# UNIVERSITA' DI NAPOLI FEDERICO II

**DOTTORATO DI RICERCA**
**BIOCHIMICA E BIOLOGIA CELLULARE E MOLECOLARE**
**XXVII CICLO**

# Development of a model based on oncolytic adenovirus loaded with L-carnosine as a drug delivery system for cancer therapy

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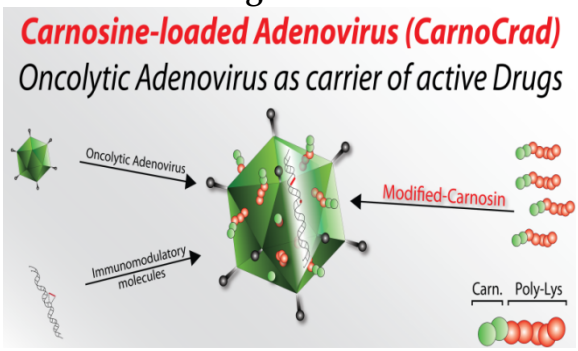


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**Academic Year 2014/2015**

# ACKNOWLEDGMENTS

My deepest appreciation to Professor Maria Assunta Bevilacqua and Dott.ssa Barbara Iovine, for welcoming me in their research group, their full support and untiring guidance.

My heartful gratitude to Professor Vincenzo Cerullo who gave me the opportunity to join his research group in Helsinki, for the patient guidance, stimulating discussions and inspiring advice.

I would also like to thank Professor Gennaro Piccialli for the persistent guidance and encouragement that he has provided during my studies.

My appreciation to Professor Paolo Arcari, coordinator of the Doctoral School for his kindness.

My sincere thanks goes to my dear friend and colleague Lukasz Kuryk for his exceptional kindness, persistent guidance and unconditional help to make my project a success. Without his help the thesis would not be done so smoothly.

My appreciation also goes to Gloria for her support and costant optimism that helped me during the darkness.

Thank you also to Mari, Cristian, Dimitri, Andrea and all people from Centre for Drug Research and Department of Pharmaceutical Bioscience (Helsinki) for their technical advice and more importantly their friendship.

To Marco, you have been there for me since the beginning of this journey and your love, encouragment, patience and support has kept me going through the good times and the bad.

Finally, thanks to my parents, Maura and Gabriele who have always believed in me and who have always supported me even from a distance

“Some things need to be believed to be seen”

-Guy Kawasaki

## Riassunto

I virus oncolitici sono un tipo virus che in maniera specifica replicano, infettano e distruggono solo le cellule tumorali. Numerosi studi clinici hanno dimostrato che il solo approccio oncolitico non può distruggere in modo efficace una grossa massa tumorale, limitando, quindi, l'efficacia di una viroterapia. La combinazione di adenovirus oncolitici con farmaci chemioterapici ha fornito promettenti risultati grazie all'azione sinergica del virus con un farmaco ed è considerato come un potenziale approccio per la terapia contro il cancro. In questo studio, abbiamo messo a punto un modello che utilizza adenovirus oncolitico come "*scaffold"* per veicolare farmaci attivi. Abbiamo, prima di tutto, sviluppato una strategia per coniugare peptidi sul capside virale, che si basa su interazioni elettrostatiche. Sulla base di risultati da noi ottenuti precedentemente, abbiamo utilizzato il dipeptide L-carnosina (β-Ala-His). L-carnosina è un dipeptide naturale con molteplici funzioni biologiche, tra le quali anche una significativa attività antiproliferativa sia in vitro che in vivo. La molecola di L-carnosina, caricata positivamente per l'aggiunta di una coda di sei lisine (Carnosina6K), è stata combinata con la superficie di un virus oncolitico, caricato negativamente, consentendo così la formazione di un complesso (Ad5D24CpG-Carnosine6K). Utilizzando differenti linee cellulari, abbiamo osservato che 100vp del complesso, rispetto ad un adenovirus non coniugato, riducono in maniera significativa la proliferazione cellulare e la produzione di ATP. Nelle stesse linee cellulari il complesso aumenta l'efficienza di trasduzione ed il titolo infettivo del vettore virale rispetto ad un adenovirus oncolitico non coniugato. Infine, l'attività oncolitica del complesso è stata analizzata in un modello xenografico di tumore del polmone e del colon. Topi nudi, nei quali cellule neoplastiche A549 e HCT-116 sono state impiantate sottocute nei fianchi, sono stati trattati a livello intra-tumorale a 0, 2 e 5 giorni con $1x10^8$ particelle virali. La crescita

tumorale è stata seguita nel tempo ed i risultati ottenuti dimostrano una riduzione del tumore nei topi trattati con il complesso rispetto a quelli trattati con il solo virus o con Carnosina6K. Successivamente abbiamo investigato i possibili meccanismi molecolari responsabili degli effetti del complesso sulla riduzione della crescita tumorale. I risultati ottenuti suggeriscono che il complesso (del virus oncolitico con la L-carnosina modificata) induce apoptosi e determina un aumento dell'autofagia (LC3), migliorando l'efficacia del processo di oncolisi mediato dall'adenovirus. In conclusione, il presente modello, una volta validato, potrebbe rappresentare una nuova strategia per il rilascio di un farmaco nella terapia contro il cancro.

## Abstract

Oncolytic viruses are viruses that are able to replicate specifically and infect and destroy only tumor cells. Many clinical studies have shown that the oncolytic approach alone could not efficiently destroy the large tumor mass, thus by limiting an efficacy virotherapy. Combination of oncolytic adenoviruses (Ads) and chemotherapeutic drugs has shown promising therapeutic results due to the synergistic action of virus and drug and is considered as a potential approach for cancer therapy. In the present study, we have optimized a model to use oncolytic adenovirus as a scaffold to deliver active drugs. First, we have developed a strategy to conjugate peptides on viral capsid, based on electrostatic interactions. We have utilized the L-carnosine (β-Ala-His) dipeptide, according to our previous results. L-carnosine is a naturally occurring histidine dipeptide, with a number of biological functions, including a significant antiproliferative activity both in vitro and in vivo. L-carnosine positively charged, consisting of a tail of six lysines (Carnosine6K), has been combined with the surface of oncolytic adenovirus that is negatively charged, to allow the formation of a complex (Ad5D24CpG-Carnosine6K).

We have observed using different cancer cell lines that complex at 100vp, is able to reduce significantly cell proliferation and ATP production compared to an uncoated oncolytic adenovirus. Finally, the oncolytic activity of the complex was tested in a lung and colon cancer xenograft model. Nude mice bearing A549 and HCT-116 cell tumors in the flanks were treated intra-tumorally and each tumor was treated with $1x10^8$ VP. The tumor growth was followed over time, our results show a reduction in tumor growth in mice treated with complex compared to the mice treated with virus alone or Carnosine6k. Later, we have investigated the molecular mechanisms underlying the effects by oncolytic virus coated with modified L-

carnosine on tumor growth reduction. Our results suggest that the oncolytic virus coated with modified L-carnosine induces apoptosis, increases autophagy (LC3), by improving the efficacy of Ad-mediated oncolysis. In conclusion, once validated the proposed model could be used as a novel drug delivery system for cancer therapy.

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## Abbreviations

Ad: Adenoviruses

AGEs: Advanced glycosylation end products

ATP: Adenosine Triphosphate

Carnosine6K: Carnosine with six lysines

CHIP:C terminus of HSC70-Interacting Protein

CN1: Serumcarnosinase

CN2: Tissuecarnosinase

CpG: Cytosine-Phosphate-Guanine

DHAP: Dihydroxyacetone-phosphate

DSL: Dynamic light scattering

ERK1/2: Extracellular signal-regulated kinase

G3P: Glyceraldehyde-3-phosphate

GM-CSF: Granulocyte-macrophage colony stimulating factor

HPF: Human fetal fibroblasts

HSP: Heat shock protein

HSV-1: Simplex Virus 1

ITRs: Inverted terminal repeats

KRAS: Kirsten rat sarcoma viral oncogene

LC3: Phosphatidylethanolamine conjugate

MAPK: Mitogen-activated protein kinase

PEG: Polyethyleneglycol

pRb: Retinoblastomaprotein

RFP: Red fluorescent protein

ROS: Reactive Oxygen Species

TLR9: Toll-like receptor 9

TLR9: Toll-like receptor9

UROD: Uroporphyrinogen decarboxylase

VSV: Vescicular Stomatitis Virus

# 1 Introduction

Cancer is a neoformation of cells that grow uncontrolled and is independent compared to the normal tissue of origin. A tumor can be caused by mutations in genes that control cell growth, alterations of genetic nature (which convert a proto-oncogene into an oncogene), or by infection by oncogenic viruses. The cell, as a result of these events, is not able to control the correct way of its growth and its differentiation. Moreover cancer is a metabolic disorder producing energy to survive when resources are limited and diverting metabolic intermediates in pathways that promote cell proliferation (Porporato et al., 2011). The tumor cells use the intermediates of the glycolytic process for the synthesis of anabolic compounds essential to cell survival. They use glucose 6-phosphate for the synthesis of glycogen and ribose 5-phosphate, dihydroxyacetone phosphate for the synthesis of triglycerides and phospholipids, and phosphoenolpyruvate to pyruvate and the formation of numerous amino acids. This anabolic activity is also favored by an increase in the expression of a particular isoform of pyruvate kinase (PKM2), responsible for the accumulation of intermediates of glycolysis.

Cancer is a major cause of death globally. Despite the improvements made in recent years in conventional cancer treatments, the number of cancer cases is still on the rise (Eaton, 2003). Although treatments have improved significantly in the last decades, conventional chemotherapy or radiotherapy still have limited effects against many forms of cancer, not to mention a lot of treatment-related side effects. Novel more effective and less toxic therapeutic strategies are needed to improve the treatments. To address these issues, numerous attempts are underway. All these are driven by an improved understanding of the molecular basis of cancer in order to improve treatment strategies. It has been showed that monotherapy with oncolytic adenovirus can't eradicate tumour mass. Therefore nowadays many effort has been put

to combine oncolytic adenoviruses with chemotherapy, radiation and immunotherapy. Cancer microenvironment is highly immunosuppressed, therefore its important to not only to focused on cancer direct lysis (oncolytic adenovirus leads to direct lysis of cancer cells), but above all immunostimulation of the host immune system (immunotherapy). Therefore new treatment modalities are highly needed.

## 1.1 *Virotherapy*

Virotherapy is the name of the approach that enables the use of viruses as therapeutic tools in the experimental medicine. This approach consists of genetically modifying viruses in a such a way that they lose some functions and acquire some other ones. It utilizes genetically engineered viruses for selective infection and killing of tumor cells while leaving normal cells relatively unarmed. They achieve this by a number of mechanisms, including direct lysis, apoptosis, expression of toxic proteins, autophagy and shutdown of protein synthesis, as well as the induction of anti-tumoral immunity.
Indeed, virotherapy can be split into three main branches:
- The development of oncolytic viruses, to selectively kill cancer cells and to be used thus as antitumor agents.
- Gene therapy, that involves the use of different viruses and techniques to deliver genes or factors that change the gene expression of the host cells and tissues to cure genetic diseases and other disorders. It is a form of molecular medicine that promises to provide new treatment for a large number of inherited and acquired human diseases and is used where conventional clinical procedures are less effective.
-Viral immunotherapy is a third branch where viral vectors deliver immune system-targeting factors to change the immune responsiveness of the hosts. Thus, new vaccines may be generated and

many diseases may be cured with the immunotherapy is an experimental approach to treat cancer.

The origin of oncolytic virotherapy comes from rare anecdotal reports of cancer patients who experience a temporary remission after contracting a viral infection. These cases usually involve patients with hematologic malignancies, associated immunosuppression and a subsequent naturally acquired infection such as influenza, chicken pox or measles. The obvious hypothesis followed that optimized intentional viral inoculation could be a cure for cancer. Clinical trials of several naturally-occurring oncolytic viruses were started back in the 1950s. Early work in the area was greatly affected from a lack of standardization of study protocols (Moore, 1954). An example, clinical trials were conducted using serum from viremic blood donors (Hoster et al., 1949; Taylor, 1953). In spite of these challenges, some of these early clinical studies established transient responses with only a few mild side effects and suggested the potentiality of oncolytic virotherapy (Norman et al., 2001). The renaissance of this field in recent years came from advances in knowledge about the interactions between virus and host at the molecular level, combined with the possibility of genetic engineering of the viruses.

Viral oncolysis (also termed virotherapy) has been studied intensively over the past two decades to develop a new strategy for cancer therapy (Pennisi, 1998).

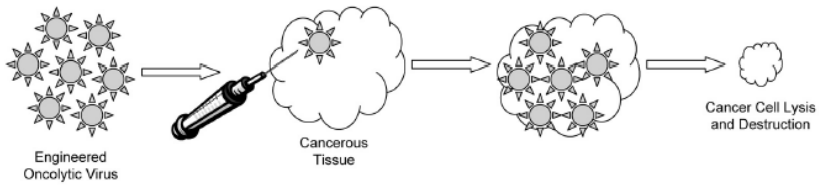


**Figure 1 Schematic diagram of oncolytic virotherapy** (Cross and Burmester, 2006). The intratumoral injection of an oncolytic adenovirus leads to the eradication of the tumor.

