and rats by provirus insertion and activation of proto-oncogene (e.g. c-*myc*, *pim*-1).

Current status and stage of the research activities

Over the past two decades significant advances have been made in gene transfer technology such as vector (gene delivery vehicle) construction, vector producer cell efficiency and scale-up processes, preclinical models for target diseases and regulatory guidance regarding clinical trial design including endpoint definitions and measurements. Thus many aspects of gene transfer technology have matured to the point of clinical and commercial feasibility.

The following information has been obtained from http://clinicaltrials.gov/

Rank	**Status**	**Study** NCT01156584
1	Recruiting	**Study of a Replication Competent Retrovirus Administered to Subjects With Recurrent Glioblastoma (GBM) Condition: Recurrent Glioblastoma** Interventions: Biological: Toca 511; Drug: 5-FC Sponsor: Tocagen Inc. (see below) Phases: Phase I / Phase II Funded By: **Industry** Study Type: Interventional Study Design: Control: Uncontrolled; Endpoint Classification: Safety/Efficacy Study; Intervention Model: Single Group Assignment; Masking: Open Label; Primary Purpose: Treatment Last Verified Date: **July 2010**

The following information has been adapted from http://www.tocagen.com/site/ (Information obtained end September 2010). Tocagen Inc.'s Controlled Active Gene

Transfer Technology (CAGT) platform is a gene transfer technology that uses a non-oncolytic, replicating virus. However, the name of the virus used cannot be obtained from the company's web page. The company states: "Based on experiments in mice, our virus is cleared from healthy cells by the immune system. However, our virus is not cleared from the cancer cells by the immune system allowing delivery of the therapeutic gene to the entire cancer. Tocagen's gene transfer approach is designed to enable the therapeutic gene to selectively destroy cancer cells while not harming healthy tissue. Toca 511 is a live virus that has the ability to carry a therapeutic gene to cancer cells". Furthermore the company provides the following: "The therapeutic gene carried by the Toca 511 virus is called cytosine deaminase (CD). Once in the cancer cell, the CD gene directs the production of an enzyme (protein) that converts the prodrug, 5-FC, to the anticancer drug, 5-FU. This is why Toca 511 is referred to as a Prodrug Activator - the gene Toca 511 delivers converts an inactive, prodrug, 5-FC, to an active drug, 5-FU, that can kill cancer cells." 5-FC, also known as flucytosine, is an approved oral antibiotic to treat serious fungal infections.

Tocagen Inc also provides the information on planned clinical trials: A Phase 1/2 Ascending Dose Trial with Extended Cohort of the Safety and Tolerability of Toca 511 with 5-FC in Patients with Recurrent Glioblastoma Multiforme. The first human study of Toca 511 with 5-FC will be conducted in subjects who have recurrent Glioblastoma Multiforme (GBM). The study will be conducted at three neuro-oncology centers in the United States. To enroll, subjects must be between 18 and 75 years of age whose tumor has recurred despite surgery, radiation therapy and treatment with temozolomide. The study will be conducted in two parts. Part one will study increasing single doses of Toca 511 delivered by intratumoral injection. Approximately 3 weeks later subjects will begin a 6-day course of oral flucytosine (5-FC). If tolerated, these 6-day courses of 5-FC will be repeated for 6 months. The focus of this study is to identify the highest safe and tolerated dose of Toca 511. MRI scan will performed every two months for 6 months to evaluate tumor size. Approximately 15 subjects will be enrolled in part one of the study.

In part two of the study, the highest safe and tolerated dose of Toca 511 identified in part one of the study, will be administered to nine more subjects. As in part one, Toca 511 will be delivered once by intratumoral injection and 3 weeks later subjects will begin oral uptake of 5-FC, following the same procedure as in part one. Part two of the study will focus on safety as well as tumor response.

Available assessment information in humans or humanized systems
The information regarding human studies is depicted in the above text.

