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“The role of autophagy in neoplastic cell response to oncolytic viruses”

Ginevra Botta

University of Naples Federico II
Dipartimento di Biologia e Patologia Cellulare e Molecolare
“L. Califano”

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Nancy Carrasco, MD

Ai miei genitori,
con affetto

“The role of autophagy in neoplastic cell response to oncolytic viruses”

TABLE OF CONTENTS

LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

G.Botta, G. Perruolo, S. Libertini, A. Cassese, A. Abagnale, F. Beguinot, P. Formisano and G. Portella
PED/PEA-15 modulates Coxsackie and Adenovirus Receptor (CAR) expression and adenoviral infectivity via ERK-mediated signals in glioma cells.
Human Gene Therapy, Pending revision

S. Libertini, A. Abagnale, **G. Botta**, C. Passaro, and G. Portella
Aurora B inhibitor AZD 1152 induces mitotic catastrophe and enhances the effects of E1A defective oncolytic adenovirus dl922-947 in human anaplastic thyroid carcinoma cells in vitro and in vivo.
Cancer Research, Submitted for publication.

F. Panariello, G. Perruolo, A. Cassese, F.Giacco, **G. Botta**, A.P.M. Barbagallo, G. Muscettola, F. Beguinot, P. Formisano, and A.de Bartolomeis
Clozapine and haloperidol affect insulin sensitivity by up-regulating Akt and Ped/Pea-15: a putative mechanism for impairment of glucose metabolism.
European Neuropsychopharmacology, Submitted for publication

S. Libertini, A. Abagnale, C. Passaro, **G. Botta** and G. Portella
Aurora B Kinase inhibitors as novel agents for cancer therapy.
Recent Patents on Anti-cancer Drugs Discovery, Submitted for publication

G. Botta, S. Libertini, A. Abagnale, C. Passaro, G. Hallden, P. Formisano and G. Portella
Autphagy protects glioma cells from oncolytic dl922-947 infection: implication for therapy
Oncogene, Manuscript in preparation.

ABSTRACT

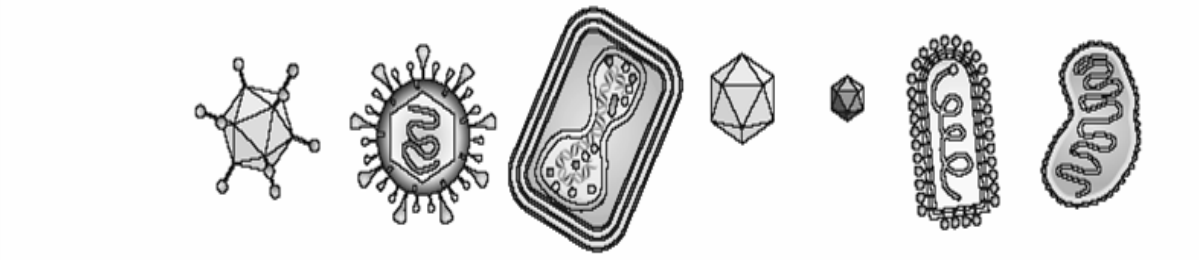
Oncolytic conditionally replicating adenoviruses (CRAds) are viral mutants able to selectively replicate in tumour cells. CRAds are considered a promising platform for cancer therapy. *dl*922-947, bearing a mutation in E1A gene and AdΔΔ, carrying mutations in both E1A and E1B genes, exhibited an antitumour effect against glioma and prostate cancer cells, respectively. Recently, it is thought that the adequate modulation of autophagy can enhance efficacy of anticancer therapy. However, the outcome of autophagy manipulation depends on the autophagy initiator, the combined stimuli, the extent of cellular damage and the type of cells. In this study, I characterized the role of autophagy in oncolytic adenovirus-induced therapeutics effects. When autophagy was inhibited at different steps by chloroquine (HCQ) or 3-methyladenine (3-MA), the cytotoxicity of *dl*922-947 and AdΔΔ in glioma and prostate cancer cells was augumented. These findings indicate that autophagy is a cell survival response in infected cells. Moreover, I showed that the oncolytic adenoviruses activated the Akt/mTOR/p70s6k pathway, that plays a central role in the negative regulation of autophagy, and, accordingly, inhibited the ERK pathway, that is a positive regulator of autophagy. Interestingly, a MEK inhibitor, PD98059, synergistically sensitized glioma cells to *dl*922-947 by increasing autophagy inhibition. These findings suggest that a disruption of ERK signalling pathway could greatly enhance the efficacy of CRAds by inhibiting autophagy.