Many different viruses have been investigated for their potential use as virotherapy agents, including influenza, Herpes Simplex Virus 1 (HSV-1), Vesicular Stomatitis Virus (VSV), Adenovirus, Vaccinia Virus, Coxsackie Virus, Measles Virus and Poliovirus. The potential of oncolytic virus, once limited by the limited knowledge of virus properties, is now surpassed by the progress in the fields of virology, immunology and cancer is no longer limited by the properties of the viruses in nature. The new oncolytic viruses are engineered to overcome limits related to efficacy and safety of these therapeutic agents.

### *1.2 Adenovirus structure, life cycle and genome*

Adenoviruses (Ads) were isolated in 1953 from human adenoid tissue samples in culture undergoing spontaneous regression (Rowe et al., 1953). Since then Ads have become the most widely used for gene delivery and therapy also due to the efficient production.

They are common opportunistic pathogens rarely associated with severe clinical symptoms in healthy adults (Lenaerts et al., 2008). It is well known that Ads are able to infect humans via the respiratory, the fecal-oral, or the ocular conjunctival routes. Illnesses are generally

mild in immunocompetent humans, but can spread and cause disease in patients with compromised immunity, such as AIDS patients and transplant recipients (Kojaoghlanian et al., 2003). Adenoviruses belong to the family of Adenoviridae, which is subdivided into four genera (Siadenovirus, Aviadenovirus, Atadenovirus, and Mastadenovirus) (Davison, A. J et al; 2003). All human adenoviruses belong to the genera of Mastadenovirus. Adenoviruses are icosahedral, non-enveloped viruses with a dsDNA genome varying between 25-45 kilobases in size with inverted terminal repeats (ITRs) at both termini and a terminal protein attached to the 5´ends (Russell, 2000). The icosahedral capsid is formed by three major proteins, of which the hexon trimers are most abundant (**Figure 2**) (Nemerow et al., 2009). Each of the twelve vertices of the capsid also contains a pentameric protein, a penton base that is covalently attached to the fiber. The fiber is a trimeric protein that protrudes from the penton base and is a knobbed rod-like structure. Each has an N-terminal tail, a shaft with repeating sequences, and a C-terminal knob domain with a globular structure. The knob domain is mainly responsible for binding the target cellular receptor and its globular structure presents a large surface for lateral and apical binding. The fiber proteins of adenoviruses from different subgroups most distinctively differ in length and ability to bend. As in many other nonenveloped viruses, Ad virions are mainly constituted of proteins and DNA, and also some carbohydrates can be found (Russell, 2000).

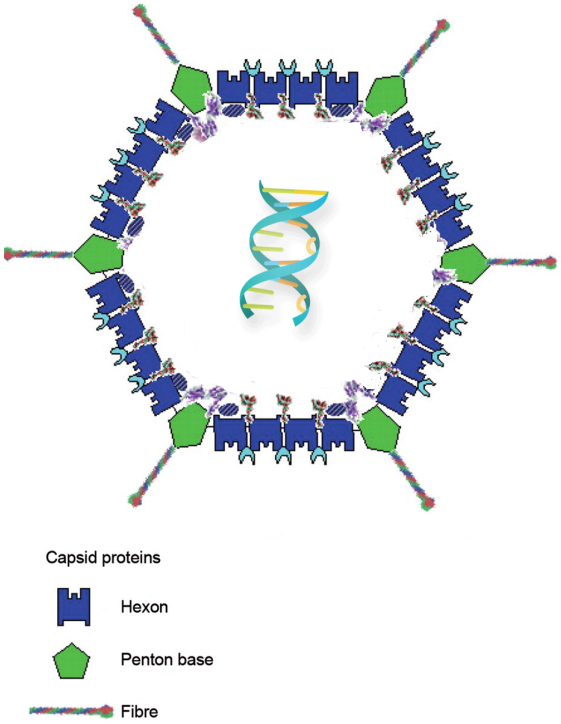


**Figure 2 Structure of Adenovirus.** Adenoviruses are non-enveloped particles of 70-90 nm in diameter with an inner nucleoprotein core. The double stranded DNA is packaged within an icosahedral protein capsid. The major protein of the capsid is the trimeric hexon that constitutes the 20 triangular faces of the icosahedron. Penton capsomeres, formed by the protein of the penton base and fiber, are localized at each of the 12 vertices of the Ad capsid and to which the 12 protruding fiber homotrimers attach(modified fromRussell 2009).

Adenovirus life cycle can be characterized by five stages: binding, entry, escape, translocation, and nuclear transport. Initially the fiber knob binds a primary receptor with high affinity and once the virus is tethered on the cell surface, low affinity binding to secondary receptors leads to internalization (Wickham et al., 1993). The internalization via dynamin dependent chlatrin-mediated endocytosis. Escape from the endosome into the cytosol occurs within minutes and is dependent on the acidification of the endosome, this triggers

changes in the adenoviral capsid resulting in the lysis of the endosome membrane. Once in the cytosol, adenoviral capsids translocate towards the nucleus along the microtubules by interacting with cellular molecular motors, such as cytoplasmic dynein. Thereafter, the adenoviral genome is transported inside the nucleus via nuclear pores (**Figure 3**).

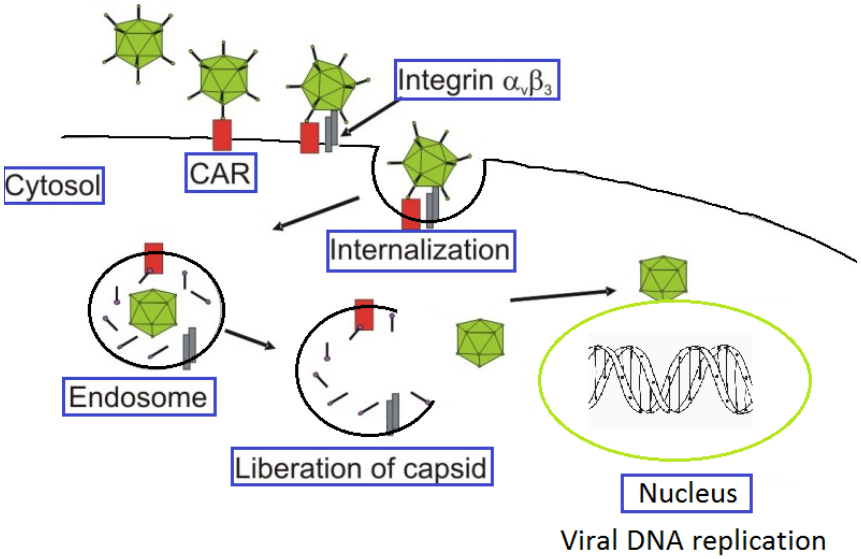


**Figure 3 Adenoviral replication cycle**. Viruses first attach to the coxsackie adenovirus receptor (CAR) followed by an interaction with cellular integrins resulting in internalization of the virus via receptor- mediated endocytosis. In the endosomes, the viral genome is released from the viral capsid and there after transported into the nucleus for DNA replication. Structural viral proteins assemble together with viral genomes in the nucleus followed by cell lysis and release of newly synthesized virions (modified from Bryan E et al; 2013).

The human Ad replication cycle has been mainly studied in Ad2 and Ad5 serotypes as models and so far it has been found equivalent to the other serotypes. The viral replication cycle is divided into two phases that are separated by the onset of viral replication. The early phase starts with the viral infection with the host cell and also includes adsorption, penetration, movement of partially uncoated virus to a

nuclear pore complex, transport of viral DNA through the nuclear pore complex into the nucleus and finally expression of an early set of genes. Early viral gene products mediate viral gene expression, DNA replication, cell cycle progression, and block of apoptosis and antagonize a variety of host antiviral measures. The late phase begins with expression of late viral genes and assembly of progeny virions.

### *1.3 Adenoviral genome*

Adenoviral transcription can be described as early and late, characterized by the expression of different viral genes. Dividing the genome into immediate early (E1A), early (E1B, E2, E3, E4), intermediate (IX, Iva), and late (L1-L5) genes (**Figure 4**). The first transcription unit to be expressed is the E1A. The E1A proteins stimulate the transcription of other early genes and modulate the expression of cellular genes involved in the transition into S-phase, making the cell more susceptible to viral DNA replication (Berk, 1986). The E1B proteins suppress cell death elicited in response to unregulated cell proliferation signals, including those mediated by E1A. The E2 gene products provide the replication machinery for viral gene products. E3 gene products are not essential for virus replication, in vitro it has been correlated with the control of various host immune responses.

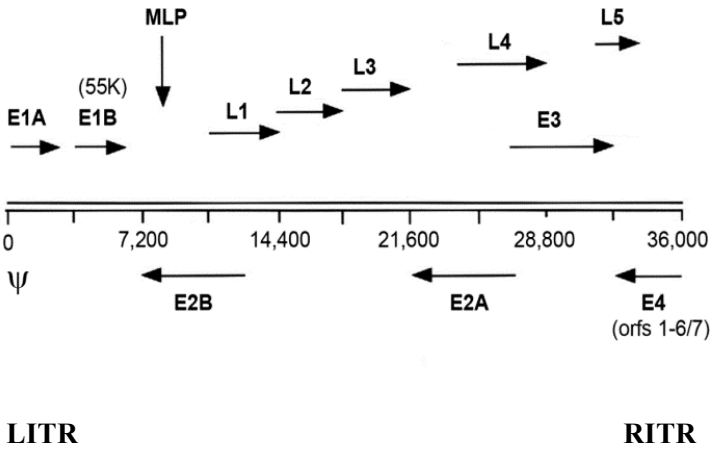


**Figure 4 Representation of adenovirus genome**. Right part: Scheme of the adenovirus genome. E = early genes, L = late genes, MLP = major late promoter, Ψ = packaging signal, which is as a starting point for virus replication, LITR/RITR = left/right inverted terminal repeats, which are crucial for complementary DNA pairing during virus replication. Modified from: (Russell 2000).

E4 gene products have been implicated in many events that occur as the late program starts. E4 regulates transcription, the transition from early to late gene expression, viral replication and assembly of virions. The late genes L1-L5 result in the production of the viral structural components and encapsidation and maturation of the particles in the nucleus (Russell et al; 2000). Ads, especially serotype 5, are the most studied thus used in gene therapy approaches, including oncolytic virotherapy. Ads are human pathogens, causing only mild respiratory and conjunctival infections. Their genome does not integrate into host DNA, resulting in low risk of mutagenesis. The limited duration of viral gene expression makes Ads suitable for gene therapy applications. Moreover these are optimal vectors where only transient gene expression is needed (e.g. cancer therapy) (Robbins and Ghivizzani, 1998; Russell, 2009). To make the adenovirus tumor-specific, a modification in the viral E1A gene (deletion 24 bp) is made. This deletion makes the expressed, defective E1A protein

unable to bind to a retinoblastoma protein (pRb). The pRb is free to bind to the E2F-1 transcription factor, preventing the cell to pass to the S-phase of cell cycle. These delta24 viruses can only replicate in cells where the pRb-E2F-1 interaction is not necessary (i.e. tumor cells). In many cancers the pRb is mutated and E2F-1 is constantly present in the cells. In these cells E2F-1 is able to activate genes that facilitate the G1/S transition and S-phase so that the viral genome can be replicated. On the other hand a deletion of E1B allows p53 to induce apoptosis in infected cells, aborting the replication and spread of the virus.

### *1.4 Mechanism of tumor selectivity for Ads*

One potential approach to treat cancer refractory is to use oncolytic viruses **(Figure 5).** Oncolytic virotherapy exploits the normal life cycle of a virus in order to kill tumor cells. Oncolytic adenoviruses are either genetically engineered leaving normal cells unharmed or naturally attenuated and non-pathogenic in humans (Post, 2002). Normal tissue is kept safe since viruses are targeted towards tumor cells leading to no replication in normal cells (Khuri et al., 2000). Ideally, oncolytic virus should also be able to spread through the circulation into distant metastases (Liu and Kirn, 2007).