Pre-clinical studies: RCR vectors assessment in in-vivo experiments

MuLV-based replication competent retroviral (RCR) vectors show significant advantages over standard replication-defective vectors in efficiency of gene delivery both in culture and *in vivo*. Logg and colleagues (Logg et al. 2001) describe the development of RCR vector (named ZAPd-GFP) that harbors an internal ribosome entry site–transgene (GFP) cassette positioned between the env gene and the 39 long terminal repeat (LTR). The ability of this vector to replicate and transmit a transgene was examined in culture and in a solid tumor model *in vivo*. After injection of 6×10^3 PFU of vector into established mammary cancer xenografts in 12 weeks-old nude mice, highly efficient spread of the transgene was observed over a period of 7 weeks. In some cases, the expression of the transgene (GFP) was detectable throughout the entire tumor. By the 49th day, the average transduction level was approximately 75%, with some tumors showing transduction levels approaching 100%. Tissue culture (explantation) of dissociated tumor tissue and subsequent examination by fluorescence microscopy revealed that host-derived stromal cells within the tumor were also transduced by the vector. This study represents the first example of the use of an RCR vector in an adult mammalian host (12 weeks old mice), and their first application to transduction of solid tumors. In addition, the experiments have been conducted to test any spread of RCR vector outside the tumors. High molecular weight DNA was harvested from spleen, lung, kidney, liver, and heart and was used, along with DNA extracted from tumors, in PCR with primers specific for the GFP transgene. Tumor samples revealed the presence of the full-length GFP transgene, but none of the non-tumor tissues or the mock-treated tumor sample. The data shows that spread of this untargeted vector appeared to be confined to the tumor tissue.

Another study by Tai and colleagues (Tai et al. 2005) shows that transduction by replication-competent retrovirus (RCR) vectors is efficient, tumor-selective, and persistent. This study demonstrates that a single dose of RCR vector expressing suicide gene (the yeast cytosine deaminase) followed by a single cycle of a prodrug (5-FC) administration was able to achieve inhibition of pre-established primary gliomas in mice without apparent damage to adjacent normal brain tissue. This resulted in a doubling of the median survival time compared to controls. Notably, also observed is a complete transduction of tumor foci that developed at multiple ectopic sites extending down to the brain stem; thus infection by the RCR vector was sufficiently persistent to follow the tumor cells even as they migrated away from the primary lesion, and continuing multiple cycles of 5-FC administration was found to achieve further long-term survival benefit. The prolonged therapeutic effectiveness of this multiple-cycle treatment regimen represents a new finding and was possible due to the ability of replicating MuLV to integrate stably into the genome of tumor cells and spread even to metastatic lesions after only a single injection. Such persistence is unique among oncolytic viruses, which generally achieve only transitory benefit. Certainly the U-87 tumor model is different from many human

gliomas. Cell line U-87 and other transplantable glioma models contain a high percentage of dividing cells, whereas in human glioblastoma multiforme only a small fraction of tumor cells are dividing at any one time. Thus, optimization of the dosage and timing of RCR and prodrug injections will certainly be required to sustain log-phase growth of the virus and achieve comparable results in human brain tumors. The authors also reported on RCR-transduced malignant gliomas in an immunocompetent in vivo rodent model (Fischer 344 rats) to further investigate this gene therapy option (Wang et al 2006). In addition, this group has recently published two more articles addressing the efficiency of RCR vectors replicative spread, and their therapeutic effect upon prodrug administration in experimental *in vivo* animal models (Lu et al. 2010; Tai et al. 2010).

Bio-selection and genetic modifications

Numerous retroviral vectors are based on MuLV since retroviral vectors are of great interest for introduction of foreign genes into eukaryotic cells. Those vectors offer several advantages: infection is non-lytic, the transduced DNA is stably integrated into host cell chromosomal DNA and expression from integrated DNA is much more efficient than from transfected DNA (e.g. plasmid DNA).

Modifications to MuLV-based vectors include the use of vesicular stomatitis virus (VSV) enveloped glycoprotein (G protein) to pseudotype the vector particles in place of MuLV *env* protein. This type of modification allows broader host range. The information on other modified vectors is depicted in the context of in vivo (animal) experiments and extensively reviewed (Dalba et al. 2007).