The observation that autophagy inhibitors increase adenoviruses antitumor activity in cancer cells suggests a novel multimodal strategy for virotherapy.

1. BACKGROUND

1.1 Oncolytic viruses in the treatment of cancer

An *oncolytic virus* (OV) is a virus used to treat cancer due to its ability to specifically infect and lyse cancer cells, while ideally leaving normal cells unharmed. These viruses are essentially tumor-specific, self-replicating, lysis-inducing cancer killers. They are self-perpetuating in cancerous, rapidly dividing tissue and will continuously infect and replicate as long as the host's cell population is permissive. Viruses which have been mutated to be dependent on certain molecular defects in cancer cells are called *conditionally replicating* (i.e. restricted to replicate only in permissive cells).
Oncolytic virotherapy is a novel promising form of gene therapy for cancer. By using a variety of viral vectors that are capable of replication specifically in tumor cells, virotherapy is finally emerging as potentially useful anticancer strategy. Numerous oncolytic viruses are currently in Phase I and II clinical testing in many different countries, showing extremely encouraging results. The first marketing approval for an oncolytic virus was granted by Chinese regulators in 2005 (Russell and Peng 2007).
Members from an increasing number of virus families are being investigated as oncolytic agents for cancer treatment. Some of these viruses are reported in table below (table 1).



(Cattaneo et al. 2008)

Table 1. **Oncolytic viruses that are currently used in cancer clinical trials.**
HSV1, herpes simplex virus 1; MV, measles virus; NDV, Newcastle disease virus; VSV, vesicular stomatitis virus.