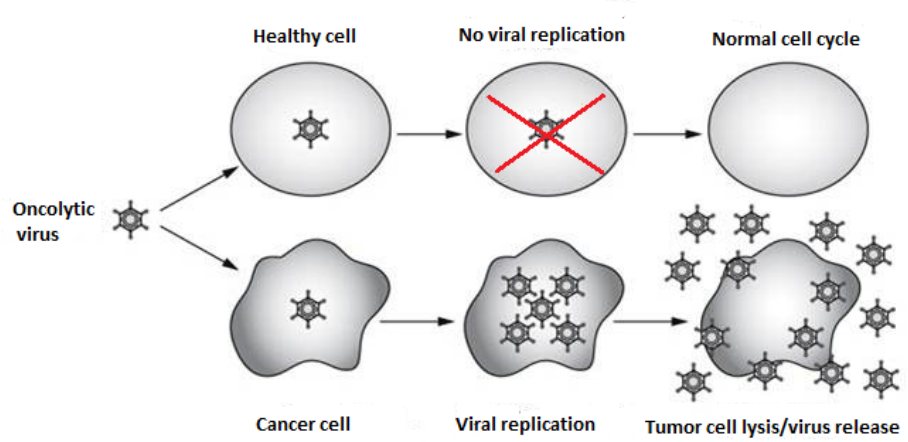


**Figure 5 Concept of oncolysis**. Oncolytic viruses are derived from human viruses via genetic modifications. Such modifications include the mutation or deletion of viral genes, or the insertion of tumor-specific promoters. Oncolytic adenoviruses infect tumor cells, replicate their genome, assemble new viral particles and kill the host tumor cell by lysis, resulting in the release of the progeny viruses. This new virus generation spreads, and starts a new cycle of virus replication and tumor cell killing. Thus, an ideal oncolytic virus represents an efficient and specific anti-cancer agent.

The most used in oncolytic cancer therapy are specific class of adenovirus, which can kill tumor cells by tumor cell-specific replication (Mathis et al., 2005; Ribacka et al., 2008).
Adenoviruses have become the most widely used and most extensively studied viruses for gene delivery/therapy purposes, being easy to manipulate and very safe in vitro and in vivo. Numerous pre-clinical studies were conducted using adenoviral vectors to transfer tumor suppressor genes or as oncolytic viruses (Alemany et al., 2000). Oncolytic adenoviruses are genetically manipulated human adenoviruses that acquired a replication phenotype in tumor cells, but show a more restricted phonotype in normal cells. Several features of wild type adenoviruses can be modified to acquire tumor replication properties (Chiocca, 2002). There are different ways in order to develop tumor specificity in oncolytic adenoviruses:

- by altering viral genes that attenuate replication in normal tissue but not in tumor cells;
- by placing viral genes that initiate viral replication under the control of promoter sequences that are active only in tumor cells;
- by the modification of viral coat proteins that function in host cell infection.

Various types of oncolytic Ads have been developed, and can be mainly classified into 2 groups. One shows tumor-selectivity via deletion of E1B-55K gene, which are dispensable for the replication of Ads in tumor cells. The second type of oncolytic Ads possess the E1 gene expression cassette driven by tumor-specific promoters. For example, by altering a region of the E1B gene, the adenovirus can selectively replicate in p53-deficient (i.e.tumour) cells and leave p53-competent cells intact. In addition E1B-55KDa mediates late-viral RNA transport and the loss of E1B-55KDa is restrict to the viral replication of tumor cells capable of taking over the RNA export function of the viral gene product (O'Shea et al., 2005).

### *1.5 Oncolytic Adenoviruses and human cancer*

Cancer is one of the leading causes of death worldwide. The discovery of new technologies has led to the development of more sensitive diagnostic methods and new types of treatment. However, conventional therapies such as radiotherapy and chemotherapy, have shown limitations related to side effects and the impossibility to prevent relapses.

Cancer cells have undergone a mini-evolution (point-mutations, larger chromosomal shifts and alterations), providing them with selective growth advantages over normal cells (Cahill et al., 1999). While cancer cells gain growth, they can simultaneously lose critical

components of the intracellular defense mechanisms and thus become fertile ground for the replication of many viruses. One strategy for the treatment of cancer is the use of oncolytic adenoviruses for cancer destruction since they have the potential to take advantage of cancer specific changes to replicate specifically in tumor cells (Pesonen et al., 2011).

Oncolytic adenoviruses are engineered to infect cancer cells and induce cell death through the propagation of the virus, expression of cytotoxic proteins and cell lysis (Mullen and Tanabe, 2003). Oncolytic viruses replicate preferentially in cancer cells and can destroy those cells at the end of replication cycles by lysis. The viral progeny are then released and they can infect neighboring cells, which results in multiple steps of infection, replication, lysis and spread throughout the tumor, all while sparing normal cells, hence toxicity is limited. The limitations in success may be due to the intrinsic biological properties of the tumor targets.

In 2005, State Food and Drug Administration in China approved the first oncolytic adenovirus as a drug for head and neck squamous cell carcinoma (Yamamoto and Curiel, 2010). This to bring the hope that oncolytic adenoviruses can be used for the treatment of cancer. The efficacy of oncolytic adenoviruses in cancer gene therapy can be increased by arming the virus with a therapeutic transgene. In this regard oncolytic adenoviruses have been engineered to express tumor associated antigen, co-stimulatory molecules, such as CD40 ligand (CD40L) and CD80, immunostimulatory cytokines, including interleukin IL-2, IL-12, IL-15, IL-23, IL-24 and granulocyte macrophage colony-stimulating factor (GM-CSF) (Pol J et al; 2014). The first generation of oncolytic adenovirus, ONYX-015 (also known as dl1520, H101 in China), is a genetically modified adenovirus with deletion of the 55 kD gene in the E1B region (Nemunaitis et al., 2001). This virus is the only approved for oncolytic therapy, it contains a deletion in the E1B gene, preventing the formation of a

functional E1B-55kDa protein. With this mutation, ONYX-015 was expected to replicate only in p53-deficient cells (Bischoff et al., 1996). A phase I study with E1B-55kDa gene deleted ONYX-015 was a proof of concept that oncolytic adenoviruses can be used for clinical oncolytic virotherapy. ONYX-015 was injected in head and neck and was well tolerated, nevertheless it was not efficient as a single agent and was combined with chemotherapy in phase II trials, and this strategy led to a better response and to a phase III study.
However it should be remembered that when used as anticancer drugs, viruses must respect strict criteria for safety, efficacy and accessibility to pharmacological study in human subjects. Specificity for cancer tissues is the key to safety, and this goal can be achieved through strategies that take advantage by tumor-specific changes of cancer versus normal cells. In cancer treatments the response to oncolytic adenoviruses can be different from one individual to another one that's why more specific and personalized approaches are required to optimize the oncolytic virus medicine.

### *1.6 The metabolism of cancer cells*

In tumor cells is observed an increase in intensity of anaerobic glycolysis and occurs the phenomenon of aerobic glycolysis: the cells produce lactic acid even in the presence of oxygen. The appearance of an intense aerobic glycolysis in tumors was considered by Warburg as peculiar of neoplastic cells; however it is now known that the same phenomenon can be observed in normal tissues to rapid turnover, in embryonic tissue, in the retina and in the nervous tissue (Casson et al., 2013). The main metabolic characteristic of cancer is the "Warburg effect" **(Figure 6)**, which is to prefer, for the production of cellular energy even in aerobic conditions, the use of the glycolytic pathway rather than that of mitochondrial respiration.

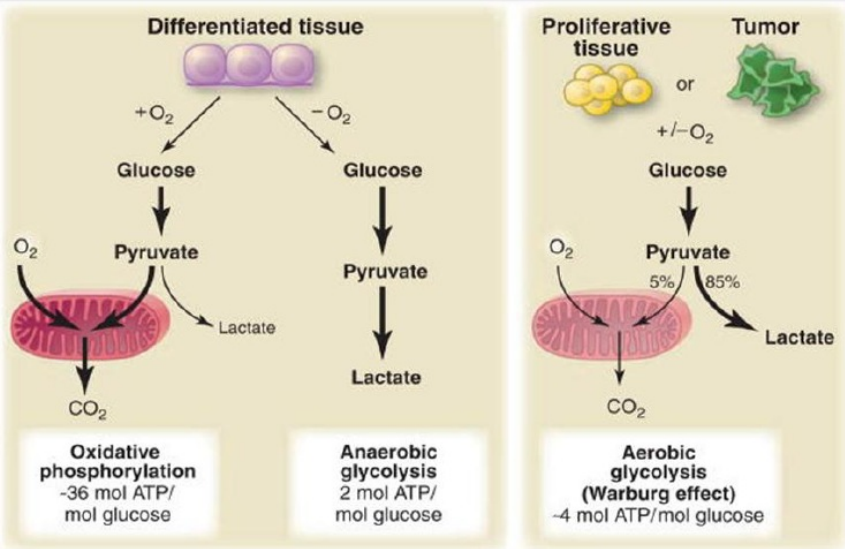


**Figure 6 The Warburg effect** (Vander Heiden et al., 2009). Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis.

To survive and grow, normal cells metabolize glucose to pyruvate, which is completely oxidized to CO2 through the Krebs cycle and oxidative phosphorylation at the mitochondrial level, generating 36 ATP for every molecule of glucose. The O2 is essential as a final electron acceptor, but when its concentration is limited, pyruvate is metabolized to lactate via anaerobic glycolysis. It is believed that the Warburg effect is dependent on the particular expression of some enzymes of the glycolytic pathway, such as hexokinase, phosphofructokinase and, above all, pyruvate dehydrogenase kinase. The most immediate consequence is that the neoplastic tissues are mainly glucose-dependent for their growth and for their energy balance, consequently the concentration of glucose that comes from outside is an index of ability for tumor cells. This explains why cancer

cells located away from the vessels undergo regressive processes and why tumors evade large amounts of glucose in the blood.

### *1.7 The natural dipeptide L-carnosine and its functions*

L-carnosine (β-Ala-His) is a naturally occurring histidine dipeptide described as a component of the meat for the first time in 1900 by Gulevitch. It is endogenously synthesized and normally found in the crystalline lens, kidney, stomach and, in particular, in the brain and in skeletal muscle in a range of concentrations of 0.3-5 mM and 2-20 mM, respectively (Babizhayev et al., 1994).

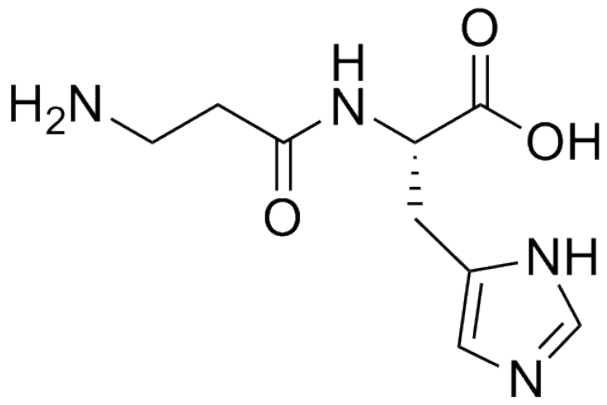


**Figure 7 Structure of L-carnosine**

L-carnosine is synthesized in the liver, by carnosine synthase from its primary constituents, histidine and b-alanine. This dipeptide is then transported through the blood to peripheral tissues, where it is absorbed. It's also introduced through the diet, in particular thanks to the meat; it is estimated that the average intake of L-carnosine roams on average between 50-250 mg / day. L-carnosine is absorbed in the

intestine unaltered, is distributed through the blood stream and used by peripheral tissues, metabolized into its constituent amino acids or filtered by the kidneys and excreted in the urine. Thanks to the presence of a β amino acid, L-carnosine is resistant to most of the known peptidases. The hydrolysis of L-carnosine is catalyzed by carnosinase. In human, two forms of carnosinase were discovered: serum carnosinase (CN1) and tissue carnosinase (CN2). CN1 was identified as a homodimeric dipeptidase with narrow substrate specificity whereas CN2 could exert dipeptidase activity on a wider range of substrates. Because of the existence of CN1 in human serum, the blood L-carnosine concentration is very low. Carnosinase present in the liver, kidney and serum is the enzyme that cleaves the L-carnosine; therefore, the two isoforms, the serum and the tissue, have different molecular properties and substrate specificity: in the liver, L and D isomers of L-carnosine are hydrolyzed with the same speed, while in serum and kidney is cleaved only the isomer L. There are a number of compounds related to L-carnosine, with whom they share part of the structure and properties, and they are: anserine, homocarnosine, omoanserine, N Acetylcarnosine, N Acetylomocarnosine and N Acetylanserine (Boldyrev, 2012).

L-carnosine performs multiple biological functions, including antioxidant activity, ability to chelate metal ions, as well as anti-inflammatory and anti-senescence properties. L-carnosine can be defined as antioxidant due to its ability to act as protection against the harmful effects of highly reactive molecules known as free radicals. The gradual accumulation of damage caused by free radicals leads to a progressive weakening of the organism and an increased susceptibility to disease. The protective action exerted by the antioxidant molecules, including L-carnosine, may therefore be of particular utility in all physiological situations in which the organism is subjected to a high oxidative stress. Cell membranes, DNA, RNA and proteins are

particularly susceptible to oxidative damage. Moreover, being extremely soluble in water, it is able to inhibit the oxidation of cell membranes due to the action of the iron, zinc, copper, hydrogen peroxide, radical oxygen (Mzhel'skaia and Boldyrev, 1998). From the chemical point of view, L-carnosine has antioxidant activity due to the presence of the peptide bond which exerts a stabilizing action against the imidazole radical, in fact the antioxidant effect of the dipeptide has proved greater than the activity of single or combined amino acids which constitute L-carnosine (Babizhayev et al., 1994).

