STRATEGIES TO ENHANCE EFFICACY OF ONCOLYTIC VIROTHERAPY

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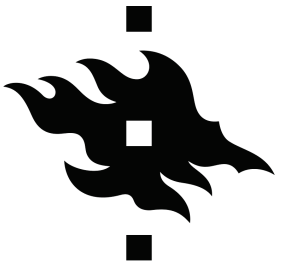

ACADEMIC DISSERTATION

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Helsinki 2016

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“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

– Maria Skłodowska Curie
(7th November 1867 – 4th July 1934)

Table of Contents

PART A

List of original publications

The thesis is based on the following original publications, which are referred to in the text by their roman numbers.

I. **KURYK, L**., HAAVISTO, E., GAROFALO, M., CAPASSO, C., HIRVINEN, M., PESONEN, S., RANKI, T., VASSILEV, L*. & CERULLO, V*. 2016. Synergistic anti-tumor efficacy of immunogenic adenovirus ONCOS-102 (Ad5/3-D24-GM-CSF) and standard of care chemotherapy in preclinical mesothelioma model. *Int J Cancer,* 139**,** 1883-93.

Patent application: **KURYK, L.**, PESONEN, S., RANKI, T., VASSILEV, L., HAAVISTO, E., VUOLANTO, A., *Combining adenovirus and chemotherapeutic agents for treating cancer*.

II. GAROFALO, M*., IOVINE, B*., **KURYK, L**., CAPASSO, C., HIRVINEN, M., VITALE, A., YLIPPERTULA, M., BEVILACQUA, M. A. & CERULLO, V. 2016. Oncolytic adenovirus loaded with L-carnosine as novel strategy to enhance the anti-tumor activity. *Mol Cancer Ther*, 15, 651-60.

III. **KURYK, L**., VASSILEV, L., RANKI, T., KARIOJA-KALLIO, A., LEVALAMPI, O., VUOLANTO, A., CERULLO, V. & PESONEN, S. 2016. Toxicological and bio-distribution profile of a GM-CSF-expressing, double-targeted, chimeric oncolytic adenovirus ONCOS-102 – support for clinical studies on advanced cancer treatment. *PLoS One (under review)*

* - an equal contribution

Personal contribution

I. I helped to design of the studies. I carried out the *in vitro* and *in vivo* studies, acquisition, analysis and interpretation of data, statistical analyses and drafted the manuscript.
II. I helped to design of the studies. I helped to carry out *in vitro* and *in vivo* studies, acquisition, analysis and interpretation of data, statistical analyses and drafted the manuscript with first co-author.
III. I carried out the analysis and interpretation of data and drafted the manuscript.

Additional publications not included in the dissertation

CAPASSO, C., HIRVINEN, M., GAROFALO, M., ROMANIUK, D., **KURYK, L**., SARVELA, T., VITALE, A., ANTOPOLSKY, M., MAGARKAR, A., VIITALA, T., SUUTARI, T., BUNKER, A., YLIPERTTULA, M., URTTI, A. & CERULLO, V. 2015. Oncolytic adenoviruses coated with MHC-I tumor epitopes increase the anti-tumor immunity and efficacy against melanoma. *OncoImmunology***,** 00-00.

HENDRICKX, R., STICHLING, N., KOELEN, J., **KURYK, L**., LIPIEC, A. & GREBER, U. F. 2014. Innate immunity to adenovirus. *Hum Gene Ther,* 25**,** 265-84.

HIRVINEN, M., CAPASSO, C., GUSE, K., GAROFALO, M., VITALE, A., AHONEN, M., **KURYK, L**., VAHA-KOSKELA, M., HEMMINKI, A., FORTINO, V., GRECO, D. & CERULLO, V. 2016. Expression of DAI by an oncolytic vaccinia virus boosts the immunogenicity of the virus and enhances antitumor immunity. *Mol Ther Oncolytics,* 3**,** 16002.

KURYK, L., WIECZOREK, M., DIEDRICH, S., BOTTCHER, S., WITEK, A. & LITWINSKA, B. 2014. Genetic analysis of poliovirus strains isolated from sewage in Poland. *J Med Virol,* 86**,** 1243-8.

KURYK, L., WIECZOREK, M. & LITWINSKA, B. 2013. Polio - A mysterious virus. *Post. Mikrobiol.,* 52**,** 43–152.

WIECZOREK, M., CIACKA, A., WITEK, A., **KURYK, L**. & ZUK-WASEK, A. 2015. Environmental Surveillance of Non-polio Enteroviruses in Poland, 2011. *Food Environ Virol,* 7**,** 224-31.

WIECZOREK, M., **KURYK, L**., WITEK, A., DIUWE, A. & LITWIŃSKA, B. 2013. The detection of enteroviruses in sewage using Caco-2 cells. *Pol J Microbiol,* 62**,** 97-100.

Additional patent application not included in the dissertation

KURYK, L., PESONEN, S., VUOLANTO, A., RANKI, T., JADERBERG, M., HAAVISTO, E., *Combining ONCOS-102 with PD-(L)1 Check Point Inhibitors for Treating Cancer.*

Abbreviations

4-HP-CP	4-hydroperoxycyclophosphamide
5-FU	Fluorouracyl
Ad	adenovirus
APC	antigen presenting cells
ASR	age-standardised rate
AST	aminotransferase
ATP	adenosine triphosphate
BCG	Bacillus Calmette-Guerin
CAR	coxsackievirus and adenovirus receptor
CCVs	clathrin-coated vesicles
CMX001	hexadecyloxypropyl cidofovir
CPA	cyclophosphamide
CPO	cyclophosphamide
CR	complete response
CRC	colorectal cancer
CTLs	cytotoxic T lymphocytes
DBP	DNA-binding protein
DC	dendritic cell
DLTs	dose limiting toxicities
EGFR	epidermal growth factor receptor
EMA	European Medicines Agency
ERCC1	DNA excision repair protein
FDA	Food and Drug Administration
GM-CSF	granulocyte macrophage colony-stimulating factor
H101	Oncorine (Ad5)
Hsp27	heat shock protein 27
HSV	Herpes simplex virus
HSV-1	HSV type 1
ICD	immunogenic cell death
IFN	interferon
IPV	inactivated poliovirus
ITRs	inverted terminal repeats
IU	infectious units
KC	Kupffer cells
M2	type 2 macrophages

MAPKs	mitogen-activated protein kinases
MDSC	myeloid-derived suppressor cells
MHC I	major histocompatibility complex I
MM	malignant mesothelioma
MPM	malignant pleural mesothelioma
mRECIST	Modified Response Evaluation Criteria in Solid Tumors
MTD	maximum tolerated dose
MV	Measles virus
NAbs	neutralization antibodies
nAChR	nicotinic acetylcholine receptor
NCD	Newcastle disease virus
NK	natural killer cells
NPC	nuclear pore complex
NS	nervous system
NSCLC	non–small cell lung cancer
oAd	oncolytic Ad
ORR	objective response rate
OS	overall survival
OTC	ornithine transcarbamoylase
OV	oncolytic virus
PAMPs	pathogen-associated molecular patterns
PD	progression disease
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PET	positron emission tomography
PFS	progression free survival
Pfu	plaque-forming unit
PKR	protein kinase RNA-activated
PR	partial response
PRRs	pattern recognition receptors
PSA	prostate-specific antigen
RECIST	Response Evaluation Criteria In Solid Tumors
RFP	red fluorescence protein
RGD	arginine-glycine-aspartic acid
ROS	reactive oxygen species
RR	response rate
RRM1	regulatory subunit of ribonucleotide reductase

SBRT	stereotactic body radiotherapy
SCLC	small-cell lung cancer
SD	stable disease
SFDA	China's State Food and Drug Administration
SoC	standard of care
STS	soft tissue sarcoma
T-Vec	talimogene laherparepvec or Imlygic (HSV-1)
TAAs	tumor-associated antigens
TILs	tumor infiltrating leucocytes
TLRs	Toll-like receptors
TNF	tumor necrosis factor
TP	terminal protein
Tregs	regulatory T cells
VA RNA	viral associated RNA
VP	viral particles
VR	virologic response
VV	vaccinia virus
XRT	external beam radiotherapy

Abstrakti

Perinteiset syöpähoidot eli leikkaushoito, sädehoito, kemoterapia tai näiden yhdistelmät, ovat kehittyneet merkittävästi. Tästä huolimatta hoidon teho voi olla heikko tietyissä syöpätyypeissä kuten esimerkiksi mesotelioomassa, keuhkosyövässä tai paksusuolen syövässä. Lisäksi hoidon tehoa voi heikentää syövän kehittämä resistenssi käytettyä hoitomuotoa vastaan, jolloin saavutettu hoitovaste voidaan menettää. Tämän takia syövän uusien hoitomuotojen kehitys tärkeää. Erityisen tärkeää on kehittää hoitoja, joilla on uusi toimintamekanismi ja joihin syövän kehittämä hoitoresistenssi muita hoitomuotoja kohtaan ei vaikuta.

Onkolyyttinen virushoito on eräs lupaava syövän hoitomuoto. Ensimmäinen länsimaissa hyväksytty onkolyyttinen virus on Imlygic (myös tunnettu nimillä T-Vec ja talimogene laherparepvec). Imlygicin hyväksyntä sekä USA:n (FDA) että Euroopan (EMA) lääkeviranomaisten toimesta on avannut uusia mahdollisuuksia syöpähoitojen kehittämisessä.

Yhden hoitomuodon käyttö syövän hoidossa on harvoin tehokasta, erityisesti silloin kun kysessä on etäpesäkkeitä muodostava tai pitkälle edennyt tauti. Useiden hoitomuotojen yhdistäminen syövän hoidossa on osoittautunut merkittävästi tehokkaammaksi kuin vain yhden hoitomuodon käyttö. Tulevaisuudessa konventionaalisten ja uusien hoitomenetelmien yhdistäminen saattaa mahdollistaa merkittävästi parempien hoitotulosten saavuttamisen.

Tässä väitöskirjassa on pyritty osoittaamaan, että yhdistämällä onkolyyttinen adenovirus kemoterapian tai biologisen yhdisteen kanssa, voidaan syövän hoitotehoa parantaa edellä mainittujen hoitojen yhteisvaikutuksen takia. Väitöskirjassa on kokoeiltu erilaisia hoitokombinaatioita, joiden tarkoituksena on ollut parantaa onkolyyttisen virushoidon tehokkuutta. Lisäksi väitöskirjassa on tutkittu adenovirusten turvallisuustekijöitä, koska geeninsiirron ja virusvektoreiden käytön turvallisuus on yleisesti erittäin tärkeää.

Väitöskirjassa on tutkittu kemoterapiaan perustuvan käypähoidon (Pemetrexed, Cisplatin, Carboplatin) ja onkolyyttisen adenoviruksen Ad5/3-d24-GM-CSF (ONCOS-102) yhdistelmähoidon tehoa ihmisen malignin mesotelioman (MM) malleissa, mitkä olivat erilaiset *in vitro*-mallit että BALB/c-ksenograftimalli. Tutkimuksessa osoitettiin, että hoidon teho käytetyissä malleissa parani, kun ONCOS-102 yhdistettiin käypähoidon kanssa verrattaessa pelkään virus- tai käypähoitoon. Yhdistelmähoito johti yhteisvaikutukseen, joka paransi hoidon tehoa.

Väitöskirjassa tutkittiin hoitotehoa myös yhdistelmällä, missä dipeptidi L-karnosiinin oli kompleksoitu onkolyyttisen adenoviruksen kanssa (virus-L-karnosiinikompleksi).

Kompleksin käyttö lisäsi tehoa käytetyissä *in vitro*- ja *in vivo*-syöpämalleissa. HCT116 paksusyöpäsolu- ja A549 keuhkosyöpäsolulinjoissa virus-L-karnosiinikompleksin käyttö tehosti viruksen transduktiota sekä lisäsi viruksen infektiivistä tiitteriä verrattuna virukseen jota ei oltu kompleksoitu. Virus-L-karnosiinikompleksia tutkittiin kahdessa *in vivo*-mallissa: keuhkosyövän ja paksusuolensyövän ksenograftihiirimalleissa. Kompleksin käyttö johti huomattavasti vähentyneeseen kasvaimen kasvuun verrattuna muihin käytettyihin hoitoryhmiin. Lisäksi väitöskirjassa tutkittiin kompleksin molekulaarista toimintamekanismia.

Virusvektoreihin liittyvä turvallisuusarviointi tehtiin käyttämällä eläinkokeita. ONCOS-102-viruksen toksisuus- ja biodistribuutiotutkimukset Syyrian hamstereissa ja BALB/c-hiirimallissa eivät indikoineet viruksen toistuvan annostelun aiheuttavan sivuvaikutuksia. Sivuvaikutuksia arvioinnissa käytettiin eläimen painoa, ruuan kulutusta, hematotologiaa, kliinistä kemiaa, histopatologiaa sekä viruksen biodistribuutiota.

Väitöskirjan tulosten perusteella onkolyyttisen viruksen yhdistäminen kemoterapiakäypähoitoon tai viruksen kompleksoiminen L-karnosiinin kanssa johtavat syöpähoidon tehoa parantavaan yhdistelmävaikutukseen. Tämä antaa vahvan perusteen tutkia yhdistelmien tehoa mesotelioomen sekä keuhko- ja paksusuolensyövän hoidossa potilailla. Lisäksi väitöskirjassa esitetyt tulokset viittaavat siihen, että adenovirusta voitaisiin käyttää muiden bioaktiivisten lääkkeiden kuljettamiseen kohteeseensa. Tämä olisi avian uusia strategia syövän hoidossa.

Abstract

Despite major advances in conventional cancer treatments by surgery, chemotherapy, radiotherapy and their combination, the outcome remains partially ineffective against numerous cancer types, for example mesothelioma, lung cancer, and colon cancer. Furthermore, due to resistance factors and the subsequent loss of response, which may occur rapidly during the conventional treatments regimes, new anti-cancer agents, presenting new mechanisms of action and lacking cross-resistance to commonly used therapies, are in high demand.

Oncolytic virotherapy is a promising anti-cancer strategy, and the approval of the first oncolytic virus, Imlygic (T-Vec, talimogene laherparepvec), in Western world by US Food and Drug Administration (FDA) and European Medicines Agency (EMA) has opened up new perspectives for improved treatment of cancer.

Single therapy is rarely successful in treating cancer, particularly in metastatic or advanced cancer, and survival rates with monotherapies alone are generally poor. The combination of multiple therapies to treat cancer has already shown significant results in the standard care of cancer. This strategy utilizes the combination of both conventional and novel therapies that can bring the future promise of cancer treatment.

In this thesis it has been hypothesized that by combining oncolytic adenoviruses (oAd) with chemotherapeutic drugs and a biological agent we could improve anti-cancer efficacy through synergistic effect against cancer. Therefore, we have tested various treatment regimes with the overall goal being the improvement of oncolytic virotherapy efficacy. Secondly, since safety issues concerning gene therapy and viral vectors are tremendously important, we have performed studies on safety issues of adenoviral vectors. In brief, we have evaluated the anti-cancer activity of combination treatment with standard of care (SoC) chemotherapy (Pemetrexed, Cisplatin, Carboplatin) and Ad5/3-d24-GM-CSF (ONCOS-102) *in vitro* and in a xenograft BALB/c model of human malignant mesothelioma (MM). We could show improved anti-tumor effects when ONCOS-102 was combined with SoC chemotherapy regimens over chemotherapy and virus alone. Combination therapy resulted in synergistic anti-cancer effect improving the therapeutic outcome. In a subsequent study we tested anti-cancer properties of the dipeptide L-Carnosine complexed with an oncolytic adenovirus (virus-L-Carnosine complex). The complex demonstrated improved anti-tumor efficacy both *in vitro* and *in vivo* in tested cancer models. In HCT116 colon and A549 lung cancer cells, the virus-L-Carnosine complex presented a higher transduction level and infectious titer over

uncoated oncolytic adenovirus. The *in vivo* efficacy of the virus-L-Carnosine complex was tested in two cancer models: i) lung and ii) colon cancer xenograft mice models. It exhibited a significant reduction in tumor growth compared to other tested groups. Additionally, we investigated the molecular mechanism underlying the effects of the complex on tumor growth reduction.

Safety assessment of viral vectors was performed in animal studies. Extensive studies on toxicity and bio-distribution of ONCOS-102 in Syrian hamsters and experiments in BALB/c nude mice indicated no side effects of repeated administration of oncolytic adenovirus. The side effects were evaluated by assessment of body weight, food consumption, hematology, clinical chemistry, histopathology and bio-distribution.

We concluded that combinatory studies utilizing oncolytic viruses with standard of care chemotherapy and an experimental virus-L-Carnosine complex showed synergistic anti-cancer efficacy, thus providing a strong rationale for clinical testing of such combinations in mesothelioma, lung and colon cancer. Additionally, our studies suggested that adenovirus could be used in future studies for delivery of other bioactive drugs as a novel strategy in cancer therapy.

PART B

1. REVIEW OF LITERATURE

1.1 Introduction

Although many kinds of treatment have been developed during the past few decades, there is still a lack of effective therapies for advanced cancers. Currently there are no curative modalities for malignant mesothelioma (Kondola et al., 2016, Boffetta, 2007), lung cancer (Gadgeel et al., 2012) and colon cancer (Sargent, 2015). Although treatments such as surgery, chemotherapy and radiotherapy can help to improve patient prognosis and increase patient life expectancy, new treatment strategies against cancer are in high demand. Efficient anti-cancer agents and their targeted delivery into the tumor mass is a key prerequisite for a successful cancer therapy (Jeanbart et al., 2014). Secondly such an approach should be safe and well tolerated for cancer patients (Bae and Park, 2011).

Cancer standard of care is commonly a combination of surgery with chemotherapy and/or radiotherapy. However, in advanced cancer patients this approach is inefficient and may cause many side effects, including severe complications and even death. Some advanced cancer patients are not responding to the standard treatment regime and undergo only symptomatic therapy (Pappagallo, 2011) without chances to recover.

Oncolytic virotherapy is emerging as a potential approach to treat cancer. It takes advantage of using viruses, which are specifically engineered to preferentially infect, replicate in and kill cancer cells instead of normal cells (Sze et al., 2013, Russell et al., 2012). However, this strategy has also disadvantages like low efficacy (Koks et al., 2015), production of anti-viral neutralization antibodies (Davis and Fang, 2005) and lack of effective antiviral drugs in case of uncontrolled virus replication (Romanowski, 2014).

Tumors are highly heterogeneous complexes of cells, which develop many mechanisms for evading the innate immune response (Marusyk and Polyak, 2010). Therefore, efficacy and antitumor responses induced by monotherapy may not be sufficient to eradicate cancer cells. Oncolytic viruses exhibit a different mechanism of action from conventional anti-cancer approaches (chemotherapy and radiotherapy), giving a possibility for additive or synergistic interactions in cancer therapy (Dilley et al., 2005, Siurala et al., 2015). Additionally, combined therapies may lead to increased efficacy without additional side effects.

1.2 Cancer

Cancer is still a leading cause of death worldwide, with 8,2 millions deaths reported by WHO in 2012. Most of the deaths each year are due to lung, stomach, liver, colorectal and breast cancer (Ferlay et al., 2015), (Figure 1). The global burden of cancer increases constantly because of the aging and rapid growth of the world's population in conjunction with an increasing habit of cancer-causing behaviors, namely: smoking, physical inactivity and ''westernized'' diets (Jemal et al., 2011).