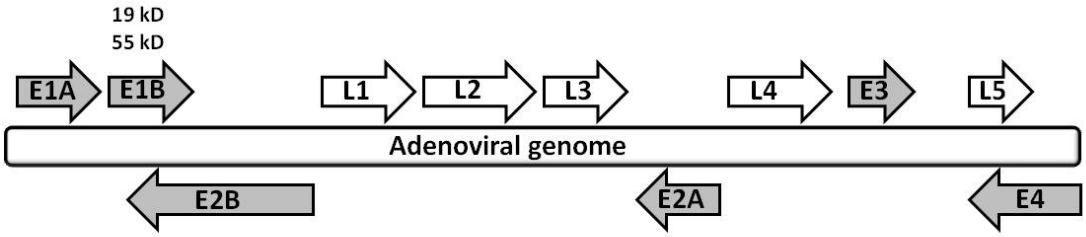
1.1.1 Oncolytic adenoviruses biology

Adenoviruses are the most widely used oncolytic viruses in cancer therapy and also the most widely described. Adenovirus is a non-enveloped, 80-110 nm diameter virus presenting icosahedral symmetry. The 51 distinct serotypes of human adenovirus have been classified into six groups (A–F) based on sequence homology (Shenk 1996). Most studies have been carried out on adenovirus serotype 2 (Ad2) and Ad5. Human adenoviruses contain a linear, double stranded DNA genome of 30-36 Kb. Adenovirus infection occurs through binding of the adenoviral fiber to cellular receptors such as the coxsackie-adenovirus receptor (CAR) or integrins. After the virus internalization through endocytosis the virus escapes the endosome and translocates to the nuclear pore complex, where the viral DNA is released into the nucleus and transcription begins. Transcription, replication and viral packaging take place in the nucleus of the infected cell. Adenoviral transcription occurs in two phases: early and late (Fields et al. 1996).
The first gene that is transcribed in the viral genome is *E1A*. Two regions of conserved sequence among E1A proteins of different adenovirus types are conserved regions 1 and 2 (CR1 and CR2). During infection, the primary mechanism by which E1A forces quiescent cells to actively cycle is by interfering with proteins of the retinoblastoma (Rb) pathway (Harlow et al. 1986; Moran 1993) and this interaction is mediated primarily by CR2. The E1A product is able to sequester Rb and release repression of E2F, allowing it to activate its target genes.
The E1B transcription unit encodes two proteins, E1B-55kDa and E1B-19kDa. The E1B-19kDa protein is a functional homologue of the proto-oncogene-encoded Bcl-2 and prevents apoptosis by similar mechanisms (Debbas and White 1993, Rao et al. 1992). The E1B-55kDa protein complexes with the amino-terminal end of p53 and inhibits its activity as a transcription factor (Kao et al. 1990, Yew and Berk 1992, Yew et al. 1994). In addition to its antiapoptotic functions, the E1B-55KDa protein facilitates the transport of viral mRNAs to the cytoplasm during the late stages of infection (Pilder et al., 1986). The E2 region encodes proteins necessary for replication of the viral genome: DNA polymerase, preterminal protein, and the 72-kDa single-stranded DNA-binding protein (De Jong et al. 2003). Products of the viral E3 region function to subvert the host immune response and allow persistence of infected cells. The E4 transcription unit encodes a number of proteins that have been known to play a role in cell cycle control and regulation of DNA replication. The viral structural proteins and the proteins necessary for assembly of the virion are encoded by genes expressed during the late phase of viral replication.

1.1.2 Genetic modifications in adenoviruses for cancer selectivity

There are different ways in order to develop tumour specificity in oncolytic adenoviruses, but the most widely-used strategy consists in the generation of replication-conditional adenoviruses. These viruses are genetically modified, by altering viral genes that attenuate replication in normal tissue but not in tumour cells. For example, some viruses have been designed to target cancer cells bearing mutations in the tumor suppressor proteins p53 or pRB. Inhibition of p53 protein activity must be blocked in normal cells in order to allow efficient viral replication. the first OV developed, *dl*1520 (ONYX-015), contains an 827-bp DNA deletion in the E1B region of the viral genome, thus lacking of E1B-p55 protein. It was hypothesized that normal cells, upon infection with *dl*1520, should generate a p53 response that leads to apoptosis, preventing *dl*1520 virus replication (Fig. 1). In contrast, tumor cells lacking a functional p53 gene should be unable to suppress viral replication. Restricting replication of *dl*1520 to p53-deficient tumor cells results in selective destruction (O'Shea et al. 2005). Similar virus to *dl*1520 is the oncolytic adenovirus (H101), bearing a E1B-55kDa gene deletion. The world's first oncolytic virus approved for the treatment of cancer patients (China).
Other CRAd have been generated by mutating the E1A region of the adenoviral genome. A second generation E1A adenoviral mutant is *dl*922-947, which carries a 24-bp deletion in E1A Conserved Region 2 (CR2) therefore, is unable to induce progression from G1 into S-phase of quiescent cells. The G1-S checkpoint is critical for cell growth progression and is lost in almost all cancer cells as a result of mutations or deletions of the RB or *CDKN2A* genes, amplification and overexpression of Cyclin D, and amplification, overexpression or mutation of the CDK4 gene (Sherr 2000). The *in vitro* efficacy of *dl*922-947 was demonstrated in a range of a cancer cell lines and this efficacy exceeded that of adenovirus 5 wild-type (Ad5wt) and *dl*1520 (Heise et al. 2000). *dl*922-947 has also been shown to have a higher oncolytic activity compared with *dl*1520 in anaplastic thyroid carcinoma cell (Libertini S. et al. 2008). A similar adenovirus, Δ24, with the same deletion in E1A-CR2, has shown activity in preclinical models of glioma (Fueyo et al. 2000). *dl*922-947 has also an impressive in vitro activity in ovarian carcinoma and was able to produce some long-term survivors in an aggressive xenograft model (Lockley et al. 2006).
D. Oberg et al. have reported on the generation of potent replication-selective mutants targeting both altered pRb (ΔCR2) and apoptosis pathways (ΔE1B19K) with intact E3-region to improve efficacy and selectively both as single agents and in combination with standard clinical therapies. They have demonstrated that cell killing potency of the AdΔΔ mutant was either superior or similar to wild type virus in prostate, pancreatic and lung carcinoma cells. Moreover, they found