Horizontal transmission and establishment in the human population

The replication competence of RCR vectors described here obviously raises questions about possible pathogenic effects resulting from spread of the vector in the host. Moloney MuLV is known to induce thymic T lymphoma in newborn mice, and many other murine retroviruses are associated with characteristic malignancies. However, most of these viruses are not pathogenic in adult mice (Rosenberg and Jolicoeur, 1997). Nonpathogenic strains of MuLV have also been described, and these may be amenable for use in the construction of replicating vectors similar to those described here. Initial studies of the activity of amphotropic MuLV in rhesus monkeys could find no evidence of pathology in infected animals over a 3-year observation period, despite severe immune suppression at the time of infection and the administration of high doses of replication-competent MuLV (Cornetta et al. 1990). A later study, however, revealed that MuLV can be oncogenic in primates under certain conditions, on the basis of the observation that 3 of 10 rhesus monkey recipients of bone marrow cells infected with replication-competent MuLV developed T cell lymphoma (Donahue et al. 1992; Anderson et al. 1993). These results suggest that while MuLV has oncogenic potential in primates, the presence of a normally functioning immune system is sufficient to prevent the realization of this potential. The tumor

microenvironment itself is known to be inherently immunosuppressive; therefore it is possible that RCR vectors spread would be facilitated within the tumor even in immunocompetent hosts, while extratumoral spread would be restricted by the immune system. Extensive knowledge of retrovirus biology suggests that no acute complications secondary to retroviral-mediated gene transfer are likely, but the possibility of long-term or unforeseen sequelae in patients suggests the need for post-treatment monitoring (Cornetta et al. 1991; Nair, 2008; Staal et al. 2008; Romano et al. 2009).

Environmental risk assessment/Biosafety

MuLVs are widely distributed in domestic and feral mice and do not appear to pose an acute health risk. Furthermore, the *in vivo* fate of amphotropic murine leukemia retrovirus was studied by Cornetta (1990) in five rhesus monkeys. This study shows that retroviruses infused intravenously into 4 animals were cleared rapidly from the circulation and subsequent viremia has not been detected. A fifth monkey was immunosuppressed and transplanted with virus-producing autologous fibroblasts in addition to an intraperitoneal injection of virus. This animal was viremic for 2 days and its lymph node cells and peripheral blood mononuclear cells were shown to be producing virus for up to 22 days post-inoculation, but subsequently has been negative after 17 months of analysis. In the 5 animals studied, clinical illness has not been identified at any time.

The following information has been adapted from http://www.tocagen.com/site/
Evaluating Toca 511 with 5-FC in Brain Cancer Patients

Based on what was learned from animal studies, physicians plan to administer a single injection of Toca 511 into the brain cancer of patients enrolled in clinical trials. This injection will be made through a small hole in the skull, called a burr hole. This procedure is very similar to what patients experience when they have a brain cancer biopsy. For 3 weeks the virus will be allowed to spread through the cancer, carrying the CD gene with it. However, it is not clear if virus-treated patient will remain hospitalized during this period.

*Patients will then take 5-FC by mouth for 6 days, and these 6-day courses of 5-FC will be repeated every month for 6 months. 5-FC (also known as flucytosine) is an approved oral antibiotic to treat serious fungal infections. Since the treatment involves a drug that is taken orally, it means that patients do not need to stay in the hospital to receive this drug.*It appears that virus-treated patients, carrying replicating retrovirus in their brain tumor mass, can freely leave the hospital, reside at home and inevitably come in contact with family and friends.