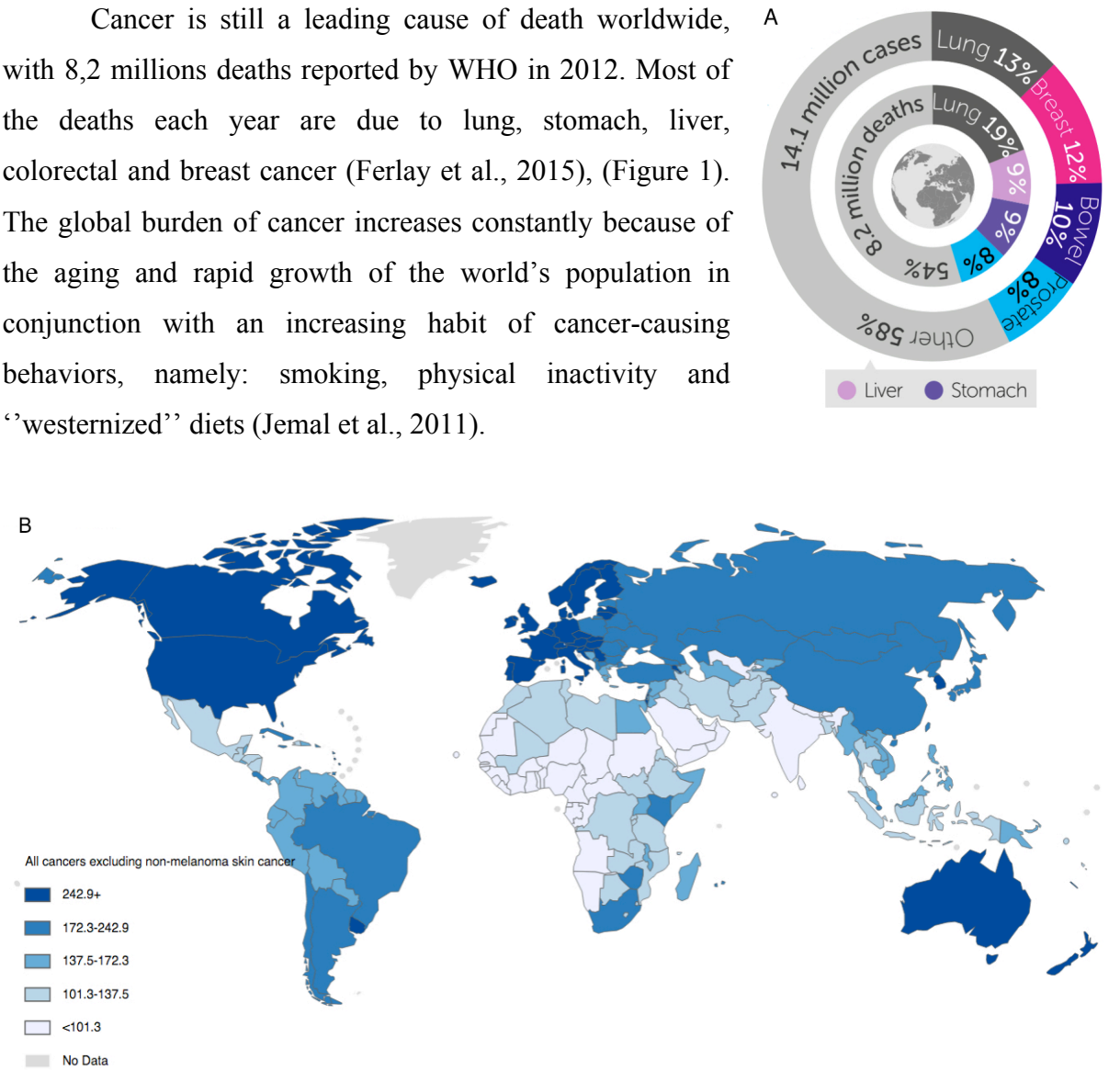

Figure 1. Cancer statistics. (**A)** Overview of worldwide cancer statistic, including both sexes. Modified from (Cancer-Research-UK, 2015); (**B)** Age-standardised rate for cancer incidence (all cancers excluding non-melanoma skin cancer, both sexes). Modified from (Ferlay et al., 2015).

1.2.1 Lung cancer

Lung cancer derives from abnormal epithelial cells located in the airways of the lungs and is the leading cause of deaths worldwide (Figure 1A). There are two major forms of lung cancer: i) non–small cell lung cancer (NSCLC), (about 85% of all lung cancers) and ii) small-cell lung cancer (SCLC, 15%), (Molina et al., 2008). Despite of the improvements in early detection methods and the treatment therapies in the last decades, non– small cell lung cancer is still often diagnosed at an advanced stage with poor prognosis and no efficient treatment

options (Ridge et al., 2013). Therefore, the prevention and treatment of lung cancer is still major unmet need. Therapy and diagnostics can be improved by a better understanding of the molecular mechanisms of the origin and evolution of this cancer type (Herbst et al., 2008) and by developing of more efficient treatment modalities. An age-standardise rate (ASR) of lung cancer incidence is presented in Figure 2. NSCLC can be divided into 3 major histologic subgroups: i) squamous-cell carcinoma, ii) adenocarcinoma, and iii) large-cell lung cancer. Smoking can cause all lung cancer types, however, it is most strongly linked with SCLC and squamous-cell carcinoma. Adenocarcinoma is the most common type in patients who have never smoked (Herbst et al., 2008).

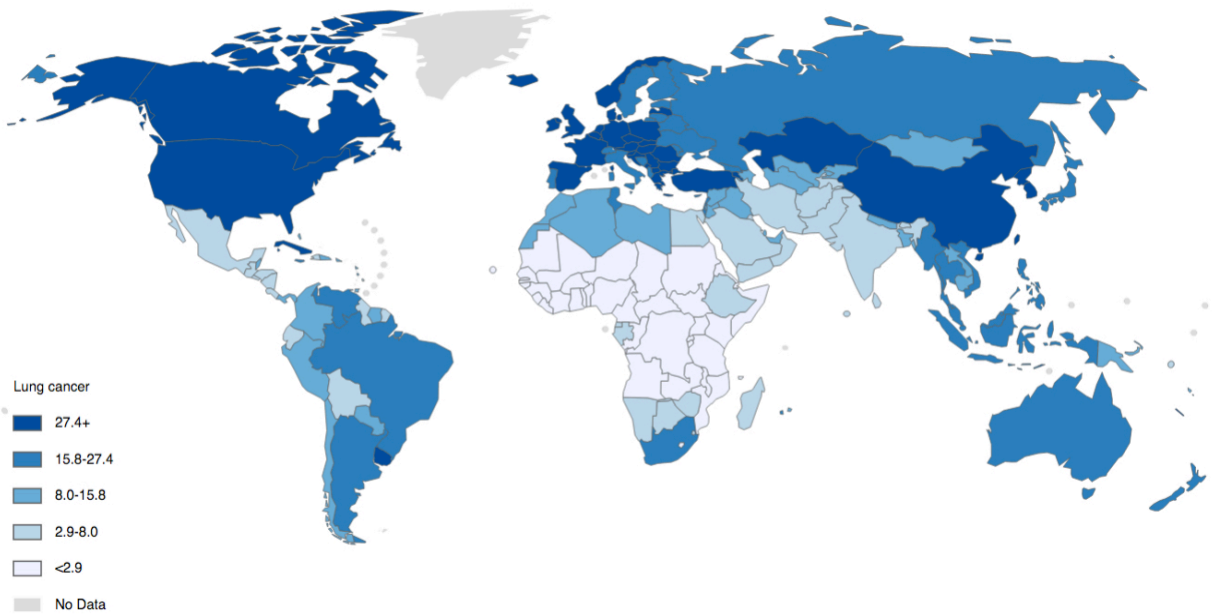

Figure 2. Age-standardised rate for lung cancer incidence (both sexes). Modified from (Ferlay et al., 2015).

Epidemiologic studies have shown the tendency of increased lung cancer risk in families with lung cancer history. Lung cancer risk and susceptibility is elevated also in rare inherited germ-line mutations in p53 (Hwang et al., 2003), retinoblastoma (Herbst et al., 2008), and other genes (Bailey-Wilson et al., 2004) as well as more common germ-line mutation in the epidermal growth factor receptor (EGFR) gene (Bell et al., 2005). More recently, an association to single-nucleotide polymorphism (SNP) variation at 15q24–15q25.1 was demonstrated. The region of the SNP variation covers genes encoding subunits of the nicotinic acetylcholine receptor (nAChR) alpha, regulated by nicotine exposure (Hung et al., 2008, Thorgeirsson et al., 2008, Lam et al., 2007). Lung cancer risk also elevates with diminished DNA repair capacity of the cell, resulting from germ-line alterations in nucleotide

excision repair genes such as DNA excision repair protein (ERCC1) (Yu et al., 2008). Increased expression of DNA synthesis and repair genes in NSCLC, like the regulatory subunit of ribonucleotide reductase (RRM1) and ERCC1, correlates with improved treatment prognosis (Friboulet et al., 2013, Zheng et al., 2007).

1.2.1.1. Current treatment options for lung cancer

Surgery, chemotherapy, and radiation or their combinations are used to treat NSCLC (Besse et al., 2014). For NSCLC that have not spread beyond the lung area, surgery is recommended to remove the cancer tissue. Surgery may also be conducted in combination with radiation therapy and chemotherapy in case of advanced cancers where these treatments are used prior to surgery to decrease tumors volume and prevent the transmission of cancer cells through the blood stream (neoadjuvant therapy). Chemotherapy regimens include Cisplatin and Carboplatin based therapies in combination with Paclitaxel (Belani et al., 2005), Gemcitabine (Gridelli et al., 2007), Docetaxel (Schiller et al., 2002), Vinorelbine (Tan et al., 2005), Bevacizumab (Herbst et al., 2007), Erlotinib (Herbst et al., 2005) and Etoposide (Rusch et al., 2007, Hanna et al., 2008, Gandara et al., 2003). In some cases, targeted therapy (erlotinib (Park et al., 2016), gefitinib (Yang et al., 2016), crizotinib, afatinib (Losanno and Gridelli, 2016)) may be used as an alternative to chemotherapy, or after chemotherapy treatment.

Surgery is recommended for cancer patients with stage I or II of NSCLC and provides the best curative outcome. Surgery with or without adjuvant chemotherapy for stages IB and II is generally recommended. Adjuvant chemotherapy after surgical resection of tumor mass provides an increase in survival at 5 years in approximately 5% of the patients. The median 5 years overall survival (OS) rates range between 45 and 70%. No beneficial treatment effect has been observed for adjuvant chemotherapy after an operation for stage I NSCLC. However, the benefit of adjuvant chemotherapy increases as the disease progresses (Pignon et al., 2008). Stereotactic body radiotherapy (SBRT) may be used in early-stage NSCLC tumors, which are smaller than 5 cm and without lymph node involvement (Simone et al., 2013). This treatment has become an effective option for inoperable patients with early stage NSCLC. Surgery is less frequently used in SCLC, which tends to transmit more quickly than NSCLC to other parts of the body. Chemotherapy is the most common treatment option for small cell lung cancer as chemotherapeutics circulate throughout the body killing lung cancer cells located in different locations. Radiation therapy is used to prevent or treat SCLC that has spread into the brain. Radiation therapy is also recommended to prevent tumor recurrence

after surgery and for those patients who are inoperative (Fruh et al., 2013, Stahel et al., 2011). Cisplatin and Carboplatin based chemotherapy in combination with Etoposide and Cyclophosphamide (Tjan-Heijnen et al., 2002) or Doxorubicin, Vincristine, and Methotrexate is used in SCLC treatment (Crivellari et al., 2007).

1.3.1. Mesothelioma

Malignant mesothelioma is an aggressive and a rare form of cancer that develops from mesothelium. MM is primarily caused by exposure to asbestos and exhibits a long latency period (Belli et al., 2009), usually >30 years. The median survival time for mesothelioma patients after diagnosis is typically only 9-12 months (Delgermaa et al., 2011). MM affects the pleura (85,5%), peritoneum (13,2%), pericardium (0,5%), and tunica vaginalis (0,8%) (Fukuoka, 2014). MM tumors are often poorly responsive to standard therapies and therefore incidence is steadily increasing worldwide (Robinson et al., 2005, Delgermaa et al., 2011, Szulkin et al., 2014, Gomez and Tsao, 2014, L et al., 2001). The low incidence of MM has for a long time limited the discovery of new drugs (Fukuoka, 2014), therefore new treatment modalities are highly needed.

1.3.1.1. Current treatment options for mesothelioma

Currently there are no curative modalities for malignant mesothelioma, however treatments such as surgery, chemotherapy and radiotherapy can help to improve patient prognosis and increase patient life expectancy (Fennell et al., 2008). Malignant pleural mesothelioma (MPM) patients should receive surgical resection (if possible), followed by adjuvant radiation therapy and either neoadjuvant or adjuvant chemotherapy (Cisplatin plus Pemetrexed for 4 cycles), (Gomez and Tsao, 2014). Several studies have used an intrapleural approach in MPM. Intrapleural therapy is meant to increase antitumor efficacy. Most clinical studies of intra-cavitary chemotherapy have tested platinum based modalities in conjunction with Pemetrexed and Doxorubicin. The median progression free survival (PFS) ranged between 7,5 and 13,6 months, while OS ranged from 11,5 to 18,3 months (Chang and Sugarbaker, 2004, Lee et al., 2002, Colleoni et al., 1996, Rice et al., 1994, Rusch et al., 1994). Hyperthermic intrapleural perfusion chemotherapy with Cisplatin, Pemetrexed and Doxorubicin has been studied as well. This approach aims to elevate tumor tissue temperatures to 42°C in order to enable chemotherapeutic agents to penetrate the tumor cells more efficiently. Clinical studies have shown that this is a feasible treatment strategy and have reported the maximum tolerated dose (MTD) of Cisplatin at 225–250 mg/m^2 (Rusch et

al., 1994, Rice et al., 1994, Lu et al., 2005, Richards et al., 2006, Gomez et al., 2013, Ratto et al., 1999).

1.4.1. Colon cancer

Colon cancer (colorectal cancer, CRC) is the most common type of malignant tumor arising from the inner wall of intestine. CRC appears randomly in the sporadic forms (85%) and in hereditary familial forms (15%), (Jasperson, 2012). It usually appears as a benign from, called a polyp. Early polyp removal prevents their transformation into cancer. Colorectal cancer can remain asymptomatic for many years before being diagnosed, since the symptoms can vary greatly according to the location of the tumor site. CRC is the third the most common tumor in men and the second in women, covering 10% of all tumors worldwide with approx. 608 000 CRC related deaths reported each year (approx. 8% of all cancer deaths). CRC is the 4th most common cancer cause of death worldwide (Labianca et al., 2013). In Europe and other western countries, it is the second leading cause of death in both males (after lung cancer) and females (after breast cancer), (O'Connell et al., 2004), (Figure 3).

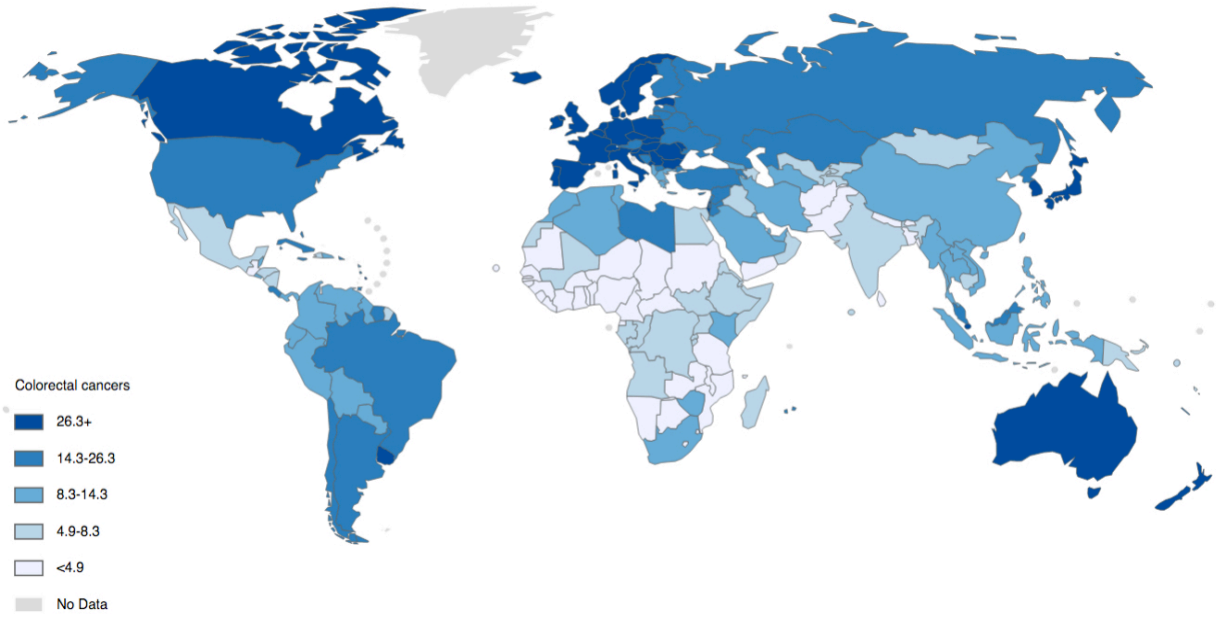

Figure 3. Age-standardised rate for colon cancer incidence (both sexes). Modified from (Ferlay et al., 2015).

1.4.1.1. Current treatment options for colon cancer

Current treatment of colon cancer depends on the location, size, and stage of the tumor as well as the health of the cancer patient. Chemotherapy can extend and refine quality of life, however, surgery is still the primary procedure for colon cancer treatment (Labianca et al.,

2013, Glimelius et al., 2013) and may be the only form of treatment required. Cancers that invade all layers of the bowel wall may continue to invade adjacent organs and local tissues or spread throughout the abdomen. Surgery aims at resecting all cancer mass, including adjacent organs, such as the uterus, ovaries or bladder. Once the disease has metastasized to lymph nodes or to distant organ (liver or the lungs), it is rarely curable by a surgical approach alone (Van Cutsem et al., 2014, Jasperson, 2012, Yeatman, 2001, O'Connell et al., 2004). Unfortunately, the majority of patients have metastatic, inoperative disease. However patient can become eligible for resection after successful chemotherapy (Van Cutsem et al., 2014). When the disease has spread to lymph nodes or to distant organ sites, chemotherapy is generally recommended in the treatment plan. Adjuvant chemotherapy is usually used for patients with nodal disease (Dukes' stage C). Current modalities utilize 6 months of adjuvant chemotherapy with S-phase-specific cytotoxic drugs, including 5-fluorouracil and leucovorin. Patients with full-thickness tumors, but without evidence of Dukes' stage B, seem not to respond to chemotherapy. However, immune modulation has been demonstrated to be beneficial in these groups of patients. Modulation may take the form of vaccination with radiated, autologous tumor cells in combination with the immune adjuvant Bacillus Calmette–Guerin (BCG), (Yeatman, 2001, Van Cutsem et al., 2014). Systemic chemotherapy includes 5-fluorouracil, leucovorin, ironotecan, and 5,10-Methylenetetrahydrofolate (Han et al., 2007, Gustavsson et al., 2015).

1.5. Adenoviruses

Adenoviruses (Ads) are non-enveloped DNA viruses of approximately 90 nm in diameter. The double-stranded DNA has a length of approximately 36,000 bp and the inverted terminal repeats (ITRs) acts as origins of replication (Figure 4). Ads are classified into 57 human serotypes and 7 different species from A to G. Their name derives from initial isolation from human adenoids in 1953 (Rowe et al., 1953).