higher viral activity in vivo (human prostate cancer xenograft) when both CR2 and E1B19K regions were deleted (Oberg 2009).
The mutation of these reported adenoviruses are reported in the Figure 1.



Oncolityc virus	Mutation
Onyx (*dl*1520)	827 bp deletion in E1B-55 kD (ΔE1B)
*dl*922-947	24 bp deletion in E1A (ΔCR2)
AdΔΔ	E1B-19kD and E1A(ΔCR2) deletions

Figure 1. Adenoviral genome and mutations

1.1.3 The role of oncolytic adenoviruses in combination cancer therapy

Although the safety and the antitumour efficacy of many oncolytic viruses alone were demonstrated in clinical trials, oncolytic virotherapy, by itself, has not been effective in complete tumor eradication. It appears that the best chance for complete tumor eradication lies in combining oncolytic viruses with current chemo- and radiation therapies or with the emerging novel biological agents. There have been several preclinical and clinical trials looking at the benefit of adding oncolytic adenoviruses to radiation therapy and chemotherapy.
The addition of oncolytic adenoviruses to traditional chemotherapy has already entered Phase II trials with promising results. One such trial evaluated the use of intratumoral *dl*1520 injection in combination with cisplatin and 5-fluorouracil therapy in patients with recurrent squamous cell carcinoma of the head and neck (Nemunaitis et al. 2000). Another Phase II trial looked at the combination of *dl*1520 with leucovorin and 5-fluorouracil in patients with gastrointestinal

carcinoma metastatic to the liver (Kruyt and Curiel 2002). *dl*1520 was also administered intratumorally to patients with unresectable pancreatic cancer in combination with intravenous gemcitabine in a Phase I clinical trial (Post et al. 2003).

Recent results from a phase III clinical trial have confirmed the ability of an oncolytic adenovirus (H101) to increase the response rate of 15 nasopharyngeal carcinoma in combination with cisplatin (Crompton and Kirn 2007; Yu and Fang 2007). All these clinical trials have demonstrated that the combination treatment enhances the oncolytic viral effects.

In addition, the chemotherapeutic drug, taxol, in combination with the E1B-deleted chimeric oncolytic adenovirus SG235-TRAIL produced a synergistic cytotoxic effect in cancer cells as well as in the gastric tumor xenograft mouse model (Chen et al. 2009).

D. Oberg *et al*. have also reported that the clinically used cytotoxic drug docetaxel synergises with the newly generated AdΔΔ mutant in killing prostate cancer cells (Oberg 2009).

Some preclinical studies in combining oncolytic adenoviruses with radiation therapy have also provided several important findings. For example, enhanced antitumor action was shown with the combination of *dl*1520 and radiation therapy in anaplastic thyroid carcinoma (ATC) cells and tumor xenograft (Portella et al. 2003), as well as in tumor xenograft models of human malignant glioma and colon cancer (Geoerger et al. 2003).