To allow efficient infection of human cells, the envelope protein of the conventional MuLV, which infects primarily rodent cells, needs to be replaced with an envelope of a virus that has the capacity to infect human cells. This 'envelope-pseudotyping' is essential for clinical gene therapy with MuLV. Often the envelope of an 'amphotropic' retrovirus isolate is used for pseudotyping. Replication-defective

derivatives of MuLV have been used frequently as gene transfer vector in clinical gene therapy. While generally safe and well tolerated, this has led to a number of cases where T-cell leukemia's developed in the recipient as result of insertional mutagenesis and activation of proto-oncogene expression. The use of replication-competent MuLV-derived vectors in cancer gene therapy is a novel approach. The uncertainty surrounding the effects of a replication-competent amphotropic MuLV led us to classify '**High**' in the relative environmental risk score.

Genus Spumavirus
Foamy virus

Pathogenicity/Disease association/Host range/Tissue tropism
Foamy viruses (FV) belong to the genus Spumavirus. They follow a replication pathway that is distinct from orthoretroviruses and have several analogies to the hepadnaviral replication strategy (Rethwilm, 2003). The infection of natural hosts by FV is regarded to be non-pathogenic (Falcone et al. 2003). However, FV DNA was detected in all analyzed organs of infected animals.

The viral receptor appears to be very widespread, which also points to the broad tissue tropism of FV (Lindemann et al. 2000).Unlike lentiviruses but similar to murine leukemia virus, Foamy viruses require dividing cells for integration and replication, (Trobridge and Russell, 2004; Bieniasz et al. 1995) which appears to be a prerequisite of a retrovirus vector to be used in cancer therapy. Thus, any selectivity for tumor cells is probably due to this particular feature of FVs.

Current status and stage of the research activities/Available assessment information in humans or humanized systems/Bio-selection and genetic modifications
Both replication deficient and replication competent vectors have been generated (Schmidt and Rethwilm, 1995; Nestler et al. 1997). In a single published study several suicide gene-expressing vector were generated and evaluated for their ability to suppress tumor growth in a human glioma xenograft mouse model (Heinkelein et al. 2005). In this study, replication-competent vectors based on the prototype strain of this virus were able to control subcutaneous U87 tumors in nude mice for up to 25 weeks. Surprisingly, they found that the administration of the particular prodrug after allowing vector replication was not advantageous in antitumor growth activity compared with vector replication alone. When the virus from which the vectors were derived was analyzed for its oncolytic activity, it was found it also exhibits the same trait.

Horizontal transmission and establishment in the human population /Environmental risk assessment/Biosafety

The results published demonstrate that vector replication is not restricted to cells in the tumor. Even in mice successfully treated with vector and the respective prodrug, the virus was not eliminated from the host. It was found to reside in various mouse tissues including the gonads.

Humans are not natural hosts of FV and a pre-existing immunity does not exist in humans (Heneine et al. 2003) However, humans can be readily and persistently infected by FV of nonhuman primate origin (Heneine et al. 2003). The infection is not transmitted to other humans, even by intimate contact, and no disease has been associated with these rare zoonotic transmissions (Heneine et al. 2003).The prototypic FV (PFV) is isolated from human tissue following probably trans-species infection by a chimpanzee virus (Epstein, 2004; Schmidt et al. 1997).

Although there is so far no evidence that FV become pathogenic and spread among humans, the risk of adaptation and development of a new human retroviral infection cannot be excluded (Switzer et al. 2004; Wolfe et al. 2004).

Foamy virus is classified as '**Low**' in the relative environmental risk score. This assignement is based on the inefficient infection of humans and the absence of pathogenicity in host and non-host species.

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APPENDIX 1

Marking, according to the parameters of Box 1, of the viruses with a 'Negligible' and 'High' relative environmantal risk score.