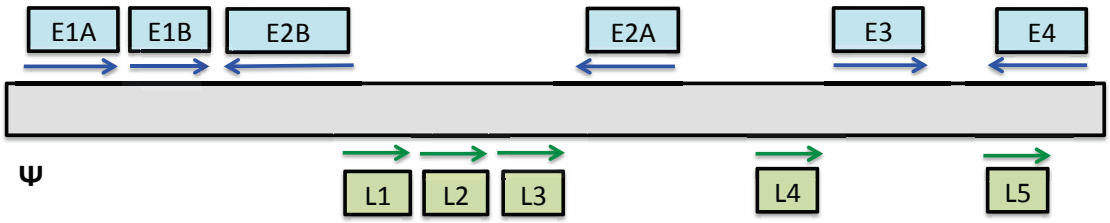

Figure 4. Schematic representation of human adenovirus genome. Early genes (E1-E4), late genes (L1-L5) and ψ replication start point. Modified from (Knipe et al., 2007).

Ads are the most intensively studied vectors in gene therapy clinical studies among all used viruses (Zamarin and Pesonen, 2015), (Figure 5). Ads have been used as non-replicating gene transfer vectors, vaccination vectors (Draper and Heeney, 2010) and as oncolytic cancer treatment agents (Zamarin and Pesonen, 2015, Capasso et al., 2015, Ranki et al., 2014a, Vassilev et al., 2015). Ads exhibit a natural lytic replication cycle (Alemany et al., 2000), their production is efficient, leading to high titer of stable viral particles (Tatsis and Ertl, 2004), and the Ad genome is easy to modify, allowing genome extension up to 105% of wild type's genome (Hermiston, 2000).

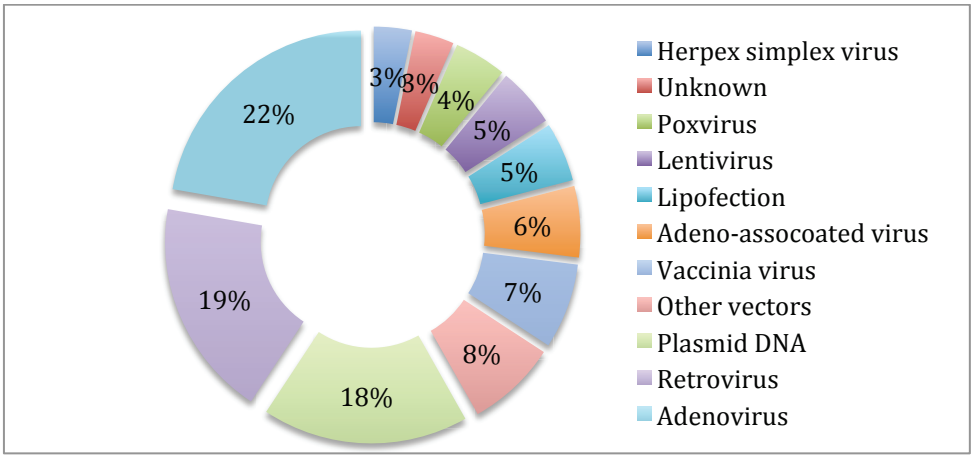

Figure 5. Vectors tested in gene therapy clinical trials. Based on database provided by the Journal of Gene Medicine (http://www.abedia.com/wiley/vectors.php, 2015).

1.5.1. Adenovirus structure

Ad consists of 87% protein and 13% DNA (Rux and Burnett, 2004). Studies made by electron microscopy (Figure 6) and X ray crystallography showed that icosahedral structure of Ad capsid consists of 20 triangular and 12 vertices surfaces. It has 252 capsomere subunits, containing 240 hexons and 12 pentons. Each hexon is surrounded by 6 subunits and each penton is in direct contact with 5 subunits and has a fiber. Capsid contains three major capsid proteins (hexon, penton base and fiber knob) (Zhang and Imperiale, 2003), four minor capsid proteins (VI, VIII, IX and IIIa), and four core proteins (terminal protein, protein Mu, VII and V), (Nemerow et al., 2009, Rawlins et al., 1984).

Adenovirus has a linear, double-stranded DNA with a terminal protein (TP) at the 5' ends. The genome consists of 5 early transcription units (E1A, E1B, E2, E3 and E4), three delayed early units (IX, IVa2, E2) and one late unit (L1-L5) (Thimmappaya et al., 1982), (Figure 4). E1A proteins activate transcription and mediate the entry into the S phase of the cell cycle (Knipe and Howley, 2013). Two E1B proteins block apoptosis in adenovirus-

infected cells. Three E2 proteins mediate DNA replication (Knipe et al., 2007). E3 proteins modulate the antiviral host response (Wold et al., 1999). E4 proteins promote adenovirus messenger RNA metabolism (Goodrum and Ornelles, 1999), facilitate viral DNA replication and inhibit host-cell protein synthesis. Late proteins (L1-L5) are capsid components (Hoeben and Uil, 2013).

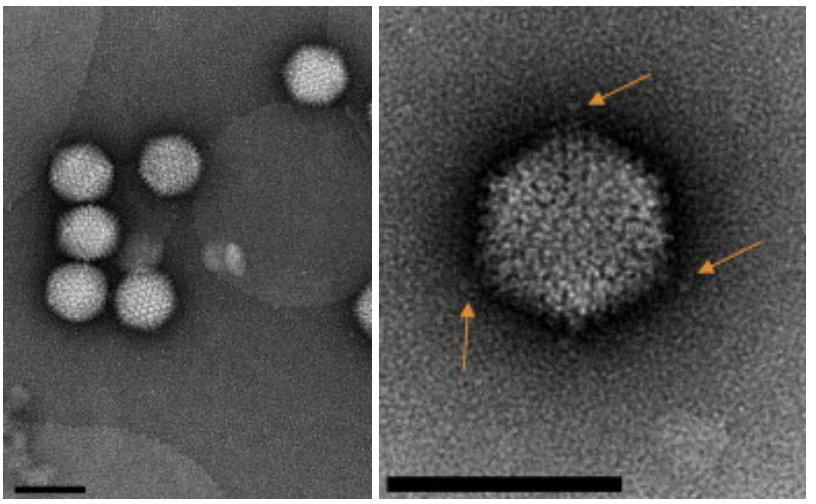

Figure 6. Electron micrographs of negatively stained viral particles of Ad5F3EGFP. Orange arrows indicate the HAdV-3 fiber. Scale bar represents 100 nm (Murakami et al., 2010).

1.5.2. Adenovirus cell entry

Adenovirus life cycle consists of two phases: i) the early phase lasting for 5 - 6 hours (cell entry) and ii) the second phase starting from the expression of late genes and assembly of the virus progeny (replication), (Figure 7). Adenovirus infection starts by the binding of the Ad fiber knob to a cell surface receptor. Most adenovirus species (subgroup A, C, E, F) utilize the coxsackievirus adenovirus receptor (CAR), (Zhang and Bergelson, 2005, Roelvink, 1999). Some Ads bind to CD46 receptor (group B, D), (Zhang and Bergelson, 2005). Ad binding is followed by receptor-mediated endocytosis facilitated by interactions between an arginine-glycine-aspartic acid (RGD) within the adenoviral penton base and cellular $\alpha_V\beta$ integrins (Wickham et al., 1994). Ad counterplays with cells by binding to CAR and moves in random motions. Next, Ad engages in acto-myosin-mediated slow drifting motions. Mechanical cues on the adenovirus increase from the slow drifts of CAR, and the stalling motions of the second virus receptor, integrins. These result in mechanical stress, which initiates the virus-uncoating program. Ad infection uptake consists of a two-step membrane penetration process. The first step is controlled by the mechanical properties of the virus and the cell. The second step increases the levels of ceramide in the plasma membrane, and gradually enhances

membrane lesions coincident with virus endocytosis (Greber, 2016). Ad replicates in the nucleus. Virus transfers its linear DNA genome, but not the viral capsid, in the nucleus (Wang et al., 2013). It uses the molecular motor kinesin-1 to dis-attach its genome from the capsid at the cytoplasmic place of the nuclear pore complex (NPC), by docking to the nucleoporin Nup214 (Strunze et al., 2011). The kinesin-associated light chain 1,2 attaches to virus capsid protein IX, and Nup358 activates the motor domain in the heavy chain. Cascade of these steps leads to the disruption of the capsid and a part of nuclear pore complex. The viral DNA is imported into the nucleus with involvement of cellular transport factors, such as importins and transportins (Flatt and Greber, 2015, Greber, 2016).

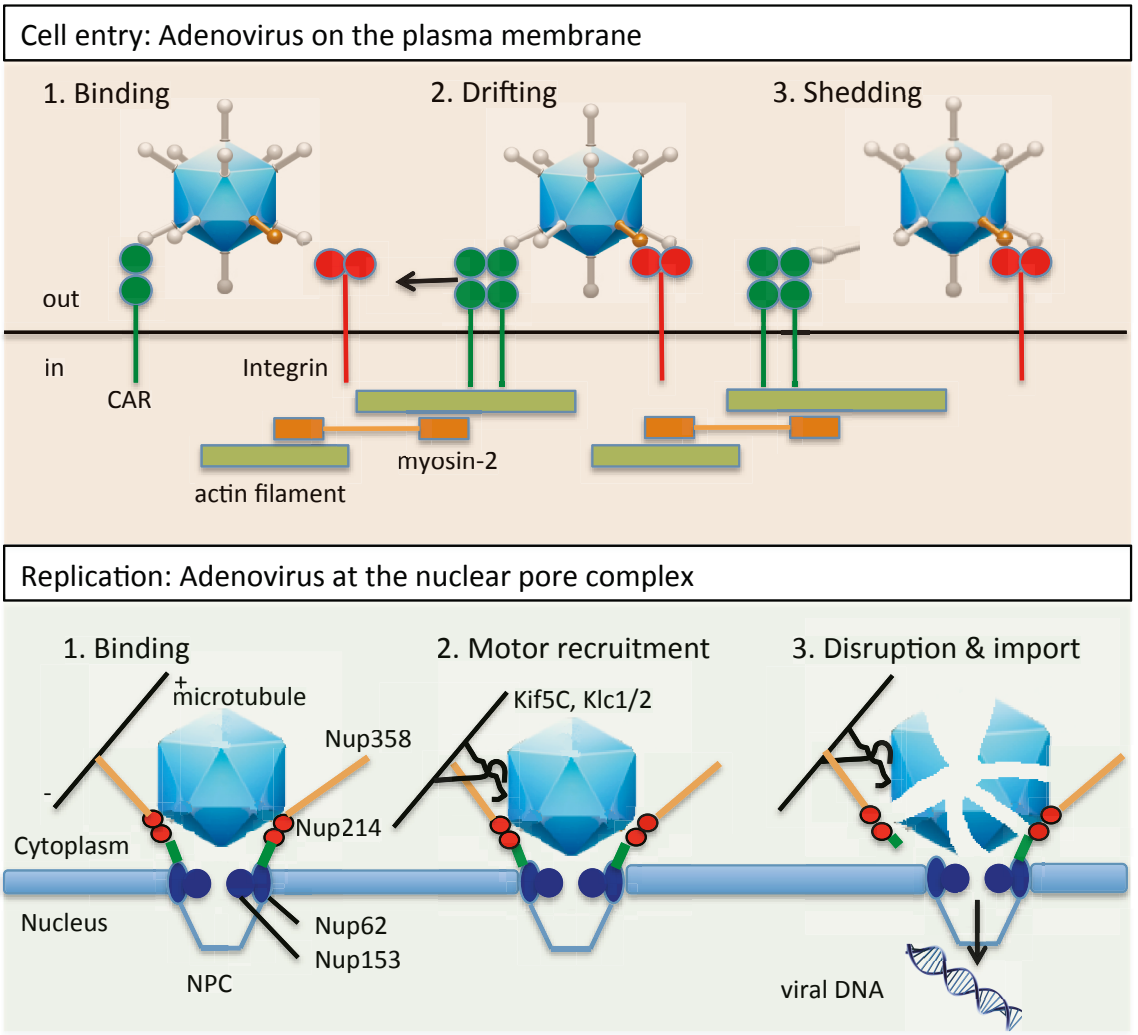

Figure 7. Adenovirus life cycle (cell and nuclear entry). (Upper panel) Acto-myosin-mediated drifting motions of Ad attached to CAR and to integrins on the cell surface result in disassembly of viral fibers. **(Lower panel)** Ad (without fibers) binds to the NPC protein Nup214 via hexon protein. The microtubule-dependent motor kinesin-1 binds to the protein IX by light chain Klc1/2. Motor turns on after heavy chain binding to Nup358 and initiate the capsid disruption. Capsid disruption deletes Nup62 from the nuclear pore, and adenoviral DNA is transferred into the nucleus. Modified from (Greber, 2016).

1.5.3. Adenovirus transcription and replication

The Ad gene transcription is launched by the production of the viral E1A transactivator, leading to a cascade of reactions. Replication is a very efficient process, where an infected cell produces approx. one million of viral DNA copies within 40 hours post infection. DNA replication begins within the inverted terminal repeats (ITRs) and requires three viral proteins encoded by E2 genes: pTP, Ad DNA polymerase and the DNA-binding protein (DBP), (Hoeben and Uil, 2013). DNA replication takes place in the nucleus and proceeds by a number of steps: i) the release of E2F upon E1A attachment to the Rb tumor suppressor, ii) inhibition of the p53 tumor suppressor by E1B- 55K, iii) inhibition of apoptosis by the Bcl-2 homologue E1B-19K. The next steps are the inhibition of cellular antiviral responses, including reservation of major histocompatibility complex I (MHC I) by E3-gp19K, leading to suppression of cytotoxic T lymphocytes (CTLs), and the production of specific gene products required for adenoviral DNA replication is activated (Knipe et al., 2007, Knipe and Howley, 2013). Translation of late genes leads to the production of adenoviral capsid proteins. Virions are released by the cell lysis. The genome is packed into the assembled capsid. This last step requires disruption of intermediate filaments (vimentin and cytokeratin), causing damage of the cell. Released virions can infect surrounding cells (Hoeben and Uil, 2013).

Ads have evolved several mechanisms allowing them to evade from the host immune system, such as the production of immune suppressive proteins. E1A neutralizes the immune response against the virus by blocking the interferon (IFN) gene activation (Routes et al., 1996) and hampers the transcription of IL-6 gene and blocks transduction of the IL-6 signaling pathway (Takeda et al., 1994). Additionally, E1A breaks cell death path induced by tumor necrosis factor (TNF, TNF-induced cell death). E1B-19k gene inhibits apoptosis during infection (Cuconati et al., 2002, Han et al., 1996, White, 2006). E3 proteins inhibit cellular (T cell-mediated) antiviral responses (McSharry et al., 2008, Hoeben and Uil, 2013).

1.6. Oncolytic adenovirus

Researchers have seen the potential of using viruses in the treatment of cancer from the beginning of their discovery, in 20th century. It has been observed that some cancer patients acquiring viral infection, like: hepatitis, influenza, measles, smallpox had tumor regression (Jessy, 2011). The first use of adenoviruses in cancer therapy was reported in 1956. 30 cervical carcinoma patients were treated with adenovirus adenoidal-pharyngeal-conjuctival virus. 26 out of 40 patients resulted in localized necrosis with mild side effects (vaginal

hemorrhage, fever, malaise). However, due to technological limitations, and lack of significant findings from clinical trials, studies on virus therapies were discontinued for many years (Kelly and Russell, 2007). Nowadays, thanks to advances in genetic engineering, an increased understanding of molecular biology, virology, and importantly, an experience in pre-clinical and clinical studies with oncolytic viruses in cancer therapies, have gave new life to oncolytic virotherapy. The use of new generation tumor-targeted oncolytic viruses has emerged as promising approach for novel cancer treatments.

Oncolytic viruses (OVs) selectively replicate and lyse cancer cells, spreading within the tumor mass, circulating into distant metastases, and not significantly harming normal cells. OVs can exhibit natural tumor-selective tropism (reovirus), (Kim, 2015) or be genetically modified for cancer cell-restricted replication (adenovirus, herpes simplex, vaccinia, Newcastle disease virus, measles). OVs are effective at inducing immune responses against themselves (anti-viral immune responses), (Pikor et al., 2015) but also to infected tumor cells (anti-tumor immune responses), (Lemay et al., 2012, Forbes et al., 2014, Kaufman et al., 2015). They can be used as adjuvants and to induce host immune responses against the tumor (Capasso et al., 2015). Oncolysis caused by OVs releases tumor epitopes along with danger signals like damage-associated molecular pattern (DAMP), OV-derived pathogen-associated molecular pattern (PAMP) molecules and pro-inflammatory cytokines (Medzhitov and Janeway, 2002, Bartlett et al., 2013, Tang et al., 2012). During the oncolysis, tumor antigens are collected by antigen presenting cells (APC), processed and presented to naïve T cells in lymph nodes. This cascade of reaction leads to the activation of tumor specific T cells, which can travel to the tumor and distant metastases and eradicate specific cancer cells. Such responses can result in long-lasting anti-tumor memory. OVs trigger also anti-viral immune responses, giving the ability of breaking the immunotolerance of tumor microenvironment and boosting an induction of anti-cancer effect. Additionally, when arming OV with specific co-stimulatory transgenes, it can trigger pro-stimulatory immune response and work as immune modulators (Liu et al., 2014, Putzer et al., 2001, Diaconu et al., 2012, Bramante et al., 2015).

Construction of oncolytic viruses in the past has been mainly focused on targeted delivery to tumor sites, enhanced replication and more potent lysis. Nowadays more attention is given to anti-tumor immunity studies (Seymour and Fisher, 2016, Prestwich et al., 2008). OVs serve as a diverse platform for immunotherapy, work as vaccines, and can be armed with immunomodulatory transgenes or combined with other anti-cancer therapies. There are two clinical approaches with OVs: i) systemic treatment and ii) local immunotherapy (Zamarin

and Pesonen, 2015), (Figure 8). Therefore, OVs are constantly receiving an increasing level of attention as anti-cancer agents in clinical studies (Seymour and Fisher, 2016). Many clinical studies with OVs are currently ongoing and more results are expected to be published in the next couple of years (Pol et al., 2016)

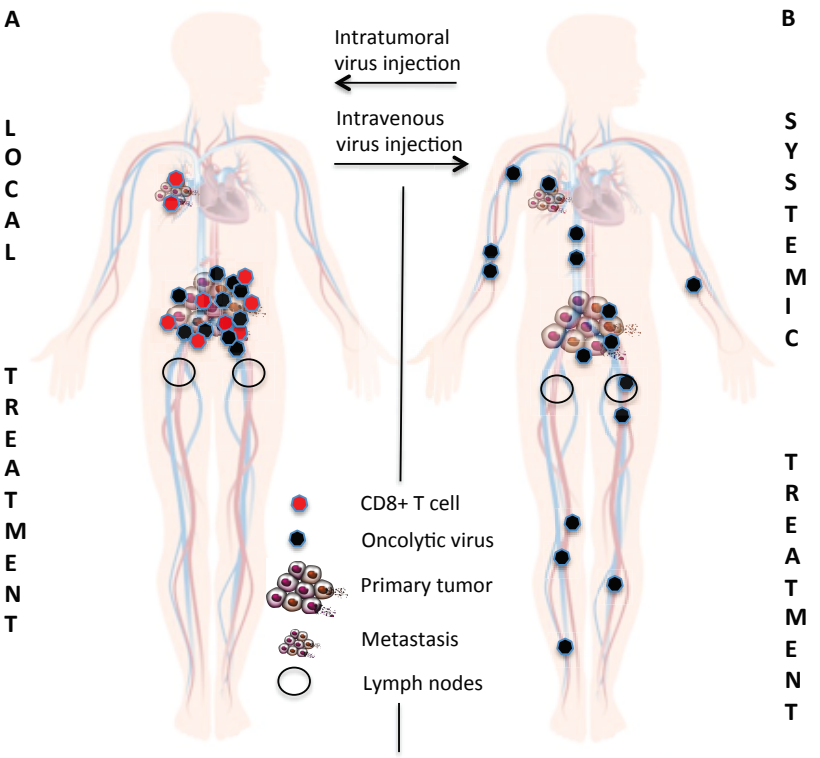

Figure 8. Two concepts of using OVs in cancer therapy. (A) Locally administered OV produce a strong ‘‘danger signal’’ leading to anti-tumor immune response against cancer cells. Additionally oncolytic viruses lyse tumor cells releasing new specific cancer epitopes. Immune activation can be improved by immune co-stimulator transgenes coded by the virus. Antigen presenting cells pick up tumor antigens and present them to T-cells in the draining lymph node. Activated tumor specific CD8+ T-cells recognize and kill cancer cells in both injected and non-injected tumor masses. **(B)** Systemic delivery aims at OVs dissemination throughout the body based on either natural or genetically improved tumor tropism. The purpose is to deliver/target the OV’s to tumors. Repeated administration of an adenoviral vector triggers specific immune reaction against the vector, resulting in neutralization of the systemically administered virus by the anti-viral antibodies. Modified from (Zamarin and Pesonen, 2015).