In the last decade, novel drugs have been developed and tested in preclinical and clinical trials, showing promising results. These novel anti-cancer agents act against specific targets which are relevant for neoplastic cells proliferation, survival, invasion and other cancer features and they could be exploited to improve the efficacy of oncolytic viruses. Indeed, some evidences are reported. For example, Libertini S. et al. have demonstrated that the combined treatment with bevacizumab, a humanized anti-VEGF monoclonal antibody with an antiangiogenic action, significantly enhances the effects of *dl*922-947 against ATC tumor xenografts, by improving viral distribution within the tumour mass. (Libertini et al. 2008). The combination treatment of cyclooxygenase-2 (Cox-2) inhibitors with vaccinia virus is proved to be more effective that either treatment alone in treating ovarian tumors (Chang et al. 2009). Some Histone deacetylase (HDAC) inhibitors, such as trichostatin A (TSA), in combination with oncolytic herpes simplex virus (HSV) have been demonstrated to improve the therapeutic efficacy of in a human glioma xenograft model in vivo (Otsuki et al. 2008), as well as the antiangiogenetic and antitumoral efficacy in animal models (Liu et al. 2008). Thus, elucidating how the oncolytic effects can be potenciated is relevant for the development of novel therapeutic strategies.

1.1.4 Oncolytic viruses and cell death

In order to find drugs that could be used for the development of novel therapeutic strategies based on the use of oncolytic viruses, the elucidations of the molecular mechanisms of virus-induced cell death is necessary. Although oncolytic viuses have been tested in clinical trials, so far oncolytic virus-induced cell death mechanisms remain to be delucidated.

Cell death induced by replicating Ads has been often referred to as apoptosis. Some studies have shown that oncolytic viruses can induce apoptosis in some cancer cells both in vitro and in vivo, and that apoptosis seems a to contribute to enhanced antitumor effect in vivo (Li et al. 2008). However, despite the known apoptosis-regulatory function of individual Ad genes, it is currently unknown whether the disruption of cancer cells at the last stage of CRAd infection, named oncolysis, always employs the basic apoptotic machinery of the host cell (Mohamed et al. 2004). Infact, a recent investigation showed that CRAds cause non-apoptotic programmed cell death in tumor cells and that Ads evolved a mechanism for disrupting the host cell at the final stage of the viral cycle, that does not require the activation of the basic apoptotic machinery. The authors infact showed that CRAd-induced oncolysis was not associated with apoptotic DNA fragmentation caused by internucleosomal DNA cleavage (Mohamed et al. 2004). In ovarian cancer cells, *dl*922-947 has been found to produce some apoptotic morphological features such as caspase-3 activation. However, other typical features of apoptosis were not reported. Indeed, a pan-caspase inhibitor had no effect on viral citotoxicity, nor do miyochondria play any determing role. Therefore, the authors concluded that in ovarian cancer cells *dl*922-947 induces a non-apoptotic programmed cell death. The peculiar form of cell death viral-induced is confirmed also by the observation that well known inducers of apoptosis (i.e. cisplatin) activate the typical apoptotic pathway in ovarian cancer cells. Moreover, few biochemical markers of necrosis are found in infected cells (Baird et al. 2008).

More evidence is now accumulating that a “programmed cell death” (PCD) can occur in complete absence of caspases, and other, non-caspase proteases have been described to be able to execute PCD. Among models of caspase-independent death programs that have been described, autophagy has been proposed, caratherised by a distinctive set of morphological and biochemical features.

Recent data suggest that some oncolytic adenoviruses are able to activate the autophagic process in cancer cells. The oncolytic adenovirus regulated by the human telomerase reverse transcriptase promoter (hTERT-Ad, OBP-301) induced tumor-specific autophagic cell death in human malignant glioma, prostate cancer and cervical cancer cells, as well as in glioma xenografts (Ito et al. 2006) and autophagy –inducing agents augment its antitumor effect on glioblastoma cells (