Recently, L-carnosine has been defined as the mixture of nutrient longevity, as experimental studies indicate that it may delay the senescence and lead to cellular rejuvenation in cultured fibroblasts human. For this reason the dipeptide L-carnosine was defined as antiaging (Calabrese et al., 2010). L-carnosine inhibits the formation of the substances mentioned under the name of AGEs, advanced glycosylation end products, ultimate result of non-enzymatic glycosylation; also called the "Maillard reaction" in which the amino groups of the amino acids react with the carbonyl groups (aldehyde and ketone) of sugars, with the production of reactive chemical entities. All this leads to the formation of cross-links between different proteins with possible formation of advanced glycation end products, which exert a detrimental effect on cell function, in addition to increasing the formation of free radicals (Reddy et al., 2005). Regarding the role of L-carnosine in cellular aging, Holliday R and McFarland GA (2000) showed that in human fibroblasts in culture, physiological concentrations of L-carnosine (20 or 30 mM) prolonged the duration of their lives and reduced the characteristic signs of aging, allowing the maintenance of a juvenile phenotype. Instead, the removal of L-carnosine from the culture medium causes the immediate resumption of the aging process. Indeed Shao et al. (2004) indicate that L-carnosine can reduce the shortening of telomeres. The

effect of the dipeptide on telomere shortening has been studied in human fetal fibroblasts (HPF). The experimental data show that cells grown in the presence of L-carnosine slow down telomere shortening compared to cells grown in the absence of L-carnosine. Aging is the accumulation of aberrant proteins, in particular those that have carbonyl groups. L-carnosine reacts with protein carbonyl groups in order to mask the deleterious effects of other molecules and facilitate their proteolytic destruction.

The Antineoplastic effects of L-carnosine were first described by Nagai and Suda, (1986) in fact they subcutaneously implanted Sarcoma-180 tumour cells into ddY mice, the day after implantation L-carnosine (50 mg/kg/day) was injected subcutaneously and the treatment was continued every second day. Comparing the treatment with L-carnosine to the treatment with saline, the authors observed that the dipeptide inhibited tumour growth and also reduced mortality. Inspired by the work of (Holliday and McFarland, 1996) who found that L-carnosine selectively inhibited the growth of transformed and neoplastic cells, (Renner et al., 2010) showed that L-carnosine inhibited the growth of cultured tumour cells isolated from human glioblastoma. The L-carnosine effect was not accompanied by apoptosis or necrosis, but rather by a reduced proliferation, probably mediated by a block of ATP production, since this dipeptide prevents anaerobic glycolysis, that is the preferential route for energy production in tumor cells. Recently it was demonstrated that L-carnosine inhibits proliferation in kidney mesangial cells and also DNA synthesis in a dose-dependent. In this cell cycle is arrested in the G1-S phase transition, this event is accompanied by a reduction of phosphorylation of ERK 1/2 and p38-MAPK, responsible of the survival and cell proliferation (Jia et al., 2009). L-carnosine has been shown to have an effect in vivo, in fact retards tumor growth in murine fibroblasts; although it is not able to completely prevent the

development of the tumor, considerably reduces the number of mitoses observed in tumor mass compared to a control sample treated only with NaCl. Tumor cells produce a greater quantity of deoxyribose, which gives them the ability to resist apoptosis, however this feature is lost after treatment with L-carnosine, for its ability to sequester deoxyribose (Hipkiss, 2011).A proteomic experiment evaluated the influence of L-carnosine on the activity of several proteins (Asperger et al., 2011). Cells isolated from patients with glioma and continuous cell lines of human glioblastoma (T98G), treated for 72h with 50mM of L-carnosine showed a significant reduction of protein in tumor progression. Among these are: the uroporphyrinogen decarboxylase (UROD), the enzyme in the biosynthetic pathway heme; the transaldolase (Taldo) involved in the oxidative pathway of the pentose phosphate, which is important in detoxification and in the production of D-Ribose. Transaldolase connects the pentose phosphate pathway intermediates to glycolysis and contribute to the survival of cancer cells. Another protein affected by treatment with L-carnosine is BAG2 (associated with Bcl-2) in turn regulated by p38 MAPK. Among the main functions of BAG2 is to inhibit the activity of the Hsp70-CHIP. The interaction of CHIP with the heat shock protein (HSP) is in the ubiquitination of the substrates and their degradation through the proteasome.

## 2 Aims of the study

Cancer is a major public health problem and new therapeutic approaches are needed. In fact, despite progress in reducing mortality due to improvements in cancer prevention, early detection and treatments, still 6.7 million deaths worldwide are caused by cancer each year (Parkin et al., 2002). In recent years considerable attention has been given to the use of natural substances as anticancer drugs. The natural antioxidant dipeptide L-carnosine belongs to this class of molecules because it has been proved to have a significant anticancer activity both in vitro and in vivo (Iovine et al., 2014). A number of biological functions have been recognized to this molecule, including antioxidant activity, ability to chelate metal ions, inhibition of protein glycosylation, anti-inflammatory and anti-senescence properties (Guiotto et al., 2005). Many authors have highlighted the anti-proliferative activity of L-carnosine, showing that this dipeptide is able to inhibit the growth of cancer cells, affecting the HIF-1alpha signaling and anaerobic glycolysis (Renner et al. 2010; Iovine et al., 2012; Iovine et al., 2014). In particular, in the laboratory were initially I have spent my doctoral fellowship, it has been demonstrated that L-carnosine reduces the levels of ROS and ATP, which are two important molecules for tumor progression and that 50 mM of dipeptide is also able to reduce the number of colonies in colon cancer cells compared to control cells not treated (Iovine et al; 2014). A major characteristic of L-carnosine, which makes this dipeptide a good candidate for new therapeutic strategies, is its bioavailability. Indeed, the cellular uptake of drug-like molecules is the major obstacle in the development of a successful cancer therapy and the aim of new anti-cancer strategies is to facilitate the uptake and intracellular accumulation of drugs into cancer cells, reducing the non-specific uptake into normal cells. From this point of view L-carnosine could represent a good adjuvant candidate for the treatment of cancer

due to its antiproliferative property and also because it is thought to be absorbed primarily via intestinal epithelium cells, for these reasons it can be used for the treatment of colon cancer (Iovine et al., 2014). However one of the limits of the use of L-carnosine is that high concentration of dipeptide is required to arrest tumor progression (Renner et al; 2010; Iovine et al; 2012) since the doses of L- carnosine used exceed those currently used in clinical settings. My first aim was to overcome this limitation studying the effect of L-carnosine at lower concentrations. To do this I joined the Immunovirotherapy lab in Helsinki where I took advantage of the new technology developed by the assistant Professor Vincenzo Cerullo. We have hypothesized that L-carnosine could be conjugated on the capsid of oncolytic viruses and this strategy would enhance the cell entry of the peptide. Oncolytic adenoviruses are a new class of anti-cancer agents with a great therapeutical potential since they can selectively kill cancer cells, in fact they can enter both normal and tumor cells but they are modified to be able to replicate only in cancerous cells leaving normal cells unharmed. Virus replication in tumor cells eventually leads to cell lyses, which allows the new virus progeny to spread to surrounding cells and even to distant metastases through circulation. Several clinical trials have revealed Ads as a safe and promising therapy for cancer (Harvey et al., 2002).

Since the combination of Ads and chemotherapeutic drugs may improve the efficacy of oncolytic virotherapy, thanks to the synergistic action of virus and drug, in the present study we have optimized a model to use oncolytic adenovirus as a scaffold to deliver active drugs. Our working hypothesis was to use an oncolytic adenovirus as drug carrier, to achieve an increase of cellular death in cancer cells treated with the L-carnosine coated oncolytic adenovirus compared to cells treated with the drug or virus alone.

The purpose of my study was to:

- Develop a new system to increase the amount of L-carnosine that is delivered to cancer cells by using viruses as carriers;

- Generate an oncolytic adenovirus loaded with L-carnosine as a drug delivery system for cancer therapy;

- Study the efficacy and the molecular mechanisms of the oncolytic virus loaded with L-carnosine compared to virus alone, in different cancer cell lines;

- Analyze the efficacy of oncolytic adenovirus loaded with L-carnosine in a lung and colon cancer xenograft model.

The possible advantages of this approach are: the synergistic action of virus and drug and consequently an increased efficacy due to an increased drug-delivery to target cancer cells. The proposed model could be used as a novel drug delivery system for cancer therapy.

# 3 Materials and Methods

## *3.1 Cell lines*

HCT-116 human colorectal carcinoma cell line was purchased from the American Type Culture Collection (ATCC) USA. The cell line was cultured at 37°C and 5% $CO_2$ in DMEM (Dulbecco's Modified Eagle Medium) purchased from Bio Whittaker Classic Cell Culture Media, Lonza - VWR International s.r.l. , supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories, North Andover, Massachusetts) and 1% penicillin/streptomycin (Gibco Laboratories). SW-480, human colorectal carcinoma cell line was purchased from the American Type Culture Collection (ATCC) USA, were cultured in RPMI 1640 medium (BioWhittaker) supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories), 1% penicillin/streptomycin (GibcoLaboratories) and 1% L-glutamine (Gibco Laboratories). A2058 human melanoma cell line was purchased from the American Type Culture Collection (ATCC) USA. The cell line was cultured at 37 °C and 5% $CO_2$ in DMEM (Dulbecco's Modified Eagle Medium) purchased from Bio Whittaker Classic Cell Culture Media, Lonza - VWR International s.r.l., supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories, North Andover, Massachusetts) and 1% penicillin/streptomycin (Gibco Laboratories). A549 lung cancer cell line was purchased from the American Type Culture Collection (ATCC) USA. The cell line was cultured at 37 °C and 5% $CO_2$ in DMEM (Dulbecco's Modified Eagle Medium) purchased from Bio Whittaker Classic Cell Culture Media, Lonza - VWR International s.r.l., supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories, North Andover, Massachusetts) and 1% penicillin/streptomycin (Gibco Laboratories).

### *3.2 Preparation of adenoviruses*

Ad5D24-CpG, a gift from Dr. Vincenzo Cerullo was generated (according to the protocol reported in Cerullo et al; 2012) recombining a CpG-rich shuttle plasmid (pTHSN-CpG1) with a plasmid containing the 24 adenovirus backbone. pTHSN-CpG1was constructed by inserting NdeI-digested CpG1 (derived by digestion from custom designed pU57-CpG1; GenScript, Piscataway, NJ) and SpeI-digested CpG2 sequences (derived by digestion from custom designed pU57-CpG1; GenScript) respectively into NdeI- and Spe-I linearized plasmid pTHSN previously generated. Viral stocks were expanded in human lung cancer cell line A549 and purified on cesium chloride gradients. Stocks were stored at -80°C after the addition of glycerol to a concentration of 50% vol/vol. The viral particle concentration was determined by $OD_{260}$-reading and standard $TCID_{50}$ (tissue culture infectious dose 50) assay on 293 cells was performed to determine infectious particle (pfu) titer. Virus was characterized by PCR and restriction enzyme analysis. Protein concentration of viral prep was determined by Breadford Assay (Biorad). Ad5D24-Rfp, kindly provided by Dr. Masataka Suzuki from Baylor College of Medicine (USA). It was generated (according to the protocol reported in (Farzad et al., 2014) using standard adenovirus preparation techniques. In order to insert RFP gene into E3 region, the monomeric RFF complementary DNA was inserted into pTHSN. pAd5D24RFP was generated by homologous recombination in E.coli between linearized pTHSNRFP and linearized pAd5D24RFP. Ad5D24RFP virus genome was transfected in A549 cells for amplification and rescue. Viral particles (vp) were calculated according the following instructions:

- Viral particle concentration (VP/ml) = A260 (blank corrected) x dilution factor x extinction coefficient.
- Extinction coefficient = 1,10E+12 viral particles / ml / A260 unit

- For VP calculations include only values which meet requirements: 1) A260 nm (raw data) should be between 0.1-2.5 OD units (linear range of spectrophotometer), 2) A260/A280 ratio should be between 1.2-1.4

### *3.3 Treatments*

L-carnosine was purchased from Sigma–Aldrich, and dissolved in PBS (Lonza-VWR International s.r.l.). A modified version of L-carnosine (Carnosine6K), featuring six additional lysines at the C-terminus, was purchased from GeneCust Europe Laboratoire de Biotechnologie du Luxembourg S.A, and dissolved in PBS (Lonza-VWR International s.r.l).

### *3.4 Complex formation*

In order to saturate the surface of the oncolytic adenovirus an excess of Carnosine6K has been used. Previous studies performed in the hosting laboratory, showed that a ratio of 1:500 virus-to-peptide protein amount is sufficient to efficiently coat the viral surface. To allow the formation of the complex between the oncolytic virus and Carnosine6K the two components need to be mixed and incubated at room temperature for 15 minutes using MilliQ $H_2O$ at pH 7.4 as buffer.