1.6.1. ONCOS-102

ONCOS-102 is a serotype 5, human, double-targeted oncolytic adenovirus with a chimeric 5/3 capsid for enhanced cancer cell transduction. It has a 24 bp deletion in the Rb binding site of the E1A gene for cancer-cell restricted replication (Koski et al., 2010). ONCOS-102 codes for human granulocyte macrophage colony-stimulating factor (GM-CSF)

for improved anti-tumor immunity. GM-CSF recruits and activates APC (Dranoff, 2002, van de Laar et al., 2012), (Figure 9). ONCOS-102 was engineered using standard cloning techniques. A fibre chimeric plasmid was cloned (pAdEasy5/3) and recombined with a shuttle vector containing a 24-bp deletion in *E1A* (pShuttleD24) resulting in pAd5/3-D24. An E3-shuttle vector pTHSN was created by including a 965-bp deletion into the E3 region to incorporate the human GM-CSF gene in place of *E3* gp19k and 6.7k. pAd5/3-D24-GM-CSF was obtained by homologous recombination in *E. coli* between pTHSN-GM-CSF and pAd5/3-D24. The genome of ONCOS-102 was rescued by digestion and transfection of A549 cells.

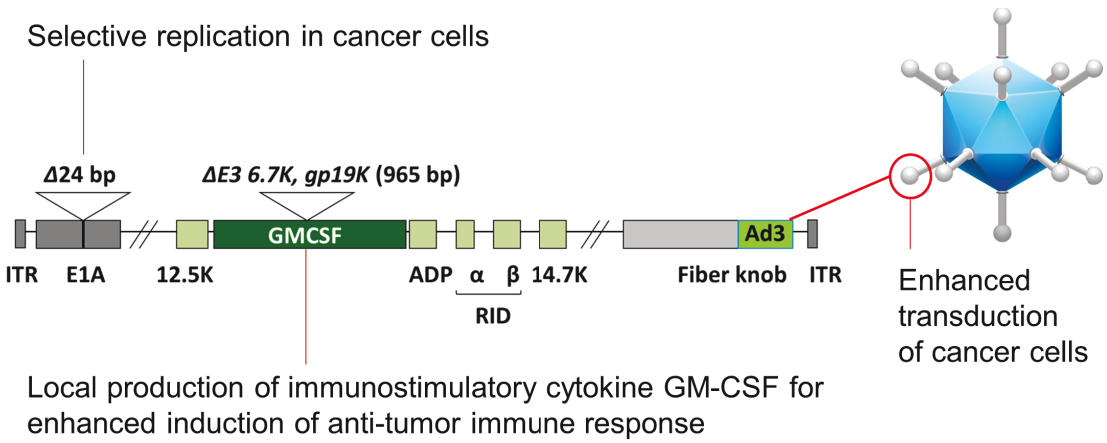

Figure 9. Structure of ONCOS-102. Compared to the wild-type adenovirus serotype 5, it has three genetic modifications. The knob of the adenovirus serotype 5 has been changed to that of adenovirus serotype 3. Thus, the ONCOS-102 uses adenovirus 3 specific receptors for transduction rather that adenovirus 5 specific receptors (CD46, Desmoglein-2, rather than CAR). A Δ24 deletion on the E1A region. The deletion is responsible of the targeting the virus replication in the cancer cells. A transgene coding human GM-CSF has been added to the E3 region. The human GM-CSF is a cytokine that enhances the immunological response towards the tumor, in which the ONCOS-102 is replicating.

1.6.1.1. ONCOS-102 mechanism of action

The mechanism of ONCOS-102 action (Figure 10) has been studied by investigating clinical samples (Ranki et al., 2016, Ranki et al., 2014a, Vassilev et al., 2015). Local injection of ONCOS-102 in the tumor induces a danger signal, stimulating the production of inflammatory cytokines, such as Il-6 and IL-8. ONCOS-102 infects tumor cells and causes ICD and releases of tumor antigens, and new virions in the tumor environment. Co-stimulatory molecule GM-CSF attracts antigen-presenting cells to the tumor. Dendritic cells (DCs) take up tumor and virus antigens and migrate to lymph nodes. There antigens are presented to naïve T Cells, resulting in T cells activation. Activated T cells identify, attack

and eradicate tumor cells expressing specific antigens. ONCOS-102 triggers systemic anti-tumor T cell responses in cancer patients by direct oncolysis of cancer cells and subsequently causing a modulation of tumor microenvironment from Th2 to Th1 type (Ranki et al., 2014b). ONCOS-102 sensitizes tumor cells to other immunotherapies by inducing a T-cell positive phenotype to an initially T-cell negative tumor mass (Ranki et al., 2014b). Treatment with ONCOS-102 has showed a prominent infiltration of TILs to tumors in 11 out of 12 treated patients (Ranki et al., 2016). Double mode of action of ONCOS-102 (direct oncolysis and induction of anti-tumor immunity) is an important factor, crucial in order to overcome the major obstacle with regards to cancer immunotherapy - suppressive tumor microenvironment (Diaconu et al., 2012).

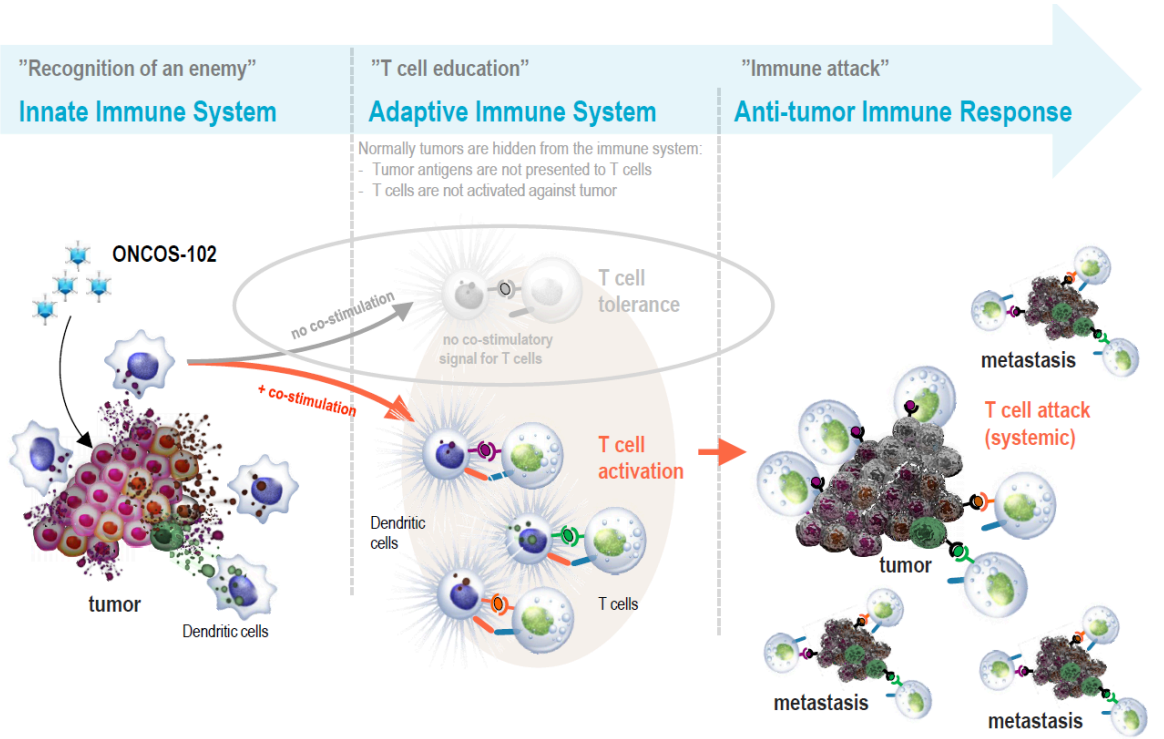

Figure 10. Mechanism of ONCOS-102 action. Local administration of ONCOS-102 induces innate and adaptive immune system leading to the development of anti-tumor and anti-virus immune responses. Activated T cells recognize, attack and kill tumor cells expressing the specific antigens. The effect is systemic (ONCOS-102 replicates in primary tumors and metastasis). The mechanism of a triple-action of ONCOS-102: 1) *Selective replication in tumour cells*. ONCOS-102 transduces and replicates in cancer cells, causing cancer cell death via lysis. New virions are released, and this process can continue as long as tumour cells are available. 2) *Activation of immune system and induction of anti-tumour response*. GM-CSF is only expressed when the virus replicates, resulting in high local concentrations in the tumour (Koski et al., 2010). GM-CSF is among the most potent stimulators of APCs, resulting in activation of cytotoxic T-cells against the tumour (Dranoff, 2003). This process is initially enhanced by the co-stimulatory response provided by adenovirus, which *per se* is a Toll-like receptor agonist and, therefore, a strong activator of the innate immune system. The 'oncolytic cancer' cell death is an immunologically active phenomenon and, in particular, helps create "danger signals" that are useful for anti-tumour

immunity (Tuve et al., 2009). Also, lysed tumour cells release tumour antigens for APCs. ONCOS-102 is an *in situ* cancer vaccine for each patient, potentially making it a personalised cancer immunotherapy. 3) *GM-CSF is a potent recruiter of NK cells.* Cancer cells have evolved a mechanism to evade immune recognition by inhibiting MHC-I synthesis (Bubeník, 2004). NK cells do not depend on MHC-1 expression for killing but instead preferentially kill MHC-I negative cells such as tumour cells. GM-CSF stimulates increased recruitment of NK cells to the tumour site.

1.6.1.2. ONCOS-102 efficacy – in vitro & in vivo studies

The efficacy of ONCOS-102 was examined in multiple cancer cell lines. The oncolytic potency of ONCOS-102 was as effective as the wild type Ad5 *in vitro* in MDA-MB-436 (human breast cancer) and A549 (human lung adenocarcinoma) cells. Effective cell killing was seen in human soft tissue sarcoma (STS) lines. Infection with 1 VP/cell resulted in more than 50% cell killing in most of the melanoma cell lines tested. In the human cell line MDA-MB-436, the combination of ONCOS-102 with cyclophosphamide (CPO) or 4-hydroperoxycyclophosphamide (4-HP-CP) increased anti-cancer efficacy. The combination of 1-10 VP/cell of with 4-HP-CP resulted in statistically significant increased cell killing compared to virus only or 4-HP-CP alone (Bramante et al., 2016, Siurala et al., 2015, Raki et al., 2008). GM-CSF secretion and functionality *in vitro* were confirmed in A549 and TF1 (human lymphocyte) cell lines.

The anti-tumour efficacy of ONCOS-102 and chemotherapy with doxorubicin and ifosfamide were studied in an immunocompetent hamster model of STS. ONCOS-102 (4.5×10^9 VP/kg), doxorubicin (1 mg/kg) and ifosfamide (30 mg/kg) were administered separately, simultaneously, or in combination with ONCOS-102 or chemotherapy. The treatment with ONCOS-102, doxorubicin and ifosfamide chemotherapy showed synergistic anti-tumour effects compared to single agent treatments. Median survival was significantly longer ($p=0.001$ or 0.002) in the delayed chemotherapy group (50 days), the simultaneous chemotherapy group (27 days), and the delayed ONCOS-102 group (27 days) than in the mock group (19 days). No major toxic changes were seen in any group. The data support the use of ONCOS-102 in combination with doxorubicin and ifosfamide for the treatment of STS (Siurala et al., 2015). ONCOS-102 (4.5×10^{11} VP/kg) displayed effective anti-tumour activity in a human melanoma xenograft NMRI nude mouse model. Complete tumour regression was observed in the group that received ONCOS-102 in combination with low-dose CPO, and near-complete tumour regression was observed in the group that received ONCOS-102 alone. Thus, ONCOS-102 is a promising treatment of melanoma.

1.6.1.3. ONCOS-102 – clinical study

ONCOS C1 (NCT01598129) was an exploratory, open label study of ONCOS-102 given in combination with low dose CPO in patients with refractory injectable solid tumours. Twelve patients (5 male, 7 female; aged 38 to 68 years [median age 63 years]) were treated with ONCOS-102: 3 patients were treated with 3×10^{10} VP/injection, 3 patients with 1×10^{11} VP/injection, and 6 patients with 3×10^{11} VP/injection. Dosing was scheduled on Days 1, 4, 8, 15, 29, 57, 85, 113, and 141. Five patients received the maximum of 9 doses of ONCOS-102, and 3 patients completed the study as planned. Patients also took oral CPO 50 mg daily throughout the treatment. The underlying cancer types differed widely across the study population with 9 different cancer types reported in 12 patients. Post baseline Response Evaluation Criteria In Solid Tumors (RECIST) evaluations were available for 10 patients. Of these, 4 patients (40.0%) had disease control (stable disease) at 3 months while all of the patients had progressed at 6 months. Responses according to immunologically relevant RECIST were consistent with the responses according to RECIST. Post baseline positron emission tomography (PET) response data were available for 10 patients. Of these, 4 patients (40.0%) had disease control. At 3 months, 1 patient had a minor metabolic response, and 2 patients had stable metabolic disease based on modified PET response. There were 5 patients with PET response data after 6 months. Of these, 1 patient had stable metabolic disease; the remaining 4 patients had progressive metabolic disease. Median progression-free survival was 2.9 months. Median overall survival was 9.3 months in the per protocol population (n=10). Exploratory immunohistochemistry analysis of tumour biopsies showed infiltration of immune cells (CD3, CD4, CD8, CD25, CD11c, CD19, CD68, CD163) into the tumour site 1 month after treatment initiation. On Day 57, CD8 cells were increased further whereas the corresponding ratio of the other immune cells was lower on Day 57 than on Day 29, and was <1 (i.e. below baseline values) for CD3, CD4, CD11, CD19, and CD68 cells. Viral genomes were detected in blood after every injection of ONCOS-102, with peak values occurring at 6 hours after injection. Neutralising antibody titres increased between baseline, Day 15 and did not return to pre-dose values in any patient. Blood samples for analysis of peripheral blood mononuclear cells (PBMCs) were evaluable for 10 out of 12 patients. Analysis of PBMCs was possible 2 patients, in whom ONCOS-102 induced a systemic anti-tumour CD8+ T-cell response. In both cases, this immune response correlated with a clinical response.

ONCOS-102 was well tolerated. No dose limiting toxicities (DLTs) and MTD were identified. There was no relationship between the dose of ONCOS-102 and the intensity of

adverse events. The most common adverse event was pyrexia. Other common adverse events, reported in few patients were chills, fatigue, injection site pain, feeling cold, decreased appetite, nausea (Grade 1 or Grade 2). Grade 3 adverse events were reported in 6 patients (pyrexia, increased alkaline phosphatase, increased aspartate aminotransferase [AST], proteinuria, hyponatraemia, anaemia, fatigue). Grade 4 adverse events were not reported (Ranki et al., 2016, Ranki et al., 2014a, Vassilev et al., 2015).

1.6.2. Combination of oncolytic viruses with other anti-cancer therapies

Tumors are highly heterogeneous mixture of cells, containing stroma cells, cancer cells, and immune cells, which stimulate tumor progression and maintain an immunosuppressive environment (Devaud et al., 2013). Tumor develops many mechanisms for evading the innate and adaptive immune response. Some tumor infiltrating immune cells have the ability to negatively regulate immune responses against the tumors due to the presence of regulatory T cells (Tregs), myeloid suppressor cells (MDSC), and type 2 macrophages (M2). Additionally, tumors themselves can promote suppression of antitumor immunity by exhibition of the NKG2D and MICA/B ligands which inhibit the functionality of natural killer cells (NK), T cell function (Groh et al., 2002) and accelerate the production of immunosuppressive CD4+ T cells (Groh et al., 2006). Soluble immune-suppressors (IL-10, histamine, hydrogen peroxidase, adenosine) produced by tumor cells can also block cytotoxic T lymphocytes (Palazon et al., 2012). Therefore, efficacy and antitumor responses induced by one treatment modality may not be sufficient to eradicate cancer cells.

Oncolytic viruses exhibit different anti-cancer mechanism compared to conventional therapies (chemotherapy, radiotherapy), allowing the possibility for additive or synergistic effect in cancer therapy. Additionally, combined therapies may lead to increased efficacy without additional side effects. Finally, since antitumor immune responses triggered by oncolytic adenoviruses may not be sufficient to eradicate tumors, additional treatment combinations are needed (Nguyen et al., 2014).

1.6.2.1. Chemotherapy combination

Combining oncolytic viruses with chemotherapeutics can accelerate stronger cytotoxic responses (Ottolino-Perry et al., 2010) and potentiate oncolysis. Chemotherapy can enhance the replication of oncolytic viruses and weaken the immunosuppressive tumor microenvironment (Nguyen et al., 2014). A clinically highly relevant strategy is therefore the

use of oncolytic viruses as adjuvants to standard chemotherapy, since most of the cancer patient have been or will be exposed to chemotherapy. Table 1 presents combined therapies, which have resulted in synergistic anti-cancer responses.

Table 1. Chemotherapy and oncolytic viruses combinations – mechanism of synergy. Based on (Tusell Wennier et al., 2012).

Oncolytic virus	Chemotherapy	Mechanism of synergy
Herpesviruses, Reovirus, Adenoviruses, Poxviruses, Paramyxoviruses	Cyclophosphamide	Suppression of the host's innate and adaptive anti-viral immune responses. Enhanced viral replication
Herpesviruses	Cisplatin	Improves viral replication by up-regulation of GADD34
Adenoviruses	Gemcitabine	E1A expression prevents drug resistance
Adenoviruses, Herpesviruses	5-FU	Up-regulation of CAR expression level
Adenoviruses, Herpesviruses	Taxenes	Virus replication is elevated in drug resistant cells (an up-regulated Raf/MEK/ERK molecular pathways)
Rhabdoviruses, Herpesviruses, Poxviruses, Adenoviruses	Histone deacetylase inhibitors	Reduction of IFN production in infected cancer cells results in enhanced viral replication. Up-regulation of CAR expression level
Rhabdoviruses, Poxviruses, Poxviruses, Adenoviruses	Rapamycin	Inhibited IFN production in infected cancer cells, increased viral replication
Poxviruses	Cyclooxygenase 2	Inhibited anti-viral antibody production, enhanced viral replication

Many preclinical studies have proven that combination therapy using OVs and chemotherapy may enhance therapeutic outcome (Siurala et al., 2015, Pandha et al., 2009, Lin et al., 2008). Indeed, China has approved the first oncolytic virus H101 (Oncorine) for the treatment of head and neck cancer. Phase III clinical studies showed a better response for H101 in combination with Fluorouracyl (5-FU), (72%) over chemotherapy alone (40%), (Garber, 2006).