Yokoyama et al. 2008). The Delta-24-RGD oncolytic adenovirus has been also shown to induce autophagic cell death in brain tumor stem cells (Jiang et al. 2007). Analysis of human glioma cells infected with a CRAd that utilizes the survivin promoter did not show evidences of apoptosis induction, whereas auto-phagosomal-mediated cell death was observed (Ulasov et al. 2009).
Baird SK et al. have shown that in ovarian cancer cells treated with the oncolytic virus *dl*922-947 the classical apoptotis does not occurre, neither evidences of pure necrosis are found, but autophagy is induced.
It is worth to note that literature data are controversial regarding the role of autophagy. Indeed, autophagy, differently from the other cell death models, can function both to enable cell survival during starvation, factors deprivation and to remove damaged organelles, or induce death in damaged cells without access to adequate survival factors (Klionsky and Emr 2000; Levine and Klionsky 2004). Thus, autophagy may have complex roles in mammals, and further investigations are required to better understand its role.

1.2 Autophagy

The word "autophagy" is derived from the Greek and means to eat ("phagy") oneself ("auto").
Cell homeostasis depends on the balance between the biosynthesis and catabolism of macromolecules. Cells respond to changes in their environment and intracellular milieu by altering their anabolic and catabolic pathways. Two major pathways in eukaryotic cells are involved in the catabolism of cellular material: the multi-enzyme proteasome system and the lysosome/vacuole (Codogno 2005).
The proteasomal degradative pathway is selective for proteins. The lysosomal system is responsible for the degradation of several classes of macromolecules and for the turnover of organelles by at least three different pathways: Cvt (cytosol to vacuole targeting pathway) (Scott et al.1996), Vid (vacuolar import and degradation pathway) (Shieh and Chiang 1998), and autophagy (Lockshin and Zakeri 2004). Whereas the ubiquitin-proteosomal system is the major cellular pathway for the degradation of short-lived proteins, autophagy is the primary intracellular catabolic mechanism for degrading and recycling long-lived proteins and organelles, by using a lysosomal degradation pathway.
Autophagy is a ubiquitous physiological process, that occurs at a basal level in most of eukaryotic cells, and this probably reflects its role in regulating the turnover of long-lived proteins (Bergamini et al. 2004). However, autophagy can represent a cellular response to both extracellular stress conditions (e.g., nutrient starvation, hypoxia, overcrowding, high temperature) and intracellular stress

conditions (e.g., accumulation of damaged or superfluous organelles and cytoplasmic components) and allows lower eukaryotic organisms, such as yeast, to survive nutrient starvation conditions by recycling. During periods of nutrient shortage, autophagy provides the constituents required to maintain the metabolism essential for survival (Lum et al. 2005).

In the developing organism, autophagy plays a role in the cellular and tissue remodeling that occurs during metamorphosis. In many tissues in the adult organism (especially postmitotic cells, e.g. neurons, cardiomyocytes), this function of autophagy is largely obsolete; however, protein and organelle turnover by autophagy plays an essential homeostatic or housekeeping function, removing damaged or unwanted organelles and proteins.(Levine and Klionsky 2004)

Autophagy is basically a non-selective process, in which bulk cytoplasm is randomly sequestered into the cytosolic autophagosome. However, in some cases it may select its target. For example, autophagy can selectively eliminate some organelles, such as injured or excrescent peroxisomes, endoplasmic reticulum (ER) and mitochondria (Elmore et al. 2001).

Depending on the delivery route of the cytoplasmic material to the lysosomal lumen, four different primary forms of autophagy are known: macroautophagy, microautophagy, chaperone-mediated autophagy (CMA), and crinophagy (Eskelinen 2005). During CMA, cytosolic proteins with particular peptide sequence motifs are delivered to lysosomes with the help of molecular chaperones. (Mjeski and Dice 2004). In crinophagy, secretory vesicles directly fuse with lysosomes, which leads to degradation of the granule contents (Glaumann 1989).