### *3.5 Zeta Potential Analysis*

Zeta potential analysis has been performed using 1x $10^{10}$ viral particles (vp). According to the complex formation protocol, the viral

particles have been mixed with Carnosine6K. Naked oncolytic adenovirus Ad5D24CpG or Carnosine6Kalone have been used as controls. All the samples have been 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 has been set on the software to allow the samples to stabilize at 25°C inside the measuration chamber. Three consecutive measurements have been performed on each sample using ZetaSizer Nano Malvern.

### *3.6 Dynamic Light Scattering analysis*

As described in the previous section, complex and controls have been diluted in 800 ul of MilliQ $H_2O$ pH 7.4. The solution has been transferred to a transparent cuvette and analysed ZetaSizer Nano Malvern. After an equilibration period of 120 seconds, three consecutive measurements have been performed on each sample.

### *3.7 Viability Assay*

Tumor cells were seeded at $1.0 \times 10^4$ cells per well on 96-well plates and maintained under appropriate condition (DMEM or RPMI 1640, completed with 5% FBS, 1% L-glutammine and 1% of penicillin/streptomycin (all from Gibco Laboratories). On the next day, L-carnosine, Carnosine6K and Ad5D24-CpG virus were diluted separately in growth media with 2% FBS, cells were treated and then incubated in 5% FBS containing media at 37 °C for 3 to 5 days. Cell viability was determined by MTS according to the manufacturer's protocol (Cell Titer 96 AQueous One Solution Cell Proliferation Assay; Promega, Nacka, Sweden). The absorbance was measured with

a 96-wells plate spectrophotometer Varioskan Flash Multimode Reader at 490 nm. The experiments were independently performed three times and each experiment contained triple replicates.

### *3.8 ATP determination Assay*

The ATP concentration was assessed quantitatively using the CellTiter-Glo Luminescent cell viability assay purchased from Promega Corp. (Madison, WI, USA, G7571) according to the manufacturer's instructions. Briefly, tumor cells were seeded at $1.0 \times 10^4$ cells per well in white opaque 96-well plate (tissue culture treated, sterile-6005181, from Packard), in 100 ul of fresh culture medium containing DMEM without phenol red (Sigma–Aldrich). After incubation for 24 h at 37 °C in 5% $CO_2$, L-carnosine, Carnosine6K and Ad5D24-CpG virus were diluted separately in culture medium with 2% FBS, cells were treated and then incubated in 5% FBS containing media at 37 °C for 3 to 5 days. Then, the ATP concentration was determined by CellTiter-Glo Reagent according to the manufacturer's protocol. The luminescence was measured with Varioskan Flash Multimode Reader. The experiments were independently performed three times and each treatment was performed in triplicate.

### *3.9 Transduction Assay*

Cells were seeded at a density of $1x10^4$cells/well in 96 well plates and maintained under appropriate condition (DMEM or RPMI 1640, completed with 5% FBS, 1% L-glutammine and 1% of penicillin/streptomycin (all from Gibco Laboratories). On the following day cells have been infected using an oncolytic adenovirus

Ad5D24Rfp encoding for the red fluorescent protein (100vp, 10vp, 1vp, 0.1vp) and complex (100vp, 10vp, 1vp, 0.1vp). Red fluorescence has been measured by Varioskan plate reader at 24h, 48h, and 72h after the treatment. The experiments were independently performed three times and each experiment contained triple replicates.

### *3.10 Determination of infectious titer*

Cells were seeded at a density of $2x10^5$ cells/well in 24 well plates, and maintained under appropriate condition (DMEM or RPMI 1640, completed with 5% FBS, 1% L-glutammine and 1% of penicillin/streptomycin (all from Gibco Laboratories). On the following day cells were infected with Ad5D24CpG (100vp, 10vp, 1vp, 0.1vp) and complex (protein plus Ad5D24CpG 100vp, 10vp, 1vp, 0.1vp). Plates were centrifuged for 90 minutes with 1000 rcf in 37 °C and incubated for 48h before staining at 37°C and 5% CO2. 48 hours following the incubation cells were fixed by adding 250 ul of ice-cold methanol per well and incubated 15 minutes. Then cells were washed three times with PBS1%-BSA solution and incubated in the dark for one hour with 1st antibody, mouse monoclonal anti-hexon 1:2000 (Novus Biological, NB600-413). After the incubation time, cells were washed three times with PBS1%-BSA and incubated in the dark for other 1 hour with 2nd antibody: Biotin-SP-conjugated goat anti-mouse 1:500 (Jackson Immuno Research, 115-065-062). After the incubation time, cells were washed three times with PBS1%-BSA and incubated in the dark for 30 minutes with extravidin-peroxidase (Sigma Aldrich, E2886). Finally cells were washed three times like as indicated earlier and treated with Dab solution (Sigma Aldrich, A7284-50ML). To quench the reaction, cells were treated with PBS 1x. The detection of infectious titer was made using microscope EVOS, each well was photographed (5pcs) at five non-overlapping

sites. Infected cells were counted using ImageJ and the infectious titer was calculated according to the following equation:

Infectious titer: $x \frac{A(well)}{A(field)} \frac{1}{l} \frac{1ml}{v}$

Where x= number of infected (stainend cells)

A(24 well)= 190 $mm^2$

A (field) = surface area of the field

L= dilution

V= volume of virus dilution applied per well

## *3.11 Analysis of apoptotic and necrotic cells*

Tumor cells were plated into 6 well plates, $2x10^5$ cells/well. Cells were treated with L-carnosine (100mM), Carnosine6K, Ad5d24CpG (100vp) or PBS for mock. The amount of apoptotic and necrotic cells were measured after 24 and 48 hours p.i. with a TACS Annexin V-FITC kit (Trevigen Inc., Gaitherburg, MD, US) and BD LSR II flow cytometer according to manufacture´s instructions. The analysis of apoptotic cells was performed by annexin V (annexin V-FITC) assay with fluorescein isothiocyanate (FITC)-labels. This method allows the analysis of the percentage of apoptotic cells. In early stages of apoptosis, phosphatidyl-serine is expressed in the membrane, and in the late phase of apoptosis, it binds annexin as propidium iodide enters into the cells. Experiments were performed three times in triplicate.

### *3.12 Western blot Analysis*

Total extracts of HCT116 and A549 cell lines were prepared 24 and 48 h after treatment with L-carnosine 100mM, Carnosine6K and Ad5D24CpG 100 MOI. Briefly, harvested cells were washed twice with ice-cold PBS and centrifuged at 700 rpm for 5 min at 4 °C. The cell pellet was resuspended with cold lysis buffer containing: Cell Lysis Buffer (NP40, Invitrogen FNN0021), Protease inhibitor cocktail (Sigma Aldrich P2714-1BTL) and PMSF (Phenylmethylsulfonyl fluoride Sigma Aldrich, P 7626), for 30 min on ice. The cell lysates were cleared by centrifugation at 13000 rpm for 10 min at 4 °C. Protein concentration was determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories). For the western blot analysis, proteins were challenged with antibodies against p62 (mouse antibody from Abnova), LC3 (rabbit antibody from Cell Signalling) and b-actin (mouse monoclonal from Santa Cruz Biotechnology). The signals were detected by using ECL kit (GE Healthcare).

### *3.13 Animal experiments*

All animal experiments were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Mice were obtained from Scanbur (Karlslunde, DK) at 5-7 weeks of age and quarantined at least for 2 weeks before the study. Health status of the mice was frequently monitored and as soon as signs of pain or distress were evident they were euthanized. For the efficacy experiment human xenografts were established by injecting $1 \times 10^6$ A549 and HCT-116 cells s.c. into the flanks of 5-7 week-old female BALB/c nude mice ($N = 5$ per group). Tumors (two tumors per mouse, ~5x5 mm in diameter) were injected i.t. with a volume of 50 µl for three times on days 0,2,5with $10^8$ VP/tumor of Ad5D24CpG and control tumors were injected with PBS

only. The formula (length × $(width)^2$ × 0.5) was used to calculate the tumor volumes. Mice were euthanized when a tumor reached an average diameter of 15 mm.

### *3.14 Statistical Analysis*

Statistical significance was determined by unpaired, two-tailed Student's *t*-test using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, US). Differences in tumor growth were analyzed with the nonparametric Mann–Whitney test (immundeficent mouse experiments). Results are presented as mean ±SEM for tumor growth and *in vitro* studies. All p values were two-sided and considered statistically significant when ≤ 0.05. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.

Therapeutic synergy was assessed using the fractional tumor volume (FTV) method (Xu et al., 2011) (Yokoyama et al., 2000) which is derived from a method originally developed by Webb (Webb 1963). Here, the expected FTV (i.e., the product of FTV values for monotherapies) of the combination therapy is divided by the observed FTV of the combination, yielding a ratio that indicates the nature of the interaction >1 indicates synergy and <1 indicates a less than additive effect). FTV for each experimental group is obtained by dividing the mean tumor volume of the experimental group by the mean tumor volume of the control group.

# 4 Results

## *4.1 Modified L-carnosine (Carnosine6K) can be attached into the capsid of an oncolytic adenovirus through electrostatic interaction*

Since the main purpose of this study was to develop a new system to increase the amount of L-carnosine that is delivered to cells by using virus as carrier, we have developed a strategy to combine virus and L-carnosine, based on electrostatic interaction. The first step for the characterization of the novel system was to study how the peptide can be conjugated to the surface of an oncolytic adenovirus. It is known that the surface of an oncolytic adenovirus is negatively charged, hence we have hypothesized that L-carnosine positively charged consisting of a tail of six lysines (Carnosine6K) can be mixed with the oncolytic adenovirus to allow the formation of the complex formed by the virus and Carnosine6K **(Figure 8).** The carrier used for our experiments is a serotype 5 conditionally-replicating adenovirus that features a 24 base pair deletion in E1A gene. This deletion makes this virus able to replicate only in cells that show a disrupted retinoblastoma pathway, i.e many cancer cells. Ad5D24CpG virus is also enriched with CpG islands to stimulate the innate immune system through toll-like receptor 9 (TLR9) (Cerullo V et al; 2012). Since the Zeta Potential is the electrical potential that exists at the shear plane of a particle and is a function of the surface charge of a particle, we have analyzed the electric surface charge (Z-Potential) of the complex to see if the addition of the Carnosine6K was able to change the negative charge of the adenovirus into positive. We observed that mixing Carnosine6K with oncolytic adenovirus (1:500) the electric surface

charge became very positive compared to the virus alone and Carnosine6K alone **(Figure 9a).** We have also analyzed the Dynamic light scattering (DSL) of the singles particles and we reported the results in function of the peptide concentration **(Figure 9b).**

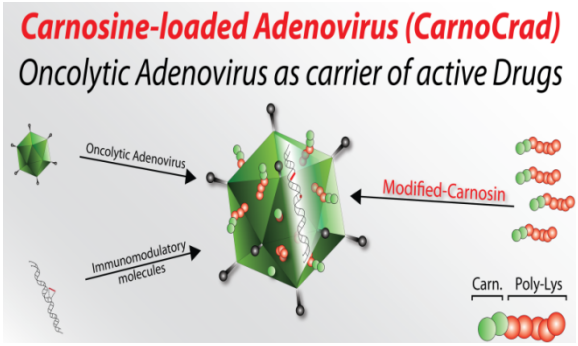


**Figure 8. Oncolytic adenovirus as a carrier for active drugs.** Our working hypothesis is to use an oncolytic adenovirus, which expressing immunomodulatory molecules, together with active peptides as a carrier for active drugs.

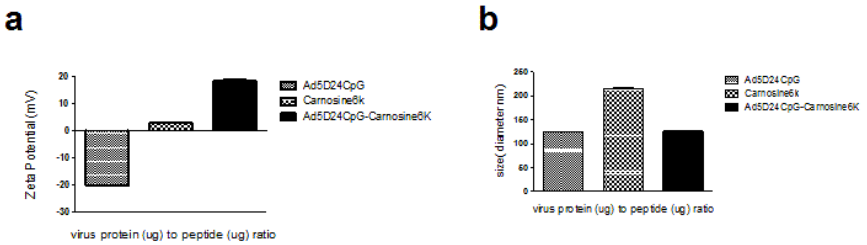


**Figure 9 a,b. Complex formation between oncolytic adenovirus and modified L-carnosine.** $1x10^{10}$viral particles were conjugated with positively charged L-carnosine (1:500). After the reaction the surface charge of the single particles was measured. Complex shows an increase in the Zeta Potential (9a). We also tested the size of the single particles and the diameter of the Carnosine6k slightly increases, however no significant aggregation is observed (9b).