Current cancer treatments are mainly based on chemotherapeutic agents. Most of these cytotoxic drugs work by inhibiting DNA replication or by disrupting microtubule structures. Cyclophosphamide (CPA) can increase the amount of OVs needed to obtain a therapeutic benefit in cancer therapy (Kambara et al., 2005). CPA inhibits DNA replication and activates cell death. The synergistic mechanism in immunotherapy is most likely related to the CPA's effect on host immune system, rather than enhancement of viral replication in cancer tissue. CPA exhibits immunosuppression activities leading to reduction of OV related toxicities. CPA also depletes Treg cells responsible for tumor-induced immune tolerance. Thus, the treatment with cyclophosphamide can sensitize cancer cells to immunotherapy (Ghiringhelli et al., 2004). Finally, OVs promote anti-cancer responses, which can be further enhanced by CPA-mediated depletion of Treg cells. Improved cancer treatment efficacy has been shown with HSV-2 and reovirus in combination with CPA (Kottke et al., 2009, Cerullo et al., 2011).

Cisplatin is a platinum based chemotherapeutic drug, acting by crosslinking of DNA and causing apoptosis in the cell. In animal studies with murine melanoma (B16F10), a synergistic anti-cancer effect has been shown when combined with reovirus. Combined therapy inhibited the OV-stimulated cytokine and chemokine assembly, however, with no impact on humoral immune responses (Pandha et al., 2009). Cisplatin has improved oncolysis of Herpes simplex virus type 1 (HSV-1) in NSCLC (Toyoizumi et al., 1999). Additionally, combination of Cisplatin with adenovirus facilitated the replication of the virus and significantly reduced the tumor progression (Cheong et al., 2008). Synergistic effects have been also reported in a MPM with NV1066 (HSV-1 based virus), (Adusumilli et al., 2006).

1.6.2.2. Radiotherapy combination

There is an increasing interest in combining oncolytic viruses with radiation therapy, as the mechanism of theses therapies is systematically better understood. It is already known that radiation-enhancement of oncolysis or viruses-caused sensitization of the cancer tissue to radiotherapy has led in synergistic anti-cancer effect in many animal studies (Ottolino-Perry et al., 2010). Combination therapy using Ad2/5 (Geoerger et al., 2003), Ad-delta24 (Idema et al., 2007), Ad-delta24-p53 (Idema et al., 2007), and Ad-delta24-RGD (Ottolino-Perry et al., 2010) with external beam radiotherapy (XRT) in a glioma cancer model led to an increase of 50%-100% in long-term survival. Furthermore, synergistic antitumor effect has been observed when combining adenovirus CV706 with XRT in prostate cancer xenograft model from 7 to 42 days after the treatment, resulting in significant tumor mass reduction and complete response (CR) in 80% of mice. Additionally, XRT combined with CV706 has reduced in serum prostate specific markers compared to monotherapy.

Viral replication is enhanced in the to presence of XRT. It was hypothesized that radiation promotes an increase in cellular GADD34 expression levels, which correlates with replication ratio. This protein protects cells against genetic disruption, including those caused by radiation. Furthermore, XRT enhances the uptake of adenoviruses due to up-regulated expression of coxsackie adenovirus receptor (CAR) and integrin (Ottolino-Perry et al., 2010).

1.6.2.3. Combination with other therapeutic agents

One of the most promising therapeutic approaches in cancer therapy are combination of OVs with immune checkpoint inhibitors. Since it's known that i) inhibition of immune checkpoints is a crucial for efficient immunotherapy and ii) immune responses stimulated by oncolytic viruses exhibit the antitumor effect, this combination can lead to potent clinical

benefits (Rojas et al., 2015), (Figure 8). There is limited preclinical data supporting this approach, however promising clinical data has been registered, such as T-Vec combined with ipilimumab and pembrolizumab. T-Vec in combination with ipilimumab in advanced melanoma patients achieved in an overall response rate (RR) of 50% and a tolerable safety profile. Patients treated with pembrolizumab, 9 out of 16 showed 56.3% response rate with the diseases control rate being 68.8% (reported at 2015 European Cancer Congress).

Adenoviruses, thanks to their efficient cell-entry mechanism, low pathogenicity for humans and well-known biology, are among the most popular delivery vectors in gene therapy (Crystal, 2014). They are commonly used as vaccine vectors as they induce innate and adaptive immune responses (Volpers and Kochanek, 2004). It has been shown that by attaching tumor-specific MHC-I-restricted peptides onto the adenoviral surface (virus works as a vector/adjuvant), it is possible to target immunity towards the tumor, causing a significantly increased anti-cancer efficacy in melanoma (Capasso et al., 2015).

1.7. Anti-cancer effect of L-Carnosine

Carnosine is a natural dipeptide (β-alanyl-L-histidine), (Figure 11), synthesized by Carnosine synthetase. Dipeptide is highly concentrated in muscle, brain tissues, and also present in lungs, kidney, and stomach (Iovine et al., 2012). Since the discovery of Carnosine in 1900, many efforts have been made to determine its biological properties (Gaunitz and Hipkiss, 2012). Some physiological functions have been described, like antioxidant (Babizhayev, 1989, Lee and Hendricks, 1997), anti-inflammatory (Lee et al., 2015), anti-senescence (Wang, 2000), and anti-cancer activity (Iovine et al., 2014).

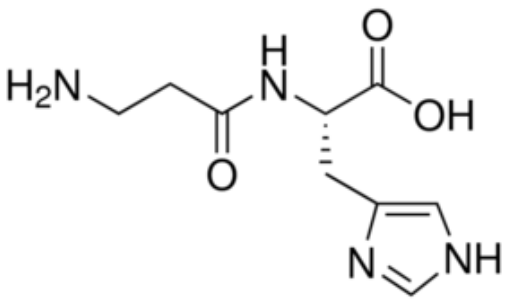

Figure 11. Structure of L-Carnosine. L-Carosine is a strong antioxidant that cleans oxygen free radicals and chelates heavy metals. It inhibits protein-protein and protein-DNA cross-links induced by hypochlorite anions and toxic aldehydes. Modified from (Boldyrev et al., 2013).

Nagai and Suda first reported anti-cancer function of Carnosine in 1986. In their experiment sarcoma-180 cells were implanted into ddYY mice, followed by the treatment with Carnosine (50mg/kg/day) every two days. Dipeptide significantly inhibited tumor progression and increased survival (Gaunitz and Hipkiss, 2012). Renner et al. reported that Carnosine reduced the proliferation of tumor cells obtained from human glioblastoma (Renner et al., 2007) by inhibiting the glycolytic energy metabolism crucial for cancer cells (Renner et al., 2010). The effects of Carnosine were also investigated in human gastric carcinoma cells. The study demonstrated that Carnosine reduced the proliferation of tumor cells by retarding Akt/mTOR/p70S6K signaling (Zhang et al., 2014). Other experiment has shown an ability of Carnosine to inhibit the growth of human HCT116 colon cancer cells. Carnosine (50-100 mM) decreased concentration of adenosine triphosphate (ATP) and reactive oxygen species (ROS), and arrested cell cycle in G1 phase. Findings presented by Iovine et al. supported the hypothesis that Carnosine could reduce HCT116 cell growth via its antioxidant properties, and its ability to affect glycolysis (Iovine et al., 2012). Additionally, the dipeptide might be considered as a potential agent for the treatment of metastatic SK-Hep-1 cells by inhibition of matrix metaoproteinase-9 expression and induction of an anti-metastatic gene, nm23-H1 (Chuang and Hu, 2008).

1.8. Clinical trials with oncolytic viruses

There has been interest in using of oncolytic viruses in cancer treatment for over decades. Previous clinical efforts have concentrated on the safety of oncolytic viruses. However, this trend is nowadays switching to studies focused on treatment efficacy. Today clinical data is available from six, the most well studied oncolytic viruses: adenovirus, reovirus, measles virus, herpes simplex virus (HSV), Newcastle disease virus and vaccinia virus. Generally speaking, these viruses have been safe and well tolerated in clinical setting (Aghi and Martuza, 2005, Pol et al., 2014, Patel and Kratzke, 2013).

OVs went through several peaks and falls of scientific activity and interests during the last century (Kelly and Russell, 2007). Jesse Gelsinger's death after the treatment with non-replicating adenovirus, affected the development of OV gene therapy too (Thomas et al., 2003). However, nowadays the interest in OVs appears to be remarkably increasing. Further evidence of clinical efficacy is still pending, as randomized clinical studies are ongoing. However, the recent approval of T-Vec by EMA and FDA gave additional attention and interest to virotherapy. T-Vec (Harrington et al., 2015) is the first oncolytic vector approved in Western countries and second in the world after Oncorine, (Liang, 2012), (approved by

China's State Food and Drug Administration SFDA in 2005). T-Vec is a genetically modified herpes simplex virus type 1 coding for GM-CSF. Neurovirulence factors ICP34.5 and ICP4 have been removed for selective virus replication and increased immunogenicity (Zamarin and Pesonen, 2015). Clinical efficacy of this virus in advanced melanoma has been demonstrated in Phase II and III of clinical trials (Kohlhapp and Kaufman, 2015). Furthermore, clinical efficacy has been demonstrated in cancer patients with pancreatic, breast and colorectal cancers (Hu et al., 2006). Oncorine is a genetically modified type 5, adenovirus, engineered by Shanghai Sunway Biotech. It obtained regulatory approval for the treatment of head and neck cancer. H101 has been constructed to remove a segment of E1B-55KD responsible for the interaction with a normal human gene p53, which is normally deregulated in many cancers (Garber, 2006).

Over the past years, dozens of oncolytic viruses have been tested in clinical trials with studies mainly concentrated on six viral species listed and introduced in Tables 2-3.

Table 2. Clinical trials with oncolytic viruses.

Virus	Vector's name	Phase	Indication	Route	Clinical outcome	Reference
Adenovirus	ONCOS-102*	I/II	Malignant Solid Tumors	i.t.	- No DLT or MTD was identified for ONCOS-102. - 4 out of 10 patients had disease control based on PET/CT scan at 3 months. - Median overall survival was 9.3 months. - High infiltration of TILs to tumors in 11 out of 12 patients. - High expression levels of genes associated with activated T_H1 type immune profile were detected. - Treatment with ONCOS-102 resulted in infiltration of CD8+ T cells to tumors and up-regulation of PD-L1.	(Vassilev et al., 2015, Ranki et al., 2014a, Ranki et al., 2016)
	Onyx-015*, # (dl1520)	I-III	Lip and Oral Cavity Cancer Head and Neck Cancer Oropharyngeal Cancer	i.v. or i.t. or i.p. or hepatic arterial	- Over 200 cancer patients have been treated. - The virus was generally well tolerated at doses of up to 2 x 10^{12} VP. - No MTD was identified by any route of administration. - Viral replication was tumor-restricted. - Anti-cancer efficacy was shown when combining Onyx-015 with chemotherapy regime.	(Kirn, 2001b, Habib et al., 2001)
	H101*	I-III	Malignant Hydrothorax Non Small Cell Lung Cancer Melanoma Brest cancer Ovarian cancer Gastric carcinoma Rectal cancer	thoracic cavity perfusion	- No DLT and serious adverse events were not observed. - Remarkable anti-cancer effect in 3 out of 15 patients (1 partial response [PR], 2 minor responses [MRs], Phase I). - CR was obtained in 3 patients, PR in 11, and objective response rate (ORR) was 30.4% (14/46, Phase II). - Enhanced anti-cancer efficacy was observed when combining H101 and chemotherapy. - ORR from combining H101 with chemotherapy was higher than in chemotherapy cohort alone (79% vs. 39.6%, Phase III).	(Liang, 2012, Yu and Fang, 2007)
	ICOVIR-7	I/II	Advanced and refractory solid tumors	i.t.	- The treatment was well tolerated with mild to moderate side effects. - ORR was seen in 9 of 17 patients. - 5 of 12 patients had stabilization or reduction in tumor size (1 PR and 2 MRs).	(Nokisalmi et al., 2010)
	KH901	I	Head and Neck	i.t.	- The treatment was well tolerated, with the main toxicity being grade 1/2. - Intratumoral administration of KH901 was associated with biological activity, however further investigation of KH90 is needed.	(Chang et al., 2014)

	OBP-301	I/II	Carcinoma, Hepatocellular	i.t.	- The treatment with OBP-301 was well tolerated. - No DLT was detected after a single intratumoral injection of OBP-301. - All patients had stable disease at the day 28. - 6 patients showed 6.6 to 34% tumor size reduction.	(Sakai et al., 2010, Yano et al., 2015)
	ColoAd1**	I/II	Resectable Colon Cancer, Resectable Non-small Cell Lung Cancer, Resectable Bladder Cancer, Resectable Renal Cell Carcinoma	i.t. or i.v. or i.p.	- no clinical data	(Di et al., 2014, Bauzon et al., 2009)
	Ad5-CD/TK_{rep}	I	Prostate	i.t.	- No DLT and the MTD was not defined. - 94% of the adverse events observed were mild or moderate. - 7 of 16 (44%) patients demonstrated a ≥25% decrease in serum prostate-specific antigen (PSA), and 3 of 16 (19%) patients demonstrated a ≥50% decrease in serum PSA.	(Thompson and Rodriguez, 2007, Freytag et al., 2003)
	CG0070	I-III	Carcinoma, Transitional Cell, Bladder Cancer	i.v.	- Grade 1–2 bladder toxicities were the most typical adverse events. - DLT was not reached. - The CR rate was 48.6%. - In the multidose cohorts the CR rate for the combined groups was 63.6% (14 of 22 patients). - In exploratory patients who received the multidose schedules had an 81.8% CR rate (9 of 11patients).	(Burke et al., 2012, Ramesh et al., 2006)
	DNX2401 (Delta-24-RGD-4C)	I	Brain Cancer, Central Nervous System Diseases, Glioblastoma or Gliosarcoma	i.t.	- DLT was not reached. - Anti-cancer efficacy: 12% of the patients had CR (remarkable complete response). - An overall median survival was 11 months.	(Lang et al., 2014)
	T-Vec (OncoVEX) **	I-III	Melanoma	i.t.	- Tolerable side-effect profile. - The only grade 3 or 4 adverse events occurred in ≥ 2% of treated patients. No fatal treatment-related AEs occurred. - Enhanced durable response rate and a trend toward superior overall survival. - T-VEC is currently being evaluated in combination with other immune therapies (ipilimumab and pembrolizumab).	(Andtbacka et al., 2015, Harrington et al., 2015, Johnson et al., 2015)
	G207	I-II	Supratentorial Neoplasms, Malignant, Glioma, Astrocytoma, Glioblastoma Head and Neck Cancer	i.t.	- The treatment was well tolerated. - The median survival time of G207 inoculation was 7.5 months. - The study showed potential anti-cancer effect of single-dose of G207 with radiation in the treatment of malignant glioma patients.	(Markert et al., 2014, Aghi and Chiocca, 2009)

Herpes simplex virus					- Additional studies with G207 in the treatment of human glioma are recommended. - G207 could be considered for the treatment of head and neck cancer.	
	1716 (HSV-1716)*	I-II	Malignant Pleural Mesothelioma, Rhabdomyosarcoma, Osteosarcoma, Ewing Sarcoma, Soft Tissue Sarcoma, Neuroblastoma, Wilms Tumor, Malignant Peripheral Nerve Sheath Tumor, Clival Chordoma, Non-CNS Solid Tumors, Recurrent Childhood Anaplastic Astrocytoma, Recurrent Childhood Anaplastic Oligoastrocytoma, Recurrent Childhood Gliosarcoma	i.t.	- No DLT was observed - Three patients remain alive and clinically stable at 15, 18 and 22 months post-surgery and HSV-1716 injection. - Microscopic evidence of tumor necrosis after HSV-1716 injection (3 patients).	(Toyoizumi et al., 1999, Mace et al., 2008, Danos, 2008, MacKie et al., 2001)
	HF10**	I-II	Refractory Head and Neck Cancer, Squamous Cell Carcinoma, Skin, Malignant Melanoma, Solid Tumor	i.t.	- The treatment was well tolerated. - 30-100% of histopathological regression in recurrent breast cancer. - SD in 3 patients, PR in 1 patient and progression disease (PD) in 2 patients.	(Kapural et al., 2015, Tan et al., 2015, Luo et al., 2012, Nakao et al., 2011)
Measles virus (MV)	MV-CEA	I	Adult Anaplastic Astrocytoma, Adult Anaplastic Oligodendroglioma, Adult Giant Cell Glioblastoma, Adult Glioblastoma, Adult Mixed Glioma,	i.p.	- No clinical or biochemical evidence of toxicity. - Safety of CNS administration of MV-CEA in glioma patients. - Median follow up, 11 months.	(Galanis et al., 2010, Zhang et al., 2012, Myers et al., 2006)
Newcastle disease virus (NCD)	PV701*	I	Recurrent Salivary Gland Cancer, Recurrent Squamous Cell Carcinoma of the Hypopharynx, Recurrent Squamous Cell Carcinoma of the Larynx, Recurrent Squamous Cell Carcinoma of the Lip and Oral Cavity, Recurrent Squamous Cell Carcinoma of the Nasopharynx,	i.v.	- No DLT was observed. - Mild flu-like symptoms were common following the first infusion. - Tumor regression was observed in a patient with anal carcinoma following palliative radiotherapy. - ORR occurred at higher dose levels, and PFS ranged from 4 to 31 months.	(Pecora, 2002, Prince et al., 2005, Lorence et al., 2007, Hotte et al., 2007)

			Recurrent Squamous Cell Carcinoma of the Oropharynx, Recurrent Squamous Cell Carcinoma of the Paranasal Sinus and Nasal Cavity, Salivary Gland Squamous Cell Carcinoma,			
Reovirus	REOLYSIN *,**	I-II	Pancreatic Adenocarcinoma, KRAS Mutant Metastatic Colorectal Cancer, Carcinoma, Squamous Cell of the Head and Neck, Metastatic Breast Cancer, Metastatic Pancreatic Adenocarcinoma, Carcinoma, Squamous Cell of the Head and Neck	i.v. or i.t.	- Treatment was tolerable and more effective compared to a single agent gemcitabine. - Up-regulation of immune checkpoint marker PD-L1. - 8 of 13 patients in the study had SD for 12 weeks or longer, (CR + PR + SD of 62%).	(Sborov et al., 2014, Mahalingam et al., 2015b, Arnberg, 2012, Villalona-Calero et al., 2015, Mahalingam et al., 2015a)
Vaccinia virus (VV)	JX-594*	I-II	Colorectal cancer, Melanoma, Lung Cancer, Ovarian Cancer, Soft-tissue Sarcoma, Brest Cancer, Neoplasma, Liver, Carcinoma	i.t.	- The treatment was well tolerated. - Anti-tumor efficacy in 3 patients. - Objective intrahepatic Modified Response Evaluation Criteria in Solid Tumors (mRECIST) was 15% and intrahepatic disease control was 50%. - Median survival of 14.1 months.	(Park et al., 2015, Parato et al., 2012, Walther and Stein, 2015, Liu et al., 2008, Heo et al., 2013)

* alone or in combination with chemotherapy
** alone or in combination with immune checkpoint blockade
# alone or in combination with radiation

Table 3. Overview of the oncolytic viruses used in clinical trials.