Canarypox virus

Virological and biological parameters:	Yes	Not known	No
The virus is replication-competent in human cells.	□	□	X
The virus produces infectious progeny virus in human cancer cells.	□	□	X
The virus produces infectious progeny virus in human noncancerous cells.	□	□	X
The virus causes viremia in humans.	□	□	X
The virus replicates unrestrictedly in humans (i.e. replication is not constrained in any way as to provide a level of biological containment).	□	□	X
The therapeutic virus can interfere with other viruses that may be present in the treated patient (for example through recombination, (re)activation, immunosuppression) as to cause adverse effects for health or spread.	□	□	X
The virus can cause persistent (chronic/latent) infection in humans.	□	□	X
The virus can cause disease in humans.	□	□	X
Parameters relating to intended clinical use:			
The virus can be shed from the patient	X	□	□
Virus shed from the patients can be transmitted to and infect susceptible host species	□	□	X
The virus can adapt itself in such a way that the capacity of the virus to spread beyond the treated patient (i.e. in human and/or non human populations) is positively affected?	□	□	X
Parameters relating to potential consequences			
The potential consequences of unintended exposure of non-target humans may be grave (for example, illness, further spread in population)	□	□	X
The potential biological/ecological consequences of unintended exposure of non-human hosts may be grave (for example, illness, further spread in population)	□	X	□
The potential economical consequences of unintended exposure of non-human hosts may be grave	□	□	X
Options for risk management			
Are vaccines available that can be used for prophylactic treatment in humans?	□		X
Are vaccines available that can be used for prophylactic treatment in the normal host species?	X		□
Are effective antivirals available that can be used for treatment of virally-infected humans?	□		X

Autographa californica Baculovirus virus

Virological and biological parameters:	**Yes**	**Not known**	**No**
The virus is replication-competent in human cells.	□	□	X
The virus produces infectious progeny virus in human cancer cells.	□	□	X
The virus produces infectious progeny virus in human noncancerous cells.	□	□	X
The virus causes viremia in humans.	□	□	X
The virus replicates unrestrictedly in humans (i.e. replication is not constrained in any way as to provide a level of biological containment).	□	□	X
The therapeutic virus can interfere with other viruses that may be present in the treated patient (for example through recombination, (re)activation, immunosuppression) as to cause adverse effects for health or spread.	□	□	X
The virus can cause persistent (chronic/latent) infection in humans.	□	□	X
The virus can cause disease in humans.	□	□	X
Parameters relating to intended clinical use:			
The virus can be shed from the patient	X	□	□
Virus shed from the patients can be transmitted to and infect susceptible host species	□	□	X
The virus can adapt itself in such a way that the capacity of the virus to spread beyond the treated patient (i.e. in human and/or non-human populations) is positively affected?	□	□	X
Parameters relating to potential consequences			
The potential consequences of unintended exposure of non-target humans may be grave (for example, illness, further spread in population)	□	□	X
The potential biological/ecological consequences of unintended exposure of non-human hosts may be grave (for example, illness, further spread in population)	□	□	X
The potential economical consequences of unintended exposure of non-human hosts may be grave.	□	□	X
Options for risk management			
Are vaccines available that can be used for prophylactic treatment in humans?	□		X
Are vaccines available that can be used for prophylactic treatment in the normal host species?	□		X
Are effective antivirals available that can be used for treatment of virally-infected humans?	□		X

Murine Leukemia virus (amphotropic)

Virological and biological parameters:	**Yes**	**Not known**	**No**
The virus is replication-competent in human cells.	X	□	□
The virus produces infectious progeny virus in human cancer cells.	X	□	□
The virus produces infectious progeny virus in human noncancerous cells.	X	□	□
The virus causes viremia in humans.	□	X	□
The virus replicates unrestrictedly in humans (i.e. replication is not constrained in any way as to provide a level of biological containment).	X	□	□
The therapeutic virus can interfere with other viruses that may be present in the treated patient (for example through recombination, (re)activation, immunosuppression) as to cause adverse effects for health or spread.	□	X	□
The virus can cause persistent (chronic/latent) infection in humans.	X	□	□
The virus can cause disease in humans.	X	□	□
Parameters relating to intended clinical use:			
The virus can be shed from the patient	□	X	□
Virus shed from the patients can be transmitted to and infect susceptible host species	□	X	□
The virus can adapt itself in such a way that the capacity of the virus to spread beyond the treated patient (i.e. in human and/or non-human populations) is positively affected?	□	X	□
Parameters relating to potential consequences			
The potential consequences of unintended exposure of non-target humans may be grave (for example, illness, further spread in population)	X	□	□
The potential biological/ecological consequences of unintended exposure of non-human hosts may be grave (for example, illness, further spread in population)	□	X	□
The potential economical consequences of unintended exposure of non-human hosts may be grave	□	X	□
Options for risk management			
Are vaccines available that can be used for prophylactic treatment in humans?	□		X
Are vaccines available that can be used for prophylactic treatment in the normal host species?	□		X
Are effective antivirals available that can be used for treatment of virally-infected humans?	X		□