### *4.2 Effect of Carnosine6K coated viruses on cell viability and ATP levels*

In order to characterize the infectivity and the cell killing activity of the complex we have performed MTS cell viability assays on four different human cancer cell lines (human lung cancer cell line A549, HCT116 human colon cancer cell line with a mutation in codon 13 of the Ras proto-oncogene, SW-480 human colon cancer cell line with a mutation in codon 12 of the Ras proto-oncogene, A2058 human melanoma cell line with a mutation in BRAF), comparing the efficacy of cell killing of the complex with an uncoated oncolytic virus, Carnosine6K alone and L-carnosine 100mM **(Figure 10a, b, c, d).** The MTS cell viability assays shows that 100vp of the complex is able to reduce cell viability even more than the virus alone in all tested cell lines ($p < 0.001$). It is known that high concentration of L-carnosine (100mM) reduce ATP levels in HCT116 cell line (Iovine et al; 2012), so we have checked the levels of ATP by ATP assays in all four cancer cell lines **(Figure 11a, 11b, 11c, 11d).** The ATP production was found significantly reduced especially in the complex 100vp compared to the virus alone and L-carnosine alone with or without six lysines.

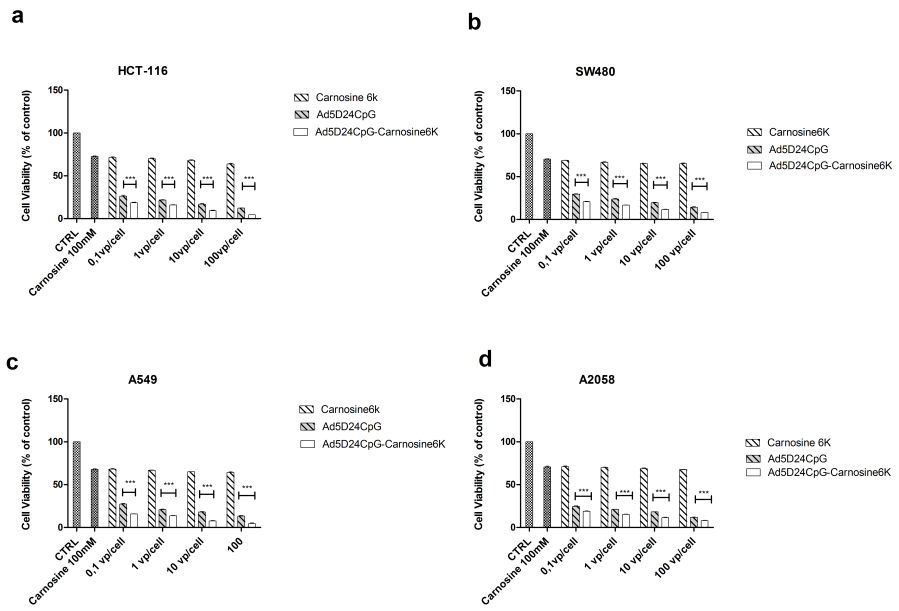


**Figure 10 a, b, c, d. In vitro killing of human cancer cell lines.** Cell viability was performed by MTS Assay on four cancer cell lines, in order to evaluate if the coating of the viral capsid with Carnosine6K could affect the biological properties of oncolytic viruses.

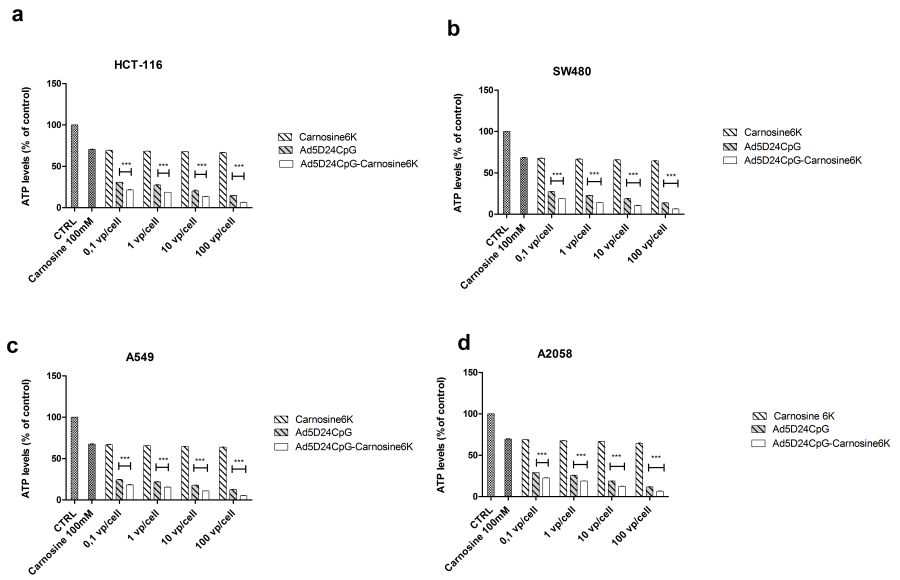


**Figure 11 a, b, c, d. Carnosine6K coated virus reduced ATP levels in human cancer cell lines.** In order to evaluate if the Carnosine6K coated virus is able to influence ATP production in human cancer cell lines the ATP levels were measured quantitatively using the CellTiter-Glo Luminescent cell viability assay.

## *4.3 Carnosine6K coated viruses display increased transduction and enhanced infectious titer*

To examine whether the presence of Carnosine6K affects the transduction efficacy of the adenoviral vector, we have performed a transduction assay using on a panel of different human tumor cell lines, using an oncolytic adenovirus expressing red-fluorescent protein (RFP) to assess the transduction efficacy of the coated complex in comparison to the naked oncolytic adenovirus. We compared the transduction efficacy of Ad5D24Rfp uncoated virus and Ad5D24Rfp-Carnosine6K complex. Reading of the fluorescent signal checked by Varioskan has been performed 24, 48 and 72 hours post-infection (p.i.) as showed in figure 12. Interestingly at 72 h p.i. the amount of

fluorescent cells is significantly higher when cells have been treated with complex in comparison to cells treated with naked oncolytic adenovirus. In fact the transduction assay shows that cells were more transduced in the complex 100 vp than in the virus alone in all four tested cell lines after 72h post-infection **(Figure 12 a, b, c, d**).

In order to determine the infectious titer based on visual quantification of cells infected with an uncoated oncolytic virus Ad5D24CpG, Ad5D24CpG-Carnosine6K complex added as a single entity and Ad5D24CpG and Carnosine6K added separately as a mix, we performed an Immunocytochemistry assay (ICC). The levels of infectious titer was higher in the complex than in the virus alone at a condition of 10vp in all four cell lines tested **(Figure 13 a, b, c, d),** especially in A549 cell line where we found that the titer is one log higher in the complex.

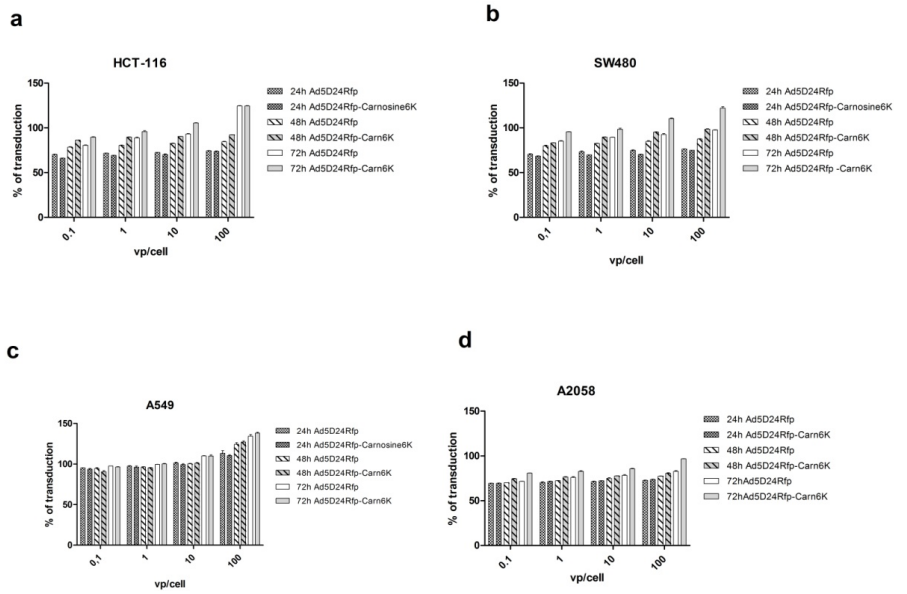


**Figure 12 a, b, c, d. In vitro transduction efficacy.** Transduction efficacy was evaluated in A549, HCT-116, SW480 and A2058 cell lines by infection with an oncolytic adenovirus encoding for the red fluorescent protein with or without

Carnosine6K. Red fluorescence was measured using Varioskan plate reader 24, 48 and 72h post infection.

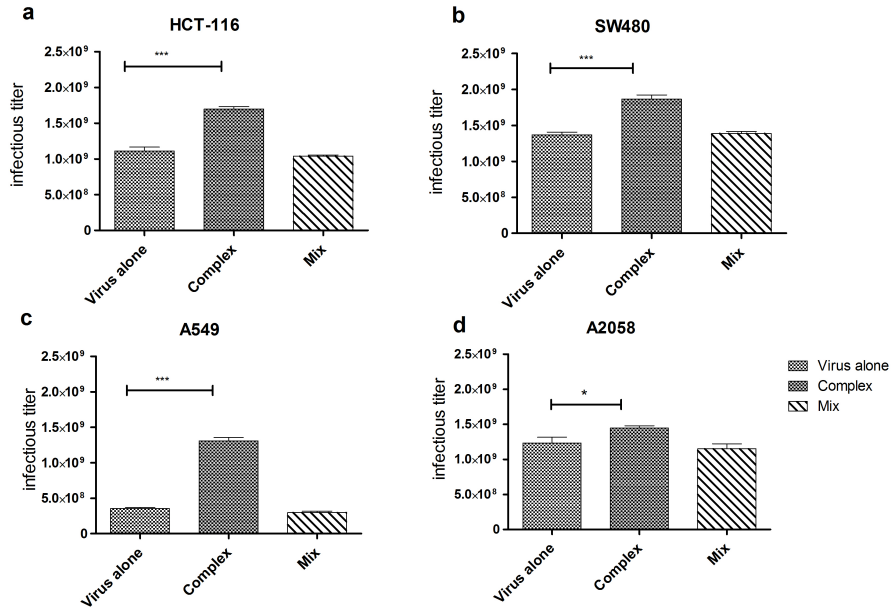


**Figure 13 a, b, c, d. Carnosine6K coated virus shows high infectious titer in different human cancer cell lines**. The infectious titer of Ad5D24CpG, Ad5D24CpG-Carnosine6K, Complex, and Ad5D24CpG-Carnosine6K mixed we performed an ICC assay. The target molecule in the assay is the virus hexon protein, an antigen associated with the viral capsule. Upon the infection, cells synthesize excessive amount of hexon proteins, which are targeted by first antibody. This mouse-origin antibody, in turn, is targeted by the second biotin-sp-conjugated antibody, which functions as a platform for extravidine peroxidase. After the addition of the stain, dab is oxidized by peroxidase, forming an insoluble brown precipitate which gives the infected cells an intense dark color.

### *4.4 Complex can induce apoptotic and necrotic cell death*

Apoptosis is a physiological response to exogenous and endogenous death signals. In particular a prerequisite for tumor growth is the inactivation of apoptotic pathways which is also responsible for therapy resistance (Sjöström and Bergh, 2001). It has been demonstrated that L-carnosine treatment at concentration 100 mM is able to induce late apoptosis only in a small number of cells (Iovine et al; 2012), thus we studied the ability of the complex to induce apoptotic and necrotic cell death. We measured the amount of Annexin-V positive, i.e. early apoptotic cells, and propidium iodide (PI), i.e. late apoptotic/necrotic cells 24 and 48 hours after the treatment in all four cell lines **(Figure 14 a, b, c, d).** The levels of fold change at 48/24 hours of both the early and the late apoptotic/necrotic cells were elevated in the complex in all tested cell lines, especially in A549 and HCT-116 cell lines, compared to the virus alone and L-carnosine alone with or without six lysines.