Virus	Nucleic acid	Characteristic
Adenovirus	DNA	• Non-enveloped, lytic double-stranded DNA virus • Genome of approx. 36kbp • Adenoviruses are classified into 57 human serotypes and into 7 different groups from A to G • Common cold-causing virus • Immunogenic • The most well studied vector in clinical studies among others (vaccination, oncolytic cancer agent, non-replicating gene transfer vector), (Zamarin and Pesonen, 2015, Draper and Heeney, 2010, Jiang et al., 2015)
Herpes		• Lytic, double-stranded enveloped DNA Herpes simplex virus type 1 • Large genome (152kbp), nonessential genes can be replaced up to 30kbp • Can establish latency • Neurovirulence tropism
Vaccinia		• Enveloped double-stranded DNA virus • Genome of 200kbp, allowing insertion of a large transgenes without loss of infectivity • Replicates within the cytoplasm of host cell • Highly lytic • Highly immunogenic • High efficiency of infection • Wild type does not infect tumor cells, however the tropism can be redirected
Measles	RNA	• Negative-stranded enveloped RNA virus • Genome of approx. 16kbp • Causing highly contagious measles disease. MV infections are mostly eradicated in developed and developing countries • Oncolytic measles virus exhibit minimal effect on normal cells (selectively replicates in cancer cells), (Russell and Peng, 2009)
Newcastle disease virus		• No pathogenic for humans • Genome of approx. 15kbp • Replication in tumor cells leads to the expression f viral proteins on the tumor cell surface • Virus stimulates the synthesis of IFN and TNF • Oncolytic activity and fast replication in cancer cells
Reovirus		• Non-enveloped double-stranded RNA virus • Genome of approx. 24kbp • Naturally targeted to tumor cells (replication occurs in cells containing RAS mutation), (Mahalingam et al., 2015b, Maitra et al., 2014) • Highly prevalent in human population, but not associated with any human disease

1.9. Safety considerations and host-adenovirus immune interactions

1.9.1. Adenovirus induced host immunity

Oncolytic viruses, e.g., adenoviruses are immunogenic and therefore the immune system detects them as pathogens (immunogens). First generations of E1/E3 deleted adeno-vectors demonstrated a limitation in potency as OVs in gene therapy due to elevated immune responses (Muruve, 2004). Additionally, Ads can exhibit acute inflammation causing a significant limitation in gene transfer efficacy, and in the worst case destruction of healthy tissue/organ, and even

patient's death (Raper et al., 2003). Ad promotes the innate immune responses causing inflammation of transduced tissues and enhanced clearance of virus (Muruve, 2004). In turn, an adaptive immune reaction can lead to production of anti-adenovirus neutralizing antibodies (Sumida et al., 2005).

1.9.1.1. Innate Immunity

Innate immunity is the first line of defense against invading pathogens, including adenoviruses. The innate immune responses are mediated through i) different pattern recognition receptors (PRRs) in the intracellular and extracellular compartments (cell surface), (Girardin et al., 2002, Wang et al., 2007), and ii) nucleotide-binding oligomerization domain/leucine-rich repeat (NOD-LRR) family of proteins located within the intracellular compartment (Muruve, 2004). The best-known family of such PRRs is Toll-like receptors (TLRs). The most important in Ads infection is TLR2, which is located on the cell surface, and IL-9, which is presented in endosomes (Appledorn et al., 2008). Toll-like receptors counterplay with numerous viral components: viral dsRNA (by TLR3), CpG motifs (by TLR9), glycoproteins (by TLR2 and TLR4), ssRNA (by TLR7 and TLR8), and intercellular viral particles (by TLR3 and TLR9). Infection with adenoviral vectors leads also to antiviral response pathways to DNA viruses. Nucleic acid recognition receptors (sensors) are grouped into RNA (RIG-I, MDA5, TLR3, TLR7, TLR8) and DNA (TLR9, DAI, AIM2), which are further divided into membrane/endosome TLRs or cytosolic. The antiviral detection response involves sensor binding to a target ligand or receptor leading to the activation of transcription factors (NF-κB, AP1, IRF3, IRF7), (Stein et al., 2012). Virus recognition launches adaptor proteins like MyD88 and TRIF (Takeda and Akira, 2015), causing a signal transduction through mitogen-activated protein kinases (MAPKs), and activation of NF-κB. Therefore, the recognition of pathogen-associated molecular patterns (PAMPs) by innate receptors leads to the activation of inflammatory cytokines (e.g., IL-5, IL-6, IL-8, Il-12, TNF-α, RANTES), type I interferon and recruitment of leucocytes. These cytokine responses recruit effector leukocytes: granulocytes, NK cells, macrophages and secrete more cytokines in order to perform cytosolic functions, and boost immune responses (Muruve, 2004), (Figure 10). Within 24 hours post infection, immune responses eradicates around 80% of Ads particles (Worgall et al., 1997).

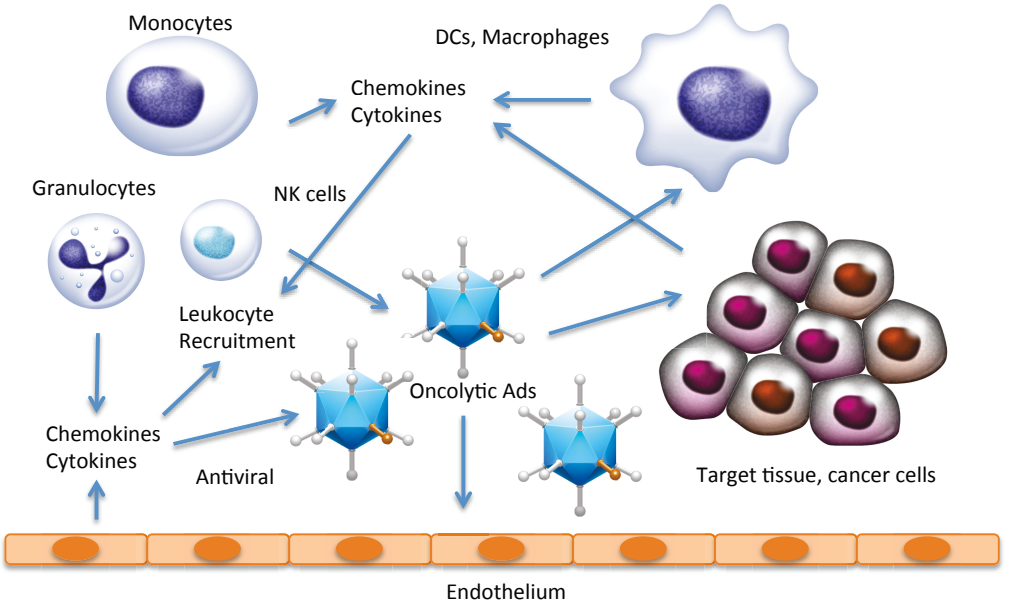

Figure 10. Brief overview of innate immune responses to Ads. Adenovirus vectors transduce many different cell types (e.g. endothelial cells, infects cancer cells). Later on vectors induce inflammatory genes like cytokines and chemokines, leading to recruitment and activation of effector cells (e.g. DCs, macrophages) to the site of infection. The effector cells play an important role in the innate response to adenoviral infection. NK cells and natural killer T cells, via interleukin IL-12 and IL-18, secrete interferon γ, crucial for the development of helper T cell type 1 (Th1) adaptive immune responses. Monocytes/macrophages also secrete antiviral cytokines and present antigens for adaptive immunity. Arrows indicate cascade of interactions between different immune cells, viral particles and cell types. Modified from (Muruve, 2004).

1.9.1.2. Adaptive Immunity

The major agent in establishing adaptive immune responses (humoral and cellular) are viral capsid components: hexon (Sumida et al., 2005), penton and fiber (Yu et al., 2013). Innate immune responses induce local inflammation and recruitment of effector leukocytes leading to activation of adaptive immune system (Muruve, 2004). The activation of the cellular immune responses occurs from four to seven days after the adenoviral infection (Muruve, 2004, Liu et al., 2000). DCs are attracted to the site of infection and play an important role between the innate and the adaptive immune system. When matured after antigen intake DCs can migrate to secondary lymphoid organs and present the antigens (viral and tumor antigens) to T cells. T lymphocytes have ability to identify peptide antigens presented by class I and class II molecules encoded MHC. In classical antigen-presentation scenario, MHC class I molecules present intracellular antigens, whereas MHC class II molecules present exogenous antigens. DCs have a specialized capacity to process endogenous proteins into the MHC class I pathway (cross-presentation). Cross presentation gives the immune system an important mechanism for creating anti-viral immunity. In MHC class II-restricted responses, DCs capture pathogen antigens and present them to MHC class II-restricted helper T cells in the draining lymph nodes. In turn, in MHC class I-restricted responses, DCs uptake antigens

that are endogenously synthesized and present the antigen to class I-restricted-cytotoxic T lymphocytes. Adenoviral antigens can be taken up by DCs and cross-presented with MHC class I molecules to CD8 T cells (Watts et al., 2003). The activation of T cells requires two signals i) antigen specific signal recognized in a context of MHC complex by the T cell receptor, and ii) additional co-stimulatory molecules (B7 (B7-1, B7-2), (Harris and Ronchese, 1999, Watts et al., 2003, Smith-Garvin et al., 2009) present on activated DCs, which bind to CD28 molecules on the T cell surface. The activated T cells can then migrate to the site of infection, and selectively eradicate infected cells. Humoral immune system plays an important role in addition to T cell responses in anti-adenoviral immunity. When B cell detects specific antigen via its B cell receptor, and activating signal from a helper T cell, it matures, proliferates, and transform into a plasma B cell, producing specific antibodies (McHeyzer-Williams and McHeyzer-Williams, 2005). B cells can also became a memory cells and together with memory T cells can mediate long-term immunity against viral infections (Russell and Peng, 2009).

1.9.2. Adverse events

Adenoviruses are common human pathogens and infect the respiratory and the intestinal tract. An infection usually causes symptoms similar to a common cold (sore throat, sneezing, headaches, cough, fever), and can lead to keratoconjunctivitis (Jhanji et al., 2015). Normally the adenoviral infection appears asymptomatic. However, it remains an important cause of mortality after blood and bone marrow transplantation (La Rosa et al., 2001, Soriano and Perales, 2012). Oncolytic adenoviruses are widely used in cancer gene therapy (Nayerossadat et al., 2012).

The risk of serious side effects following adenovirus administration is rare. However, a gene therapy by adenoviral vectors has caused major adverse effects and death of some patients (Marshall, 1999, Raper et al., 2003). Jesse Gelsinger, an 18-year-old patient became the first person that died because of multi-organ failure after his participation in gene therapy research at the University of Pennsylvania. Gelsinger suffered from a metabolic disorder called ornithine transcarbamoylase (OTC) deficiency - inability to metabolize ammonia. In 1999, Gelsinger was given a corrective OTC gene coded by a first generation replication-defective adenovirus. Intraperitoneal administration of high amount of viral particles (1×10^{11} plaque-forming units [pfu]/kg) caused the systemic activation of immune response, leading to a cytokine storm. Within 2 hours, the patient developed fever and signs of liver dysfunction. Next the patient fell into a coma and suffered multi-organ failure (Vorburger, 2002, Raper et al., 2003, Jenks, 2000, Savulescu, 2001). Gelsinger died 4 days after receiving the adenoviral injection. However, the i.t. administration of up to 1×10^{12} viral particles is learned to be safe for patients. The most common

side effects with adenoviral treatment are reported to be light fever, chills, local pain, and diarrhea. Even in cases where an adenoviral load was high and targeted in the liver, only one event of hepatotoxity and no symptoms of hepatitis were detected (Vorburger, 2002). Also a treatment of cancer patient with ICOVIR-7 was well tolerated ($2x10^{10}$ - 1×10^{12} VP). All patients experienced grade 1 or 2 side effects (fatigue, fever, anemia, chills, abdominal pain). The correlation between viral dose and the severity of side effects has not been identified. No grade 4 to 5 side effects were observed during the treatment (Nokisalmi et al., 2010).

In preclinical studies, the adenoviral vector DNA has been found in the liver, skeletal muscle, heart, brain, lung, pancreas, and tumor tissue (Volpers and Kochanek, 2004). When viruses are administered i.v., most of the viral vectors accumulate in the liver. Liver toxicity was highlighted after patient's death after intra-hepatic administration of adenovirus. Importantly, route of virus administration plays a crucial role in virus toxicity and bio-distribution. When virus is administered i.v., Kupffer cells (KC) uptake the vector, leading to necrosis of these cells.

1.9.3. Anti-adenovirus drugs

Adenovirus can lead to acute or lethal infection in immunocompromised persons (Echavarria, 2008). Therefore, the development of novel and more efficient antiviral drugs are in high demand. Nowadays there are available few anti-virus agents against i) herpes, ii) hepatitis, iii) influenza (Razonable, 2011) and iv) HIV viruses (Zhan et al., 2016, Dolgin, 2014). Although lacking FDA approval for treatment for adenovirus infection, there are currently two antiviral drugs available for use in adenovirus therapy: Cidofovir and Ribavirin (Waye and Sing, 2010). Cidofovir is an acyclic nucleoside phosphonate drug with anti-viral properties against DNA viruses (Hoffman et al., 2001). Cidofovir inhibits Ad replication. In turn, Ribavirin is more potent drug towards Ad infections. Like Cidofovir, Ribavirin is a nucleoside analogue drug but its mode of action is still controversial (Waye and Sing, 2010). An example of promising anti-adenovirus drug currently being tested in clinical studies is hexadecyloxypropyl Cidofovir (CMX001), a lipid conjugate of Cidofovir. It exhibites antiviral activity against double-strand DNA viruses, such as Ad 3, 5, 7, 8 and 31 (Toth et al., 2008). CMX001 is orally administrated and is designed to cross the intestinal wall. It binds to target cell before being cleaved to release Cidofovir for antiviral action. A Phase I clinical trial has demonstrated the CMX001 safety profile and bioavailability (NCT00780182). Additionally, another study reported higher antiviral efficacy of CMX001 than Cidofovir, importantly with less toxicity to treated patients (Lion et al., 2003). The drug was also tested in Phase II trials where its efficacy in immunocomprised patients with CMV infection was evaluated (NCT00942305). In this study, 13 immunocompromised patients received CMX001. Virologic

response (VR) was evaluated as a 99% drop from baseline or undetectable adenovirus DNA in patient's serum. No serious side effects were reported to CMX001 during this study. CMX001 may be a promising therapeutic treatment modality for the treatment of severe adenovirus disease in immunocompromised patients (Florescu et al., 2012).

2. AIMS OF THE STUDY

Oncolytic virotherapy is a promising approach to treat cancer. However, this strategy also has disadvantages – mainly insufficient efficacy. This thesis was based on the hypothesis that by combining oncolytic adenoviruses with chemotherapeutic drugs and biological agents, we could improve anti-cancer efficacy through synergistic interactions against cancer.

- **AIM I**: To improve anti-cancer efficacy by combining oncolytic adenoviruses with standard of care chemotherapy (Manuscript I)
- **AIM II**: To improve anti-cancer efficacy by combining oncolytic adenoviruses with experimental anti-cancer agent (Manuscript II)
- **AIM III**: To perform safety assessment of adenoviral vectors (Manuscript I, III)

3. MATERIALS AND METHODS

Materials and methods are described in more detail in the original publications.

3.1. Cell lines (I, II)

Characteristics of the cell lines used in the studies are described in Table 4.

Table 4. Description of the cell lines.

Cell line	Description	Provider/Origin	Study/Manuscript
Jl-1 (ACC 596)	Human epithelioid mesothelioma	DSMZ[1]	I
MSTO-211H (ACC 390)	Human mesothelioma	DSMZ[1]	I
NCI-H226 (H226, CRL-5826™)	Human mesothelioma	ATCC[2]	I
HCT-116 (ACC 581)	Human colon carcinoma	DSMZ[1]	II
A549 (CCL-185™)	Human lung carcinoma	ATCC	II
CCD-112Sk	Human skin fibroblasts	Provided by Dr Santos[3]	II

[1]Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany)
[2]American Type Culture Collection (Manassas, VA, USA)
[3]Helder A. Santos (Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland)

All cell lines were cultured under the conditions recommended by the cell providers.

3.2. Viruses (I-III)

Characteristics of the viruses used in the studies are described in Table 5. Adenoviruses were propagated on A549 cell line and purified on cesium chloride gradients. Viral particle (VP) concentration of adenoviruses was assessed by measuring the absorbance at 260 nm. The number of infectious units (IU/ml) was assessed by immunocytochemistry assay (ICC) on A549 cells. Virus constructs were checked by PCR for the presence of transgenes and genetic modifications (d24, GM-CSF, knob modifications). Details on virus engineering are described in the original publications or in provided references.

Table 5. Description of the viruses used in the studies.

Vrus	Description	Source	Study/Manuscript
Ad5/3-d24-GM-CSF (ONCOS-102)	Human adenovirus, chimera 5/3 (serotype for 3 knob domain), deletion of 24bp in E1A region, coding for human GM-CSF	Targovax Oy (Helsinki, Finland)	I, III
Ad5-d24-RFP	Human adenovirus, serotype 5, deletion of 24bp in E1A region, coding for the red fluorescence protein (RFP)	Provided by Dr Suzuki[1]	II
Ad5-d24-CpG	Human adenovirus, serotype 5, deletion of 24bp in E1A region, coding for the CpG	IVT, HU (Helsinki, Finland)	II

Ad5/3luc1	Human non-replicating serotype 5 adenovirus expressing a reporter firefly luciferase transgene	Targovax Oy (Helsinki, Finland)	III

[1]Dr Masataka Suzuki (Baylor College of Medicine, TX, USA)

3.3. Anti-cancer agents (I - III)

Anti-cancer agents used in the studies are described in Table 6.

Table 6. Anti-cancer agents used in the studies.

Name	Description	Provider	Study/Manuscript
Pemetrexed (Pemetrexed Disodium)	Pemetrexed is a chemotherapeutic drug that prevents the DNA and RNA formation required for the growth and survival of both normal cells and cancer cells	Santa Cruz Biotechnology, Inc., Dallas, TX, USA	I
Cisplatin	Cisplatin is a class of platinum anti-cancer drugs. It crosslinks DNA leading to the cell apoptosis	Santa Cruz Biotechnology, Inc., Dallas, TX, USA	I
Carboplatin	Carboplatin belongs to the class of platinum anti-cancer drugs. Interacts with DNA causing disruption in cell division and mitosis	Santa Cruz Biotechnology, Inc., Dallas, TX, USA	I
Cyclophosphamide	A chemotherapeutic drug, forms DNA crosslinks, leading to cell apoptosis. Importantly Cyclophosphamide launches beneficial immunomodulatory effects in adaptive immunotherapy	Sigma Aldrich, Germany	III
L-Carnosine	Natural dipeptide (β-alanine and L-histidine) exhibiting anti-proliferative and antioxidant properties. Endogenously synthetized in brain, kidney, skeletal muscles	Sigma Aldrich, Germany	II
L-Carnosine-6K	A modified version of L-Carnosine featuring 6 additional lysines at the C-terminus	Gene Cust Europe Laboratoire de Biotechnologie du Luxembourg S.A, Luxembourg	II

3.4. *In vitro* studies (I, II)

Description of *in vitro* assays, experiments and techniques performed in these studies are presented in Table 7.

Table 7. Description of *in vitro* studies.