Differently from CMA, both micro- and macroautophagy involve vescicular traffic and differ with respect to the pathway by which cytoplasmic material is delivered to the lysosome but share in common the final steps of lysosomal degradation of the cargo with eventual recycling of the degraded material. Microautophagy is a form with few features. In this pathway, the membrane of the lysosome/vacuole directly invaginates material derived from the cytoplasm to form an internal vacuolar vesicle. It involves the engulfment of cytoplasm directly at the lysosomal surface, by invagination, protusion, and/or septation of the lysosomal limiting membrane (Levine and Klionsky 2004).

At present, the most prevalent and well characterized form of autophagy is macroautophagy.

The notable difference between macroautophagy and microautophagy is that in the latter the cytoplasm is directly up taken into the lysosome/vacuole (Wang and Klionsky, 2004).

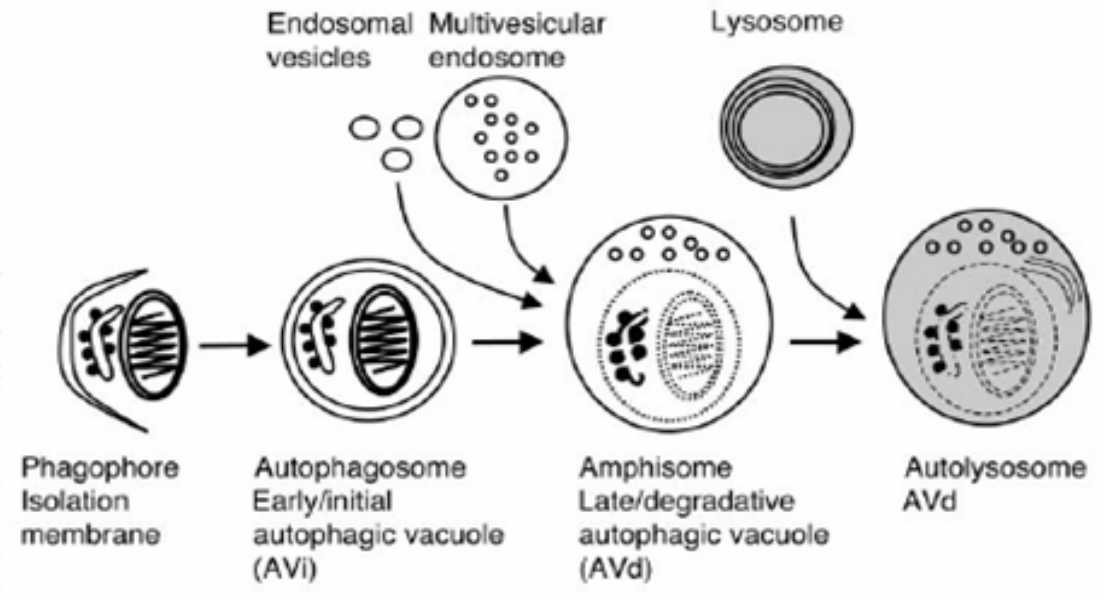
In contrast to microautophagy, macroautophagy involves the formation of cytosolic double-membrane vesicles that sequester portions of the cytoplasm. Fusion of the completed vesicle (autophagosome) with the lysosome results in

the delivery of an inner vesicle or autophagic body into the lumen of the degradative compartment (Levine and Klionsky 2004).

There is still debate on the origin of autophagosome membranes. Initially, double-membrane structures were believed to be derived from the ribosome-free region of the rough endoplasmic reticulum (Yokota et al.1993), but now it is generally accepted that they might originate from a pre-existing membrane structure called a phagophore, a poorly characterized organelle (Stromhaug et al. 1998), or could be formed *de novo* (Noda et al. 2002).