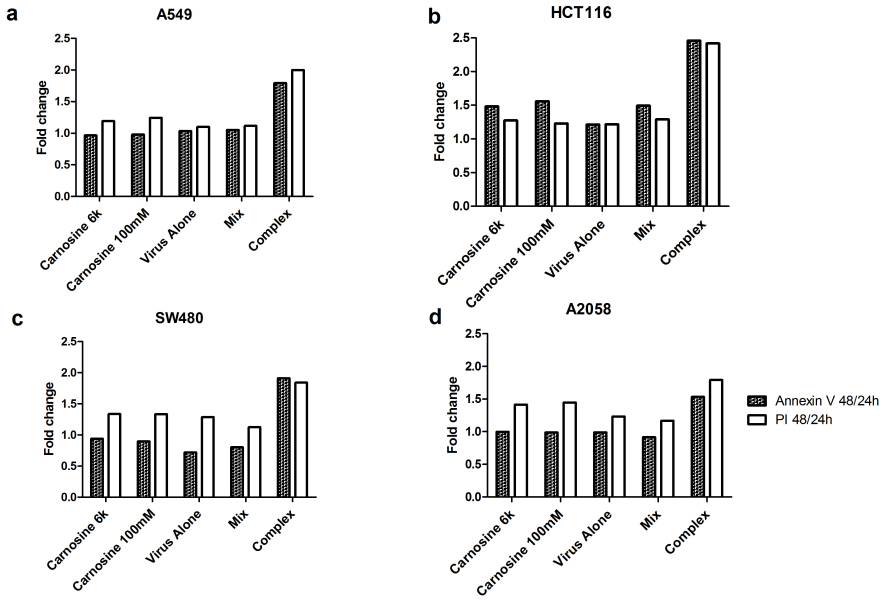


**Figure 14 a, b, c, d. Induction of apoptotic and necrotic cell death.** Early apoptotic and late apoptotic or necrotic cells are presented as fold change at 48/24 hours after infection. The amount of early apoptotic and late apoptotic or necrotic cells 24 and 48 hours after virus infection were analyzed by flow cytometry from A549, HCT-116, SW480, A2058 cell lines. FITC-labeled Annexin-V was used to indicate the early apoptotic cells and PI (propidium iodide) the necrotic or late apoptotic cells.

## *4.5 Complex induces autophagy activation*

We have investigated whether oncolytic adenovirus loaded with Carnosine6K can induce autophagy in HCT116 and A549 cell lines. One hallmark of autophagy activation is the formation of cellular autophagosome containing LC3-II. In the mammalian the precursor form of LC3 is post-modified into two forms, LC3-I and LC3-II. LC3-I is localized in the cytosol, then, it is cleaved by cysteine protease Atg4 to generate lapidated form LC3-II that specifically localizes in

autophagosomal membranes. The amount of LC3-II or the LC3-II/LC3-I ratio can be used to estimate the abundance of autophagosomes before they are destroyed through fusion with lysosomes (Mizushima and Yoshimori). In fact, the LC3-II conversion and integration into autophagosomal membrane, is one of the major sign of the activation of the autophagic process. We have analyzed the activation of autophagy in HCT116 and A549 cell lines. Cells were infected with an oncolytic adenovirus loaded with Carnosine6K respect to adenovirus alone or mix. Proteins were collected at 0, 24, 48 hours post treatment. An immunoblotting assay was used to reveal the two active species of LC3, the upper band corresponding to LC3-I (19Kd) and lower band corresponding to LC3-II (17Kd)(Kabeya, 2000). As shown in **Figure 15 (a, b)** in HCT116, complex promotes autophagy by increasing the conversion LC3-I to LC3-II, 24 and 48h post-infection. However, the best results were obtained 48h post-infection where the complex respect to adenovirus alone induces a complete conversion of LC3-I in LC3-II. In addition, in A549 complex promotes the conversion of LC3-I to LC3-II, 24h post-infection, respect to adenovirus alone or mix, while we could not find a different ratio LC3-II/LC3-I after 48h. Meanwhile we have evaluated by western blotting analysis the intracellular levels of p62 **(Figure 16)**, a protein that is degraded through autophagy, and we have found that protein levels were decreased in both cancer cells.

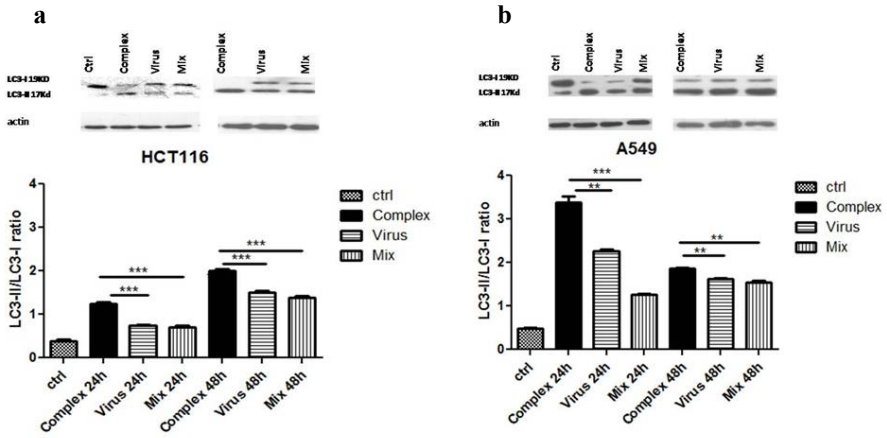


**Figure 15 a, b Induction of autophagy**. LC3 II/LC3 I ratio in HCT-116 and A549 cell lines. Cells were infected with an oncolytic adenovirus loaded with Carnosine6K respect to adenovirus alone or mix. Proteins were collected t 24 and 48 hours post treatment. An immunoblotting assay was used to reveal LC3.

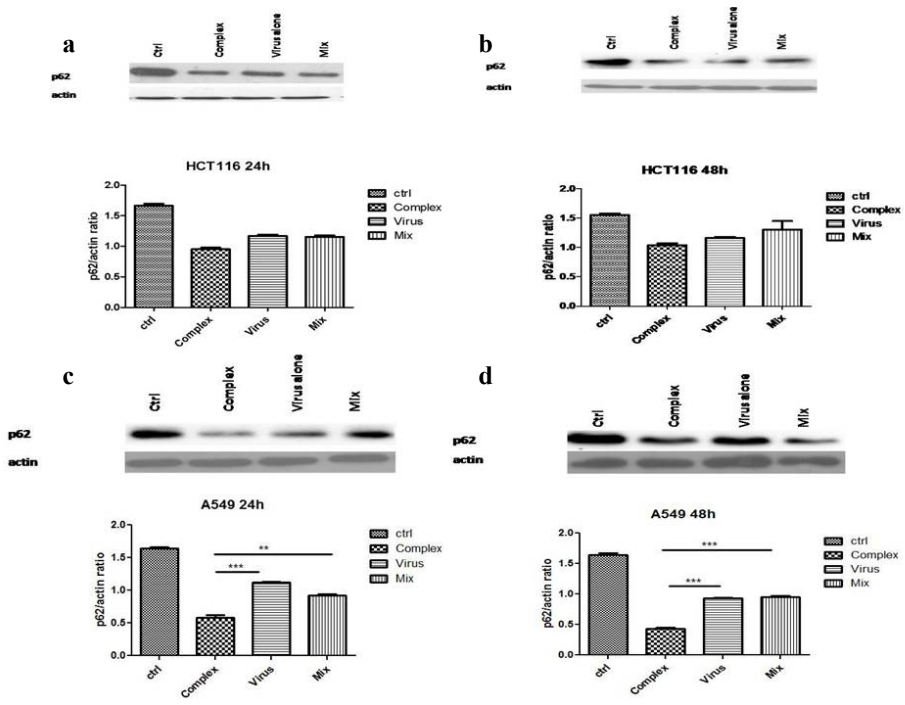


**Figure 16 a, b, c, d. Analysis of the intracellular levels of p62.** p62/actin ratio was in A549 and HCT-116 cell lines. Cells were infected with an oncolytic adenovirus loaded with Carnosine6K respect to adenovirus alone or mix. Proteins were collected at 24 and 48 hours post treatment. An immunoblotting assay was used to reveal p62.

## *4.6 Complex displays increased efficacy in vivo in a xenograft model of lung and colon cancer*

The oncolytic activity of the complex was tested in a lung and colon cancer xenograft model. Nude mice bearing A549 and HCT-116 cell tumors in the flanks were treated intratumorally with $1x10^8$ VP/tumor of viruses (or PBS) on days 0, 2, 5 and the tumor growth was followed over time. We observed that the tumor growth was

significantly ($p < 0.001$ at day 18) reduced in both complex treated mice compared to the control virus and carnosine6k alone (**Fig. 17a, b**). The intratumoral administration of the complex resulted in a clear tumor reduction and most of the tumors were fully cured within 18 days after the first virus injection. In tumor growth follow-up studies we observed that tumor growth of mice treated with complex was significantly suppressed compared to the mice treated with virus alone or mix.

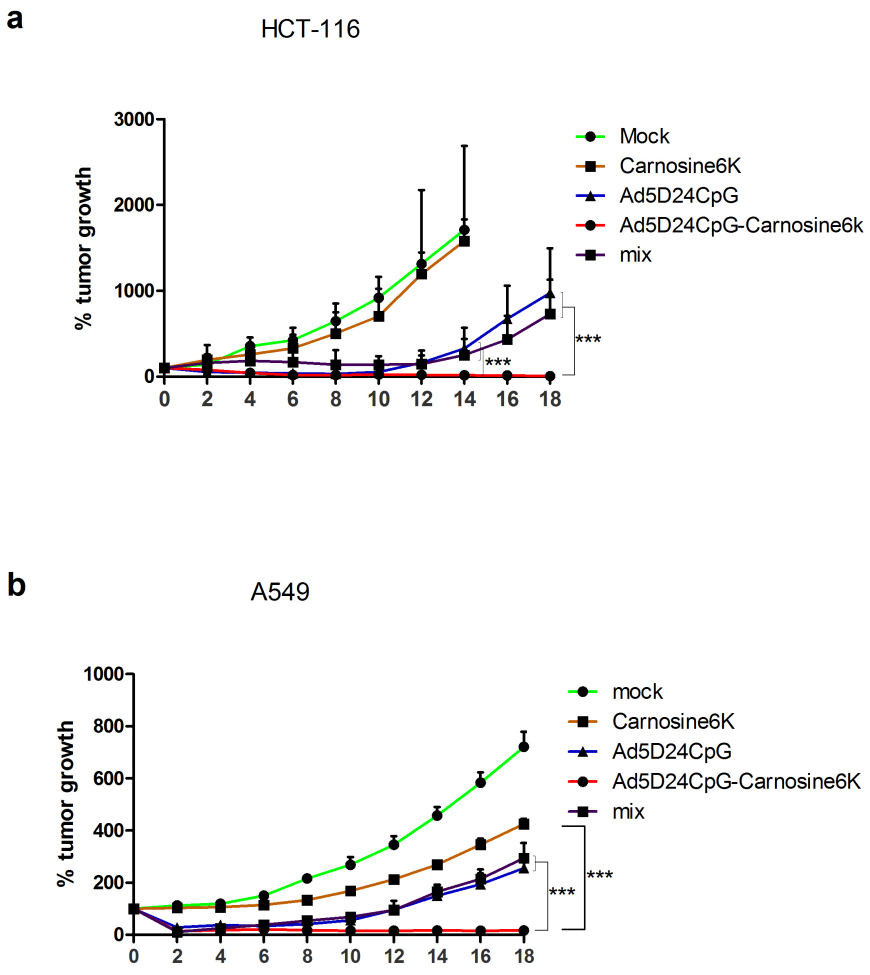


**Fig 17 a, b. In vivo efficacy of the complex.** Nude mice bearing A549 and HCT-116 tumors were treated intratumorally with PBS (mock) or with $1x10^8$vp/ tumor of Ad5D24CpG or Ad5D24CpG-Carnosine6K virus on days 0, 2 and 5. Tumor growth was measured over time.