Assay	Description	Study/Manuscript
Cell viability assay (MTS)	Cells were seeded at 1×10^4 cells per well on 96-well plates. After overnight incubation the cells were infected with ONCOS-102/Ad5-d24-CpG/complex (Ad5-d24-CpG with L-Carnosine and L-Carnosine-6K) with a viral particles/cell ratio	I, II

	of 0,1/1/10/100 (VP/cell). The virus and chemotherapeutic agents were diluted in media containing 5% FBS. Pemetrexed, Cisplatin and Carboplatin were tested at the following sub-optimal, previously selected concentrations of 0,625 mg/ml, 0,0026 mg/ml, 0,0625 mg/ml (H226 cells); 0,625 mg/ml, 0,0006 mg/ml, 0,0019 6 mg/ml (Jl-1 cells); 0,083 mg/ml, 0,0026 mg/ml, 0,0625 mg/ml (MSTO-211H cells), respectively. Cell viability was determined 1/2/3 days later by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) according to manufacturer's instruction (Promega, Madison, WI)	
Analysis of apoptotic and necrotic cell death	Cells were seeded onto 6 well plates at $2x10^5/5x10^5$ cells/well. Cells were infected with 10 VP/cell of ONCOS-102/Ad5-d24-GM-CSF/complex (Ad5-d24-CpG with L-Carnosine and L-Carnosine-6K) and supplemented with chemotherapeutics according to the treatment scheme. The amount of apoptotic and necrotic cells was measured 24/48 hours later with a TACS Annexin V-FITC kit (Trevigen Inc., Gaithersburg, MD) according to manufacturer´s instructions by flow cytometer (LSR II, BD, Franklin Lakes, NJ)	I, II
Immunogenicity of tumor cell death	**CRT exposure**: Mesothelioma cells were seeded in duplicate onto 6 well plates at $5x10^5$ cells/well. Cells were infected with 10 VP/cell of ONCOS-102 and/or with chemotherapeutic agents according to the treatment combinations. 24 (H226, Jl-1) and 48 (MSTO-211H) hours later cells were harvested and stained with 1:1000 diluted rabbit polyclonal anti-Calreticulin antibody (Abcam, Cambridge, UK) for 40 min at 4°C and subsequently with 1:100 diluted Alexa-Fluor 488 secondary antibody (Invitrogen, Carlsbad, CA) and analyzed by flow cytometry (LSR II, BD, Franklin Lakes, NJ) **HMGB-1 release**: mesothelioma cells were seeded in triplicate onto 96 well plates at $1x10^4$ cells/well and infected with 10 VP/cell of ONCOS-102 and/or with chemotherapeutic agents according to the treatment combinations. 72 hours later, supernatants were collected and HMGB-1 was measured with an Elisa kit according to manufacturer's instruction (MBL International, Woburn, MA) **ATP release**: mesothelioma cells were seeded in triplicates onto 96 well plates at $1x10^4$ cells/well and treated as mentioned above. Supernatants were collected after 48 (Jl-1, MSTO-211H) and 72 (H226) hours and analyzed with ATP Determination Kit according to manufacturer's protocol (Promega, Madison, WI) for luminometric analysis (Varioscan Flash, ThermoFisher Scientific, Waltham, MA)	I
Immunocytochemistry assay	Cell lines were seeded in 5 replicates onto 24 well plates at $2x$ $x10^5/3x10^5$ cells/well and treated with different schemes according to treatment. 24 hours later, supernatant was aspirated and cells were fixed by incubation with ice-cold methanol for 15 minutes. The determination of the adenovirus infectivity was based on visual quantification of viral hexon protein in infected cells. Cells were stained with 1:2000 diluted mouse anti-hexon antibody (Novus Biologicals, Littleton, CO) for 1 hour at RT in the dark and subsequently with 1:500 diluted Biotin-SP-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA) for 1 hour at RT in the	I, II

	dark. Subsequently the Extravidin-peroxidase was added at 1:200 and incubated for 30 minutes at RT in the dark (Sigma-Aldrich, Germany). Finally, the infected cells were visualized by adding the stain: DAB up to 5 minutes (Sigma-Aldrich, Germany). For each 5 replicates (wells) 5 images of non-overlapping fields was acquired using an AMG EVO XL microscope (ThermoFisher Scientific, Waltham, MA). Infectivity data is presented as the average number of spots in 5 wells	
Receptor expression studies	CAR, CD46 and DSG2 expression level in H226, JL-1 and MSTO-211H cells was assessed by staining with mouse monoclonal anti-CAR antibody (Santa Cruz Biotech, Dallas, TX); mouse monoclonal anti-CD46 antibody (Abcam, Cambridge, UK) and subsequently with 1:2000 diluted Alexa-Fluor 488 secondary antibody (Abcam, Cambridge, UK); mouse monoclonal anti-DSG2 antibody (Abcam, Cambridge, UK) and subsequently with 1:2000 diluted Alexa-Fluor 488 secondary antibody (Abcam, Cambridge, UK) respectively for flow cytometry analysis (LSR II, BD, Franklin Lakes, NJ)	I
Transduction assay	Cells were seeded at a density of $1x10^4$ cells/well in 96 well plates. On the following day cells were infected using Ad5-d24-RFP (100 VP, 10 VP, 1 VP, 0,1 VP) and virus-L-Carnosine complex (100 VP, 10 VP, 1 VP, 0,1 VP). Red fluorescence was measured by Varioskan plate reader (Varioscan Flash, ThermoFisher Scientific, Waltham, MA) at 24h, 48h, and 72h after the treatment	II
qPCR	qPCR for adenovirus E4 copy number was carried out (primer FW: 5´-GGA GTG CGC CGA GAC AAC-3´, primer RV: 5´-ACT ACG TCC GGC GTT CCA T-3´, probe E4: 5´-[6FAM] -TGG CAT GAC ACT ACG ACC AAC ACG ATC T-[TAMRA] -3´). Total DNA was extracted from tested cells using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Samples were analyzed using LighCycler qPCR machine (LighCycler 480, Roche, Basel, Switzerland). Actin serves as a reference mRNA Total RNA was extracted from HCT116 and A549 cells by using the RNeasy mini kit (Qiagen, Hilden, Germany) and was used to synthesize cDNA. The cDNA was then amplified (Bio-Rad Laboratories) using iQTM SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Actin served as the reference mRNA. The primer sequences were as follows: IL-8 forward: 5'-AGACAGCAGAGCACACAAGC-3' IL-8 reverse: 5'-ATGGTTCCTTCCGGTGGT-3' Actin forward: 5'-CCTCACCCTGAAGTACCCCA-3' actin reverse 5'-TCGTCCCAGTTGGTGACGAT-3'	II
Virus-L-Carnosine complex formation, Zeta Potential and Dynamic light scattering analysis	The virus-L-Carnosine complex was formed by mixing oncolytic adenovirus and Carnosine-6K at ratio 1:500 and incubated at room temperature for 15 minutes using MilliQ H_2O at pH 7.4 as buffer. Zeta potential analysis was performed using $1x10^{10}$ viral particles. All the samples were diluted in a volume of 800 ul of MilliQ H_20 at pH 7.4 and injected with a 1 ml syringe in the capillary flow cell to measure the electric surface charge of the particles. An equilibration time of 120 seconds was set on the software to allow the samples to stabilize at 25°C. Dynamic	II

	light scattering analysis was performed with a ZetaSizer Nano, Malvern (Westborough, MA)	
Western blot analysis	Total extracts of HCT116 and A549 cell lines were probed with antibodies against p62 (2 µg/ml), (mouse monoclonal antibody, Abnova), LC-3 rabbit polyclonal antibody (1:100), (Cell Signaling Technologies, Inc), heat shock protein 27 (Hsp27), (1:1000), (Abcam) and β-actin (1:200), (Santa Cruz Biotechnology). The signals were detected by using the ECL kit (K-12045-D50, Advansta, CA)	II

3.5. Preclinical *in vivo* studies (I-III)

Animal protocols were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland (I, II). Study III was performed in compliance with the OECD Principles of Good Laboratory Practice (GLP) C (97)186/Final, Directive 2004/10/EC. All animals were quarantined for at least one week, and their health status was monitored daily. Animals were euthanized according to local animal care rules and to humane end-point guidelines.

Description of *in vivo* experiments and study-related analysis are presented in Table 8.

Table 8. Description of preclinical *in vivo* experiments and study-related assays.

Animal study	Description	Study/Manuscript
Human mesothelioma xenograft model	The NCI-H226 cells in 50 µl were injected into both flanks (6E+06/flank/mouse). Tumors were let to grow 8 days prior to the treatments. Viruses were administered on every 6 days. One group received ONCOS-102 only, two groups received ONCOS-102 and chemotherapy (Pemetrexed + Cisplatin or Pemetrexed + Carboplatin) simultaneously, while two other groups received ONCOS-102 priming followed by combinatorial treatment of chemotherapy (Pemetrexed + Cisplatin or Pemetrexed + Carboplatin) and ONCOS-102 in 3-day cycles. Mock animals were treated with 0,9% saline. ONCOS-102 was diluted into 0,9% saline and injected intratumorally at a dose of $5x10^7$ VP per tumor (two tumors per animal). Injections were given in a fan-like pattern to ensure even distribution throughout the tumor. Pemetrexed, Cisplatin, and Carboplatin were diluted in 0,9% NaCl and administrated intraperitoneally at doses of 10 mg/kg, 1,5 mg/kg, and 8 mg/kg, respectively. The injection volume was 100µl per chemotherapeutic agent	I
Human GM-CSF ELISA	Protein extracts and previously collected serum were analyzed for human GM-CSF concentration using ELISA (Abcam, Cambridge, UK) according manufacturer's instructions	I
qPCR	qPCR for adenovirus E4 copy number was carried out (primer FW: 5´-GGA GTG CGC CGA GAC AAC-3´, primer RV: 5´-ACT ACG TCC GGC GTT CCA T-3´, probe E4: 5´-[6FAM] -TGG CAT GAC ACT ACG ACC AAC ACG ATC T-[TAMRA] -3´). Total DNA was extracted from BALB/c nude	I

	murine samples (tumors, livers, blood) using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Subsequently isolated DNA was analyzed for adenoviral E4 copy number normalized to murine beta-actin (liver, blood) and human beta-actin (tumor), respectively ((primer FW: 5´-CGA GCG GTT CCG ATG C-3´, primer RV: 5´-TGG ATG CCA CAG GAT TCC AT-3´, probe murine beta-actin: 5´-[6FAM)-AGG CTC TTT TCC AGC CTT CCT TCT TGG-(TAMRA)-3´; (primer FW: 5´-CAG CAG ATG TGG ATC AGC AAG-3´, primer RV: 5´-CTA GAA GCA TTT GCG GTG GAC-3´, probe human beta-actin: 5´-[6FAM)- AGG AGT ATG ACG CCG GCC CCT C-(TAMRA)-3´). Samples were analyzed using LighCycler qPCR machine (LighCycler 480, Roche, Basel, Switzerland)	
Human xenograft model of lung cancer	The oncolytic activity of the Ad5-d24-CpG and virus-L-Carnosine complex was tested in a lung cancer xenograft model. Nude mice bearing A549 cell tumors in the flanks ($1x10^6$ cells/flank injected subcutaneously) were treated intratumorally with $1x10^8$ VP/tumor (or PBS) on days 0, 2, 5 and the tumor growth was followed over time	II
Human colon cancer xenograft model	The oncolytic activity of the Ad5-d24-CpG and virus-L-Carnosine complex was tested in a colon cancer xenograft model. Nude mice bearing HCT-116 cell tumors in the flanks ($1x10^6$ cells/flank injected subcutaneously) were treated intratumorally with $1x10^8$ VP/tumor (or PBS) on days 0, 2, 5 and the tumor growth was followed over time	II
Western blot analysis	Total extracts of HCT116 and A549 cell lines were probed with antibodies against Hsp27 (1:1000), (Abcam, Cambridge, UK) and β-actin (1:200), (Santa Cruz Biotechnology, Dallas, TX, USA). The signals were detected by using the ECL kit (K, Advansta, CA)	II
Toxicity and bio-distribution studies – Syrian hamsters	The study was carried out with 300 hamsters (Syrian hamsters), divided into nine test groups – three groups for bio-distribution and six groups for toxicity analysis. Animals were sorted according to the body weight, and allocated to the dose group. Hamsters received ONCOS-102 in NaCl solution by intracardial, intraperitoneal or subcutaneous injections. Additionally, one group was administered with intraperitoneal injections of Cyclophosphamide in dose of 20 mg/kg. The control animals were administered with NaCl solution without ONCOS-102 in the same volume and way.	III
Hematology and clinical chemistry	Blood samples for hematology (Manuscript III, Table S1) and clinical chemistry (Manuscript III, Table S2) were collected on scheduled time. The animals were fasted for approximately 12-18 hours before blood sampling, but water was provided *ad libitum*. Blood samples were drawn under ether anesthesia from the retro-orbital venous plexus into tubes containing K3-EDTA (hematology), sodium citrate (for coagulation), and into TAPVAL (without anti-coagulant for serum clinical chemistry). Blood samples for coagulation, serum chemistry and analysis of antibodies were centrifuged (4000 rpm for 15 min and 6000 rpm for 10 min, respectively) and serum was transferred into plastic tubes	III
	Whole organs or samples of the collected tissues were preserved in 4% neutral buffered formaldehyde. The eyes, optic nerves,	

Necropsy	testes and epididymitis were fixed in Davidson's fluid and then moved to 4% neutral buffered formaldehyde. Histopathology was performed on days 29, 190 and 256 for selected organs and tissues. Histopathological examination was performed. Tissues from all animals were wax embedded, cut at a nominal thickness of approximately 5µm, stained with haematoxylin and eosin (HE) and examined microscopically	III
Neutralization antibody assay	Ad5/3luc1, a non-replicating serotype 5 adenovirus expressing a reporter firefly luciferase transgene, was used to indirectly quantify blocking activity of the serum on virus infectivity of permissive cells in culture. Known concentrations of rabbit anti-Ad5/3 NAbs were used to quantify the assay	III
qPCR	The number of adenoviral copies (E1 region) and hamster Gapdh sequence were determined in DNA samples. Samples of feces were isolated using NucleoSpin kit, samples of urine and buccal swabs were isolated using NucleoSpin Blood DNA isolation, and serum samples were isolated using NucleoSpin Blood DNA isolation kit according to the manufacturer´s instructions (Macherey-Nagel, Bethlehem, PA). In tissue samples the concentration of adenoviral sequence (primer FWE1: 5´-TCC GGT TTC TAT GCC AAA CCT-3´, primer RVE1: 5´- TCC TCC GGT GAT AAT GAC AAG A-3´, probe adenoE1: 5- ATC GAT CCA CCC AGT GAC GAC-3) was normalized by the concentration of hamster's Gapdh sequence (primer FWGapdh: 5´- CAC CGA GGA CCA GGT TGTC T-3´, primer RVGapdh: 5´-CAT ACC AGG AGA TGA GCT TTA CGA-3´, probe Gapdh: 5-CAA TGC CAG CCC CAG CATC A-3) or by the total amount of DNA	III

4. RESULTS

This chapter summarizes the main results of the thesis. More details can be found in the original publications (I-III).

The scope of this thesis was to: 1) improve efficacy of oncolytic adenovirus in cancer therapy by combining it with other treatment modalities: i) chemotherapy (I) and ii) experimental biological agent (II). The second objective 2) was to perform a viral safety assessment (I, III), (Figure 12).

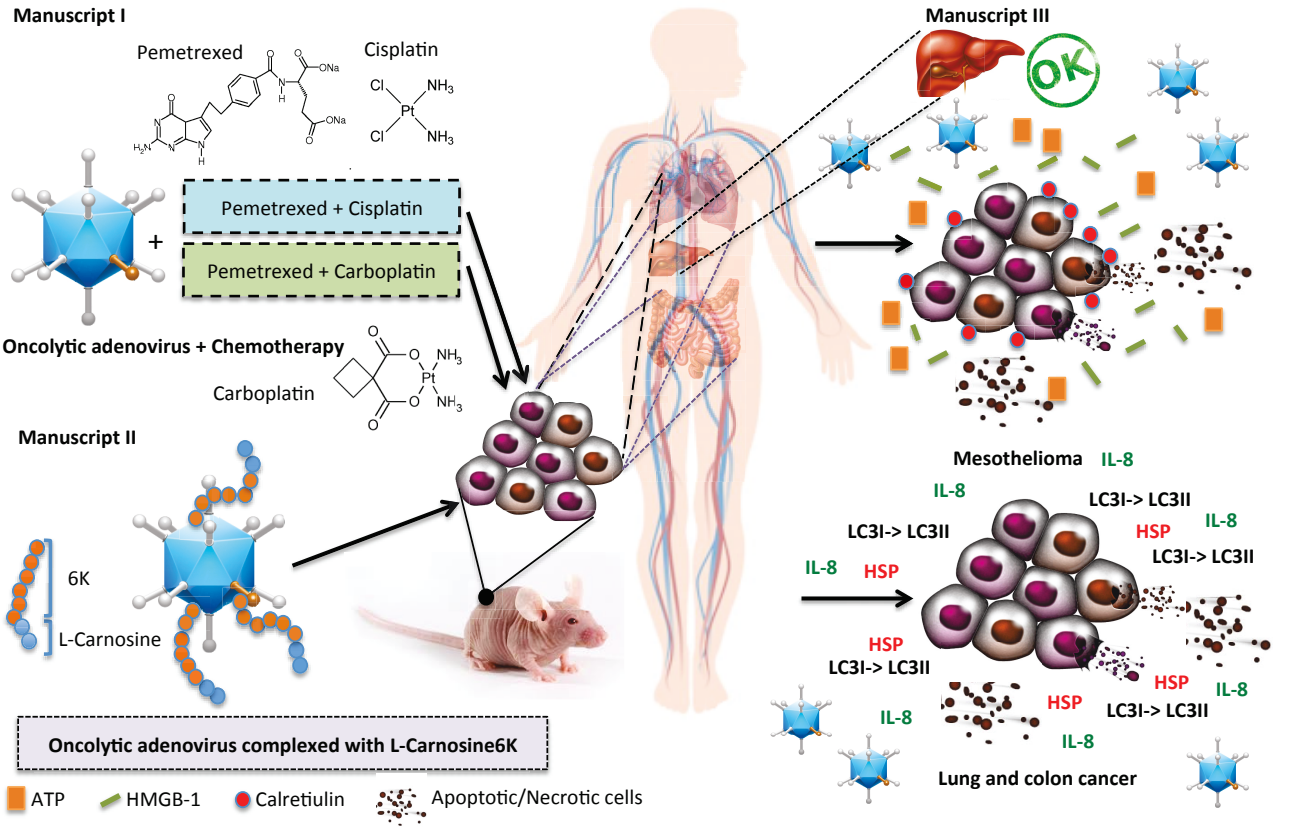

Figure 12. Brief overview of the studies carried out and presented in this thesis. Manuscript I describes synergistic anti-cancer efficacy *in vitro* and *in vivo* by combining ONCOS-102 with standard of care chemotherapy (Pemetrexed+Cisplatin or Pemetrexed+Carboplatin). Manuscript II presents enhanced anti-cancer efficacy *in vitro* and *in vivo* by combining oncolytic adenovirus with L-Carnosine in colon and lung cancer model (II). Manuscript III describes safety assessment of adenovirus vectors in Syrian hamsters and nude BALB/c mice (toxicology, biodistribution, Nabs, GM-CSF production).

4.1. Improved anti-cancer efficacy by combining oncolytic adenoviruses with standard of care chemotherapy (I)

Combinatory studies with oncolytic adenovirus and chemotherapeutic agents in mesothelioma therapy were carried out both *in vitro* and *in vivo*. Oncolytic properties of ONCOS-102 were tested in three mesothelioma cell lines *in vitro* (Fig. 3A, I). JL-1, MSTO-211H and H226 cells appeared to be relatively resistant to oncolysis as 10 VP/cell killed 18%, 24% and 11% of

cells, respectively, in 3 days. JL-1 and H226 cell lines were more resistant to chemotherapy-mediated cytotoxicity compared to MSTO-211H cells (Pemetrexed + Cisplatin or Pemetrexed + Carboplatin). Incubation with chemotherapeutics killed only 10% of JL-1 and 11-12% of H226 cells in 3 days. In contrast, 63% and 73% of MSTO-211H cells were killed by day 3 in culture with Pemetrexed + Cisplatin or Pemetrexed + Carboplatin, respectively. Compared to the results observed in the single-treatment (virus alone and chemotherapy alone), the combination of ONCOS-102 with chemotherapeutics significantly increased cytotoxicity in H226 ($p<0.05$) and JL-1 ($p<0.001$) cells (Fig. 1A, I).