Maraba Virus

Virological and biological parameters:	**Yes**	**Not known**	**No**
The virus is replication-competent in human cells.	X	□	□
The virus produces infectious progeny virus in human cancer cells.	X	□	□
The virus produces infectious progeny virus in human non-cancerous cells.	□	X	□
The virus causes viremia in humans.	□	X	□
The virus replicates unrestrictedly in humans (i.e. replication is not constrained in any way as to provide a level of biological containment).	□	X	□
The therapeutic virus can interfere with other viruses that may be present in the treated patient (for example through recombination, (re)activation, immunosuppression) as to cause adverse effects for health or spread.	□	X	□
The virus can cause persistent (chronic/latent) infection in humans.	□	X	□
The virus can cause disease in humans.	□	X	□
Parameters relating to intended clinical use:			
The virus can be shed from the patient	□	X	□
Virus shed from the patients can be transmitted to and infect susceptible host species	□	X	□
The virus can adapt itself in such a way that the capacity of the virus to spread beyond the treated patient (i.e. in human and/or non-human populations) is positively affected?	□	X	□
Parameters relating to potential consequences			
The potential consequences of unintended exposure of non-target humans may be grave (for example, illness, further spread in population)	□	X	□
The potential biological/ecological consequences of unintended exposure of non-human hosts may be grave (for example, illness, further spread in population)	□	X	□
The potential economical consequences of unintended exposure of non-human hosts may be grave	□	X	□
Options for risk management			
Are vaccines available that can be used for prophylactic treatment in humans?	□		X
Are vaccines available that can be used for prophylactic treatment in the normal host species?	□		X
Are effective antivirals available that can be used for treatment of virally-infected humans?	□		X

Seneca Valley virus

Virological and biological parameters:	**Yes**	**Not known**	**No**
The virus is replication-competent in human cells.	X	□	□
The virus produces infectious progeny virus in human cancer cells.	X	□	□
The virus produces infectious progeny virus in human non-cancerous cells.	□	X	□
The virus causes viremia in humans.	□	□	X
The virus replicates unrestrictedly in humans (i.e. replication is not constrained in any way as to provide a level of biological containment).	□	X	□
The therapeutic virus can interfere with other viruses that may be present in the treated patient (for example through recombination, (re)activation, immunosuppression) as to cause adverse effects for health or spread.	□	X	□
The virus can cause persistent (chronic/latent) infection in humans.	□	X	□
The virus can cause disease in humans.	□	X	□
Parameters relating to intended clinical use:			
The virus can be shed from the patient	□	X	□
Virus shed from the patients can be transmitted to and infect susceptible host species	□	X	□
The virus can adapt itself in such a way that the capacity of the virus to spread beyond the treated patient (i.e. in human and/or non-human populations) is positively affected?	□	X	□
Parameters relating to potential consequences			
The potential consequences of unintended exposure of non-target humans may be grave (for example, illness, further spread in population)	□	X	□
The potential biological/ecological consequences of unintended exposure of non-human hosts may be grave (for example, illness, further spread in population)	□	X	□
The potential economical consequences of unintended exposure of non-human hosts may be grave	X	□	□
Options for risk management			
Are vaccines available that can be used for prophylactic treatment in humans?	□		X
Are vaccines available that can be used for prophylactic treatment in the normal host species?	□		X
Are effective antivirals available that can be used for treatment of virally-infected humans?	□		X