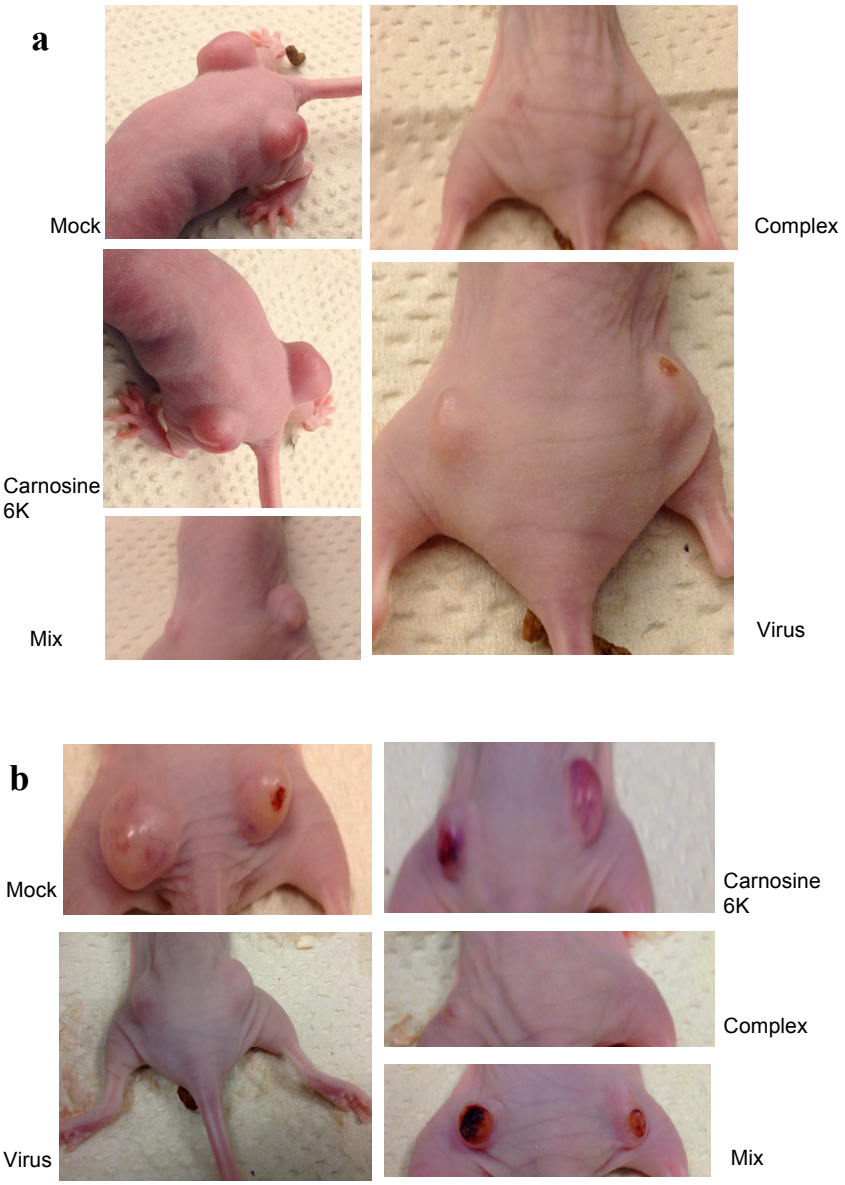


**Figure 18a Xenograft HCT-116 cancer mouse model, Figure 18b Xenograft A549 cancer mouse model**. Nude BALB/c mice bearing A549 and HCT-116 tumors were treated intratumorally with PBS (mock) or with $1x10^{8}$vp/ tumor of

Ad5D24CpG or Ad5D24CpG-Carnosine6K virus on days 0, 2 and 5. Tumor growth was measured over time.

# 5 Discussion

The ability of virus to kill cancer cells has been known for more than a century (Kelly and Russell, 2007). Their antitumor potency is due to several mechanisms, including direct lysis, apoptosis, expression of toxic proteins, autophagy, arrest of protein synthesis and induction of antitumoral immunity. Oncolytic adenoviruses are modified in order to be able to selectively kill cancer cells. Normal tissue is kept safe since viruses are targeted in different ways towards tumor cells leaving normal cells unharmed (Khuri FR et al; 2000). Although much is known about oncolytic adenoviruses, improvements to enhance their efficacy are still needed. Since every tumor and patient is unique, different researches have thought to arm oncolytic adenovirus coupled with chemotherapy (Siurala et al., 2014). One way to enhance gene delivery to tumor tissue is to create modified adenoviruses capsid for targeted cancer delivery. An example is Ad5/3-D24-GMCSF which is a chimeric oncolytic adenovirus, coding for granulocyte-macrophage colony-stimulating factor (GM-CSF), where fiber 5 knob was replaced by serotype knob 3 domain. This virus was modified to selectively replicate in p16/Rb defective cells, including human cancer cells. Ad5/3-D24-GMCSF, also called ONCOS-102, showed safety and efficacy in cancer patients both as a single agent and in combination with cyclophosphamide or low-dose pulse temozolomide (Koski et al., 2010). Another strategy to enhance transduction efficacy and prolongate the circulation time of the viruses is to attach the adenoviral surface by adding Polyethylene glycol (PEG). The covalent attachment of PEG to the viral capsid reduces hepatotoxicity and cytotoxicity in mice (Wonganan and Croyle, 2010). Furthermore, the major obstacle in the development of an effective cancer therapy is the cellular uptake of drug-like molecule. For this reason, the new anti-cancer strategies aim to deliver and facilitate the uptake and intracellular accumulation of drugs into cancer cells, while

reducing the non-specific uptake into normal cells. From this point the natural dipeptide L-carnosine could represent a good adjuvant candidate for the treatment of colon cancer for its bioavailability and because it is absorbed primarily via intestinal epithelium cells (Son et al 2004). In addition, recently the antioxidant dipeptide L-carnosine it has been the subject of study by our and others research group, for its significant anticancer activity both in vitro and in vivo. In fact, it has been reported that L-carnosine could inhibit mesangial cell proliferation by modulating cell cycle progression and affecting the proliferation of tumor cells in mouse (Jia et al., 2009). Furthermore, it has been demonstrated that the effect of the dipeptide on proliferation of tumor cells is related to an inhibition of ATP production and consequently to an anaerobic glycolysis (Renner et al; 2010). In addition, we have recently observed that treatment with 50-100mM of L-carnosine inhibits the proliferation of human colorectal carcinoma cells by affecting the ATP and Reactive Oxygen Species (ROS) production (Iovine B. et al., 2012). We have also found that L-carnosine reduces the HIF-1α protein level affecting its stability and decreases the HIF-1 transcriptional activity (Iovine B. et al., 2014). In order to enhance the antineoplastic properties of L-carnosine, reducing the high concentration of peptide required to arrest tumor progression, and to improve the viral transduction and replication, we have developed a new treatment strategy against cancer.

The present study is focused on the development of a model to use an oncolytic adenovirus as a scaffold to deliver active drugs.

First, we have developed **a strategy** to conjugate peptides on viral capsid, **based on electrostatic interactions.** L-carnosine can be modified by adding a tail of six lysines, named as Carnosine6K, and finally attached to the negatively charged viral capsid. To proof the concept of our novel strategy, we carried out in vitro studies to analyze the electric surface charge. We have found that Carnosine6K

positively charged, can be combined with the surface of oncolytic adenovirus that is negatively charged, allowing the formation of **a complex (Ad5D24CpG-Carnosine6K).** The result was a change of charge from negative in the virus condition into positive in the complex condition.
Then, we have evaluated the **biological function** of the complex to better characterize the infectivity and the cancer cell killing activity. We have found, by performing a viability assay, that modified Carnosine6K (8mM) can reduce **cancer cell viability** as well as L-carnosine (100mM) in all tested cancer cell lines (A549, HCT-116, SW480, A2058). In addition, we have demonstrated that 100vp of the complex (Ad5D24CpG-Carnosine6K) is able to reduce cell viability even more than the uncoated oncolytic virus. Previously, Iovine et al. have observed that treatment with 50-100mM of L-carnosine decreased the levels of ATP in all four cancer cell lines analyzed. Therefore, we have evaluated **ATP levels**, by comparing each other Carnosine6K, the virus alone or the complex. The complex reduced ATP production, especially 100vp (80-90%), more significantly than uncoated oncolytic virus (12%) or Carnosine6K.

Next we have also investigated whether the presence of Carnosine6K affects the transduction efficacy of the oncolytic adenovirus by performing a **transduction assay**. We compared the transduction efficacy of Ad5D24Rfp uncoated virus with Ad5D24Rfp-Carnosine6K complex. We have found that the amount of fluorescent cells is significantly higher in cells that have been treated with Ad5D24Rfp-Carnosine6K complex, especially using 100vp of complex at 72 h post-infection, compared to cells treated with naked oncolytic adenovirus. In addition, we have showed, by an immunocytochemistry assay, that also **the infectious titer** of the complex was higher at a condition of 10vp, compared to naked oncolytic adenovirus or a mix with virus and Carnosine6k added separately. These results demonstrated that virus alone or with

Carnosine6k added separately in a mixture have the same infectious titer, suggesting stronger infectivity properties of the complex. Interestingly we found that in A549 cell line the titer is one log higher in the complex compared to the other conditions. However further investigation on this mechanism are needed. The inactivation of apoptotic pathways is mainly responsible for tumor growth. Therefore from the therapeutic point of view, it is necessary to induce apoptosis in cancer cells in order to improve the efficacy of cancer therapy. It has been shown that L-carnosine treatment at a concentration of 100 mM induced late apoptosis only in a small number of cells (Iovine et al., 2012). According to these observations, we have evaluated the ability of the complex to induce **apoptotic and necrotic cell death.** We have found that Carnosine6K is able to induce apoptosis and necrosis, as well as L-carnosine 100mM, in all four cell lines that we have analyzed. However, we found that the complex is able to induce apoptosis and necrosis, especially in A549 and HCT116 cell lines.

Finally, we have investigated the possible **molecular mechanisms** underlying the synergistic effect between Carnosine6K and the oncolytic adenovirus. For this purpose we have investigated whether oncolytic adenovirus loaded with Carnosine6K can induce **autophagy** in HCT116 and A549 cell lines. One hallmark of autophagy activation is the formation of cellular autophagosome containing LC3-II. In the mammalian the precursor form of LC3 is post-modified into two forms, LC3-I and LC3-II. LC3-I is localized in the cytosol and it is then cleaved by cysteine protease Atg4 to generate lapidated form LC3-II that specifically localizes in the autophagosomal membranes. The amount of LC3-II or the LC3-II/LC3-I ratio can be use to estimate the abundance of autophagosomes before they are destroyed through fusion with lysosomes (Mizushima et al., 2001). In fact, the LC3-II conversion and integration into autophagosomal membrane, is one of the major sign of activation of the autophagic process. We have found that an oncolytic adenovirus coated with Carnosine6K increases the

autophagy by promoting the conversion of LC3-I to LC3-II and decreasing levels of p62 protein. The p62 protein is degraded during autophagy and may serve to link ubiquitinated proteins to the autophagic machinery, to enable their degradation in lysosome. Since p62 accumulates when autophagy is inhibited and decreases when autophagy is induced, the analysis of its levels may be used as marker to study the autophagic flux, in response to pro-autophagic stimuli (Puissant et al., 2012). Our results support the idea that Carnosine6K and an oncolytic adenovirus, delivered as a single entity have **a synergistic effect**. These results suggest that the enhanced oncolytic potency of complex is, at least in part, due to the fact that the L-carnosine modified, as Carnosine6K, increases cancer autophagy by improving the efficacy of Ad-mediated oncolysis. Some authors have reported that some oncolytic viruses can reverse the autophagic pathway, by promoting their replication (Jackson et al. 2005). However, as a single therapeutic agent an oncolytic adenovirus could not efficiently destroy the large tumor mass in patients (Kirn, 2001; McCormick, 2003; Zhao et al., 2003). In fact adenoviruses alone induce autophagy activation, even if there are many evidences that chemotherapeutic agents can increase cancer cell autophagy, by improving the efficacy of oncolytic virotherapy (Koski et al., 2010).

In the end we have investigated **the efficacy in vivo of the complex in human colon cancer and lung cancer** xenograft model. Horii Y et al. demonstrated that the intraduodenal administration of L-carnosine decreases splenic sympathetic nerve activity (splenic-SNA) and inhibits the proliferation of a human colon cancer cell line HCT116 implanted into athymic 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 proliferation. Starting from these results we have evaluated that the intratumoral administration of the complex resulted in a clear tumor reduction and most of the tumors were fully

eradicated within 18 days after the virus injection. In tumor growth follow-up studies we observed that tumor growth of mice treated with complex was significantly suppressed compared to the mice treated with virus alone. These results are in agreement with the synergistic effect between Carnosine6K and oncolytic adenovirus that we have found in vitro.

## *5.1 Conclusions*

Cancer is still a leading cause of death worldwide. Although many kinds of treatment have been developed during the past decades, there is still a lack of effective therapy for advanced cancer. Many studies have shown that oncolytic adenoviruses have been used in clinical trials and in cancer treatments (Koski et al., 2010; McCormick, 2003; Crompton and Kirn, 2007). Oncolytic virotherapy is emerging as a potential approach to treat cancer, using viruses which are specifically engineered to selectively infect, replicate in and kill cancer cells without causing damage to normal cells. In this study we are proposing a novel strategy using oncolytic adenovirus as a scaffold to deliver active drugs. We have demonstrated that the dipeptide L-carnosine can be modified by adding a tail of polylisines on the viral capsid through an electrostatic interaction between positively charged Carnosine6K and the viral capsid which is negatively charged. Furthermore, we have shown in vitro and in vivo that the complex Carnosine6K with oncolytic adenoviruses (Ad5D24CpG-Carnosine6K) has a synergistic effect. In addition, the complex decreases cell viability of human cancer cells enhances the transduction efficacy and the infectious titer. We have also investigated the possible molecular mechanisms underlying the synergistic effect between Carnosine6K and the oncolytic adenovirus, and we have observing that an oncolytic adenovirus loaded with L-Carnosine6K can induce autophagy in both HCT116 and A549 cancer

cell lines. The in vivo efficacy of the complex was performed in human colon cancer and lung cancer xenograft model. The intratumoral injection of the complex has efficiently reduced tumor growth and most of the tumors were fully eradicated within 18 days after the treatment. In conclusion, the results described in this study encourage the use of Ad5D24CpG-Carnosine6K for new therapeutic protocols for the treatment of cancer and in particular human colon cancer and lung cancer. Moreover the use of oncolytic adenoviruses loaded with active drugs could be used as a novel drug delivery system for cancer therapy.

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