In line with the cell viability results, the number of apoptotic H226 and JL-1 cells was generally low in all treatment groups, but a combination treatment slightly increased the number of apoptotic cells in comparison to monotherapies (Figure 1 B and 1 C, I).

Markers for immunogenic cell death, such as the exposure of calreticulin to cell surface and the extracellular release of ATP and HMGB1, were measured from mesotheolioma cell cultures after exposure to ONCOS-102, chemotherapeutic agents, or combination of both. The most immunogenic tumor cell death (the highest number of extracellular HMGB1, ATP and CRT positive cells) was triggered by treatment with ONCOS-102 + chemotherapy (Pemetrexed + Cisplatin or Pemetrexed + Carboplatin) in H226, JL-1 and MSTO-211H cells (Fig. 2, I).

In order to study the synergy of oncolytic virus and chemotherapeutics on anti-tumor treatment, we performed animal studies. Subcutaneous human mesothelioma H226 tumors were treated according to the treatment regime presented in Table 1 (I). Tumors appeared to be refractory against standard chemotherapeutics (Pemetrexed + Cisplatin, Pemetrexed + Carboplatin), as none of the treatments significantly reduced tumor growth (Figure 4A, I). Chemotherapy alone was the most inefficient treatment modality against mesothelioma. One animal treated with ONCOS-102 + Pemetrexed + Cisplatin showed a complete tumor regression (both tumors) by day 21. In addition, one animal treated with ONCOS-102 priming + Pemetrexed + Cisplatin showed a complete regression of both tumors by day 45. Indeed, this regimen was the most effective with 97% of initial tumor size at day 60 vs. 473% (mock), 563% (Pemetrexed + Cisplatin) and 672% (Pemetrexed and Carboplatin). Additionally, in all combination regimes (ONCOS-102 + chemotherapy) we observed the most significant anti-tumor activity compared to other groups (e.g. initial tumor size: 97% [virus priming + Pemetrexed + Cisplatin], 138% [virus + Pemetrexed + Cispaltin] vs. 206% [virus alone], 473% [mock], 563% [Pemetrexed + Cisplatin] at day 60), (Fig. 4A, I). Importantly, ONCOS-102 combined with chemotherapeutics showed a strong synergistic anti-tumor effect ($R>1$) on day 21, 48 and 60 (Table 2, I).

4.2. Improved anti-cancer efficacy by combining oncolytic adenoviruses with experimental anti-cancer agent (II)

Anti-cancer properties of Carnosine-6K complexed with oncolytic adenovirus was tested *in vitro* and *in vivo* in: i) colon and ii) lung cancer models.

We showed that Carnosine-6K coated viruses (complex) displayed increased transduction efficacy and enhanced infectious titer over virus and Carnosine-6K alone in colon and lung cancer cells *in vitro* (Figure 2, II). Additionally, Carnosine-6K coated adenovirus induced the strongest apoptotic/necrotic cell death in tested cell lines (Figure 4, II). We found that virus-L-Carnosine complex exhibited the most potent anti-cancer properties by enhancing viral replication, inducing autophagy and affecting the expression of Hsp27. In HCT116 cells, Carnosine-6K loaded Ad5D24CpG improved antitumor efficacy by enhancing the viral replication and inducing autophagy. In turn, in A549 cells the complex exhibited the most potent anti-cancer effect through down-regulation of Hsp27, leading to lower expression of IL-8 (Figure 5-6, II).

The oncolytic activity of the virus-L-Carnosine complex was also tested in a lung and colon cancer xenograft models. Nude mice bearing A549 and HCT-116 cell tumors in the flanks were treated intratumorally with $1x10^8$ VP/tumor (or PBS) on days 0, 2, 5 and the tumor grow was followed over time. We observed that the tumor growth was significantly ($p < 0.001$ at day 18) reduced in virus-L-Carnosine complex treated mice compared to the control virus or Carnosine-6K alone (Figure 7 A, 7 B, II). We then calculated the therapeutic synergy between Ad5D24CpG and Carnosine-6K using Fractional tumor volume (FTV) method. We found synergistic effects in A549 mouse treated with complex compared to mice treated with mix (virus and L-Carnosine administered separately). In HCT-116, we found a synergistic effect in both virus-L-Carnosine complex and mix condition, however, the effect was stronger in mice treated with the complex (Figure 7 C, 7 D and Supplementary Table 1, II). Recent studies underline that the Hsp27 expression strongly correlates with poor survival in patients with rectal cancer (Vidyasagar et al., 2012, Tweedle et al., 2010). For this reason we evaluated the expression of Hsp27 in protein extracts obtained from xenograft tumors. Surprisingly, we found that the expression of Hsp27 was dramatically reduced in both xenograft tumors after intratumoral administration of the virus-L-Carnosine complex. (Figure 7 E, 7 F, II).

4.3. Safety assessment of adenoviral vectors (I, III)

Safety assessment was done in different preclinical setups: i) GLP toxicological and biodistribution study before the human clinical trial (III), and ii) as part of the combination study of ONCOS-102 with SoC chemotherapy in a mesothelioma model (I).

The study (III) was carried out in 300 hamsters divided into nine test groups – three bio-distribution groups and six groups for analysis of toxicity. Hamsters received the tested dose of ONCOS-102 in NaCl solution by intracardial, intraperitoneal or subcutaneous injections (Table 1, III). Additionally, one group was administered twice a week with intraperitoneal injections of Cyclophosphamide in dose of 20 mg/kg. The control animals were administered with NaCl solution without ONCOS-102 in the same volume and way. No adverse effects were observed of repeated administration of ONCOS-102 on clinical signs including body weight (Figure 1-2, III), food consumption (Figure 3, III), hematology and clinical chemistry parameters (Table S4 and S5, III), histopathology (Table 2, III) and bio-accumulation (Figure 4-7, III) in the course of 6-month administration and following 3- month recovery period.

In study (I), the weight of the nude mice was followed throughout the experiment (Figure S1 B, Figure S2 B, Figure S3, I). A minor weight loss was observed in the following groups: Pemetrexed + Cisplatin (10% of decrease at day 48, 11% at day 60), ONCOS-102 priming Pemetrexed + Cisplatin (9% of decrease at day 48, 12% at day 60), Pemetrexed + Carboplatin (6% at day 60), (Figure S3, I).

5. DISCUSSION

5.1. Improved anti-cancer efficacy by combining oncolytic adenoviruses with other therapeutic agents

Improved anti-cancer efficacy by combining oncolytic adenoviruses with standard of care chemotherapy

The thesis shows that ONCOS-102 in combination with SoC chemotherapy mediates ICD in a preclinical setting. Combination increased immunogenic cell killing *in vitro*, further suggesting that the virus-induced ICD plays a part in the antitumor T-cell activation observed in humans treated with ONCOS-102 (Vassilev et al., 2015, Ranki et al., 2014a, Ranki et al., 2016). The molecular mechanisms underlying this phenomenon are still unknown, several hypotheses can however, be suggested. The production of adenovirus E1A proteins has been shown to sensitize cancer cells to chemotherapy-induced cell killing. Chemotherapeutics have also been shown to induce immunogenic cell death resulting in induction of anti-cancer immunity and anti-cancer efficacy (Liikanen et al., 2013), which is in line with our observations, where calreticulin exposure and release of ATP and HMGB1 were highest when treating with the ONCOS-102 combined with SoC chemotherapy.

The data also show that combinatory therapy results in synergistic anti-cancer effects and is the most effective treatment regime against mesothelioma tested in this setting. The findings are in line with reported results where ONCOS-102 combined with doxorubicin exhibited a synergized antitumor effect against soft STS in Syrian hamsters (Siurala et al., 2015). Similar results have been shown by combining Ad5/3-delta24 with gemcitabine, resulting in synergistic effects against ovarian cancer *in vitro* and *in vivo* (Raki et al., 2005).

In numerous preclinical studies, the combination of Pemetrexed with Cisplatin has shown activity against human non-small cell lung cancer cells, suggesting potential efficacy in mesothelioma (Vogelzang et al., 2003, Rusch, 2003). In the Phase III trial conducted by Vogelzang et al, an improved overall survival was seen when patients were treated with the chemotherapeutics combination versus single drugs. Despite the improved efficacy by the chemotherapeutic combination, MM is still a lethal disease (Belli et al., 2009). The combination of oncolytic viruses with chemotherapeutic drugs has a potential for enhanced anti-cancer killing efficacy and induction of anti-cancer immunity. Combining ONCOS-102 with chemotherapy can overcome the major obstacle of immune suppressive microenvironment in tumors (Diaconu et al., 2012) due to the immunogenic tumor cell death (Kepp et al., 2011, Wong et al., 2015, Siurala et al., 2015, Kroemer

et al., 2013) and subsequent mediation of anti-cancer immune responses (Gilboa, 1999, Liikanen et al., 2013, Kepp et al., 2011).

Thesis results provide a strong rationale to test the combination of ONCOS-102 and Pemetrexed with Cisplatin/Carboplatin in clinical settings. This strategy could be a potential future treatment option for mesothelioma patients. Study of other chemotherapy combinations could also be beneficial.

Improved anti-cancer efficacy by combining oncolytic adenoviruses with experimental anti-cancer agent

In the second study, we found that the complex formed by the interaction between Carnosine-6K and adenovirus is able to induce apoptosis and necrosis *in vitro* at significantly lower concentrations of Carnosine-6K. Interestingly, the complex exhibited the most potent anti-cancer effect compared to other tested modalities, perhaps because Carnosine-6K used the virus as a carrier to maximize cell entry. This hypothesis was suggested by *in vitro* cell viability assay, where the virus L-Carnosine complex had the highest infectivity titer and transduction rate compared to other gest groups.

It has been reported that the intraduodenal administration of L-Carnosine inhibits the proliferation of HCT116 cells in BALB/c nude mice. *In vivo* data revealed that 1mg/ml of L-Carnosine solution given in the drinking water from 6 to 22 days inhibited tumor growth (Horii et al., 2012). Our studies show that intratumoral administration of the virus-L-Carnosine complex results in significant synergistic suppression of tumor growth compared to other test groups. Most of the tumors were fully eradicated within 18 days after injection. We also found that the expression of Hsp27 dramatically reduced after the treatment with the virus-L-Carnosine complex formulation in both xenograft tumors. This is a promising result as recent studies suggest Hsp27 as a molecular target for inhibition in cancer therapy (Kim and Kim, 2011). The observed synergistic anti-cancer effects with the virus-L-Carnosine complex were based on enhanced autophagy, and on expression of Hsp27. Indeed, it has been reported that adenovirus induces cell lysis through autophagy in order to use autophagy-related vacuoles for the egress (Jiang et al., 2011). LC3I to LC3II conversion observed in HCT116 and in A549 cells has shown that the complex increases autophagy, which strongly promotes virus replication in colon cancer cells (Rodriguez-Rocha et al., 2011, Cheng et al., 2013). In agreement with these results, we found that in the HCT116 cell line, a significant increase of viral particles was detected by qPCR after complex infection.

The heat shock protein expression seems to play a crucial role not only in tumor cell survival and proliferation but also in viral replication and in mediating the viral infection signaling

(Glotzer et al., 2000). Few studies support the idea that cells with higher HSPs expression might have more favorable environment for virus replication (Wang et al., 2010). HSP overexpression may change the expression level of certain genes, including those responsible for adenovirus life cycle in cells, such as CAR, which affects the infection and replication of adenovirus. It is known that HCT116 cells express high level of Hsp27 (Hayashi et al., 2012). Indeed, we found that Hsp27 levels were higher in HCT116 compared to A549 cell line. The high basal expression of Hsp27 might favour the environment for virus replication and also exert anti-apoptotic function, helping the virus to form new virus particles in an early phase of virus-L-Carnosine complex infection. These results are in agreement with the hypothesis that HSPs expression enhances the oncolytic effect of replicative adenovirus (Wang et al., 2010).

Since it is known that in lung tumor cells Hsp27 expression is correlated with cancer cell resistance against apoptosis (Lelj-Garolla et al., 2015), we hypothesized that the replication of the virus-L-Carnosine complex in A549 cells can lead to reduction of Hsp27 levels. Down-regulation of Hsp27 is able to reduce IL-8 expression (Rajaiya et al., 2012). Endogenous expression of IL-8 has been found in various human cancers, including colon and lung cancers. Evidence shows that IL-8 biological activity may contribute to cancer progression, and in other circumstances induce an anti-tumor response. In our studies, a significant decrease of IL-8 mRNA take place only in A549 cells, which over-expresses EGFR. As IL-8 is involved in tumor cell proliferation via EGFR, the IL-8 decrease might explain the better antitumor effect of the complex (Shi et al., 2014, Diaz et al., 2010).

This therapy could be a potential future treatment of lung and colon cancer. Additionally, this virus-platform can be used in future studies for delivery of other bioactive drugs, and antibodies, as well as a novel strategy in cancer therapy.

5.2. Safety assessment of adenoviral vectors

Treatment with ONCOS-102 did not cause any major adverse effects on body weight, food consumption, hematology and clinical chemistry parameters in tested groups compared to control animals during and after the treatment period. These results are in line with extensive clinical and non-clinical evidence demonstrating the good safety profile of adenoviruses. Hundreds of cancer patients have been treated with replication competent serotype 5 adenovirus, Onyx-015, in numerous clinical trials (Nemunaitis et al., 2001, Kirn, 2001a). Onyx-015 was generally well tolerated at doses of up to 2×10^{12} viral particles by intratumoral, intraperitoneal, hepatic arterial and intravenous administration. No DLTs were identified by any route of administration. Flu-like symptoms have been the most typical toxicities (Kirn, 2001a). Similar findings with no DLTs have

been reported in a Phase I study of 18 melanoma patients receiving combination therapy with T-VEC and anti-CTLA-4 antibody (Turnbull et al., 2015). No dose-limiting toxicity or MTD has been noticed as well in Phase I clinical trial of intratumoral infusion of reovirus for the treatment of recurrent malignant gliomas (Kicielinski et al., 2014). Thousands of patients have also been treated in numerous trials testing adenovirus and importantly all studies were without any major virus-related complications, and the assessment of DLT and MTD has not been identified in any of the trials (Wollmann et al., 2012). The safety of adenoviral cancer gene therapy has been very well studied and concluded as a patient-safe therapy (Koski et al., 2009, Freytag et al., 2007, Lubaroff et al., 2009). Our obtained findings agree with the previous studies that adenovirus administration is safe.

6. SUMMARY

We tested various treatment combinations with the overall goal being improvement of efficacy of oncolytic virotherapy (I, II) and safety studies of adenoviral vectors (I, III). With the first goal in mind, we studied two setups: i) oncolytic adenovirus + SoC chemotherapies and, ii) oncolytic adenovirus complexed with L-Carnosine.

In study (I) we tested the anti-cancer activity of combination treatment with SoC chemotherapy (Pemetrexed, Cisplatin, Carboplatin) and ONCOS-102 in xenograft BALB/c model of human MM. We showed that oncolytic adenovirus is able to induce ICD of human mesothelioma cell lines *in vitro* and anti-tumor activity in H226 MPM xenograft model. Chemotherapy alone showed no anti-tumor activity in the mesothelioma model. However, a synergistic anti-tumor effect was seen when ONCOS-102 was combined with chemotherapy regimens.

In study (II) we have tested anti-cancer properties of the biological drug L-Carnosine complexed with oncolytic adenovirus. The virus-L-Carnosine complex demonstrated improved anti-tumor efficacy *in vitro* and *in vivo*. In HCT116 colon and A549 lung cancer cells the complex presented a higher transduction level and infectious titer over uncoated oncolytic adenovirus. The *in vivo* efficacy of the complex was tested in lung and colon cancer xenograft models, in which it exhibited a significant reduction in tumor growth over virus and L-Carnosine alone. Additionally, we investigated the molecular mechanisms underlying the efficacy of virus-L-Carnosine complex and found that it induced apoptosis in both cells lines by enhancing viral replication and affecting the expression of Hsp27.

The second aim of the thesis was to perform a safety assessment of adenovirus vector in i) GLP toxicological and bio-distribution study of repeated administration of ONCOS-102 in hamsters (III), ii) and as part of the combination therapy study of ONCOS-102 with SoC chemotherapy in a mesothelioma model (I). The study (III) was carried out in 300 hamsters organized into nine test groups – three bio-distribution groups and six groups for analysis of toxicity. Repeated administration of ONCOS-102 showed no side effects on clinical signs including body weight, food consumption, hematology, clinical chemistry parameters, histopathology and bio-accumulation in the course of 6-month administration and following 3-month recovery period. Study (I) did not show any major adverse effects of combined administration of ONCOS-102 with SoC chemotherapy on clinical signs, such as body weight and bio-distribution, in the 2-months administration period in tested mice.

7. CONCLUSIONS

Study results show synergism *in vitro* and *in vivo* and provide a strong rationale to test the combination of ONCOS-102 and Pemetrexed with Cisplatin/Carboplatin in clinical settings. This treatment strategy could be a potential future treatment for mesothelioma patients. Additionally, tested combinations of ONCOS-102 and SoC chemotherapy seem to be a very promising setups for clinical development, supporting the study of other chemotherapy combinations with the virus in different cancer types (I).

The virus-L-Carnosine complex demonstrated significant anti-tumor efficacy both *in vitro* and *in vivo* compared to the virus or Carnosine-6K alone in colon and lung cancer models. This strategy could be a potential future treatment of lung and colon cancer. Additionally, this virus-platform can be used in future studies for delivery of other bioactive drugs, antibodies, and as novel strategy in cancer therapy (II).

All obtained findings indicate that studied adenoviral vectors and combination therapies are safe. Thus, it is possible to use these treatment strategies in clinical settings, without added safety concerns (I, III).

8. FUTURE PROSPECS

We are entering an exciting stage in the development of onco-immunotherapy. The discovery of checkpoint inhibitors such as anti- CTLA-4, PD-1 and PD-L1 resulted in tremendous tumor regression in cancer patients. However, it has been well reported that the blockade of immune checkpoints alone is seldom curative (Ai and Curran, 2015), but it has the capacity to synergize with other approaches to activate anti-cancer immune responses (Vile, 2014). One of the most exciting and scientifically well-justified combination could be oncolytic viral therapy with checkpoint inhibitors. Indeed, a few clinical trials combining the oncolytic HSV T-VEC with anti CTL-4 anti PD-1 have been proposed, however, no results have been published yet.

Oncolytic virus immunotherapy is also entering an exciting phase of their application, the FDA and EMA have recently approved T-VEC. The safety and anti-cancer efficacy of T-VEC as a monotherapy has been shown in many clinical trials (Kaufman et al., 2016, Ott and Hodi, 2016). Combination therapies, utilizing conventional and novel therapies, will be the future promise of cancer treatment. It has already been reported that oncolytic viruses have the potential to present additive or even synergistic effects with various treatment modalities (radiotherapy, chemotherapy, biological drugs), (Siurala et al., 2015, Li et al., 2007, Dilley et al., 2005). Importantly, localized oncolytic virotherapy can overcome tumor resistance to immune checkpoint blockade therapy and enhances anti-cancer immune responses (Zamarin et al., 2014). Numerous candidates of new immune checkpoint inhibitors are currently under clinical development targeting molecules other than CTLA-4 (Wolchok and Saenger, 2008) or PD-L1/PD-1 (Mahoney et al., 2015, Ott et al., 2013, Nghiem et al., 2016). It would be very interesting to check if synergy could be established with antibodies targeting LAG-3 or TIM-3 with oncolytic adenoviruses. Additionally, triple combinatory studies with oncolytic adenoviruses, chemotherapy and checkpoint inhibitors should be studied as well. Finally, currently tested treatment options in cancer therapies are shifting to tailored personalized medicine. However, there is lack of specific biomarkers indicating the proper therapeutic response in many cancer types, giving a window for the discovery of unmet needs.

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Part C

Original Publications