Autophagosomes undergo a stepwise maturation process including fusion events with endosomal and/or lysosomal vesicles (Dunn WA Jr 1994). Autophagosomes that have fused with endosomes are called *amphisomes* or *intermediate autophagic* vacuoles. The term *autolysosome* refers to an autophagosome or amphisome the has fused with a lysosome. The term *autophagic vacuole* refers to an autophagosome, amphisome or autolysosome. Morphologically, autophagic vacuoles can be further classified into early or initially autophagic vacuoles (AVi), containing morphologically intact cytosol or organelles, and to late or degradative autophagic vacuoles (AVd), containing partially degraded cytoplasmic material. During the maturation process the segregated cytoplasm, still engulfed by the inner limiting membrane, is delivered to the endo/lysosomal lumen. Both the cytoplasm and the membrane around it are then degraded by lysosomal hydrolases, and the degradation products are transported back to the cytoplasm, where they can be eventually reused for the metabolism (Eskelinen 2005) (Fig. 2).

So, the maturation of autophagosomes in mammalian cells is a multi-step process including several fusion events with vesicles originating from the endo/lysosomal compartment. Lysosomal membrane proteins and enzymes are present in both late endosomes and lysosomes, indicating that these protein can be delivered to autophagic vacuoles during fusion with either of them. In agreement with experimental data, autophagosomes (AVi) do not contain lysosomal membrane proteins or enzymes, while both amphisomes and autolysosomes (AVd) do (Tanaka Y et al. 2000).



Eskelinen et al. 2005

Figure 2. A schematic presentation of the formation and maturation of autophagosomes in mammalian cells.

1.2.1 Autophagy inhibitors

Several autophagy inhibitors have been developed acting at different autophagic steps. During maturation autophagic vacuoles become acidic (Punnonen et al. 1992). In mouse epatocytes the pH values of AVi and AVd are estimated to be 6.4 and 5.7, respectively. It is suggested that acidification begins before the delivery of lysosomal enzymes, via fusion with vesicles containing lysosomal membrane proteins and proton pumps, but no lysosomal enzymes (Dunn 1990). Infact, the vacuolar H^+-ATPase is known to mediate the acidification process, leading to the formation of acidic vescicular organelles (AVOs). This process is inhibited by *bafilomycin A1*, a specific inhibitor of the lysosomal proton pump. It has been demonstrated that bafilomycin A treatment inhibits fusion of autophagosomes with both endosomes and lysosomes, suggesting that acidification of autophagic vacuoles, and/or endo/lysosomes, might be needed for fusion (Mousavi et al. 2001). Other drugs inhibit the maturation of autophagic vacuoles and thus cause their accumulation in mammalian cells. *Chloroquine* (HCQ) is a lysosomotropic agent that as a weak base attracts to lysosomes, which compromises their normal degradation and recycling capacity, thus also resulting in an inhibition of autophagy. Another important mediator of the fusion event are microtubules, because treatment of cells with microtubule-destabilizing drugs blocks

autophagosomes maturation. The microtubule inhibitor *vinblastine* causes accumulation of mainly early autophagic vacuoles in hepatocytes, by inhibiting the fusion of autophagosomes with lysosomes and probably also with endosomes. Another microtubule inhibitor, *nocodazole*, causes accumulation of intermediate or late autophagic vacuoles in fibroblasts (Eskelinen et al. 2002). Cells treated with *cytochalasin D*, an agent that disrupts actin filaments, display a significant reduction in autophagosomes formation (Blankson et al. 1995), whereas the microtubule stabilization mediated by a new antitumor drug, *taxol*, increases the fusion of amphisomes with lysosomes (Bursch et al. 2000).
Inhibition of lysosomal enzymes, such as *cathepsins D, B* and *L*, also causes accumulation of late autophagic vacuoles. *Leupeptin*, that blocks the activity of lysosomal proteases, has been reported to inhibit degradation of segregated cytoplasm, causing accumulation of autophagic vacuoles (Eskelinen 2005)
Aother early autophagy inhibitor is 3-methyladenine (3MA) (Shintani and Klionsky 2004).
The multi-step autophagic process and the main autophagic inhibitors are summarysed in figure 3.

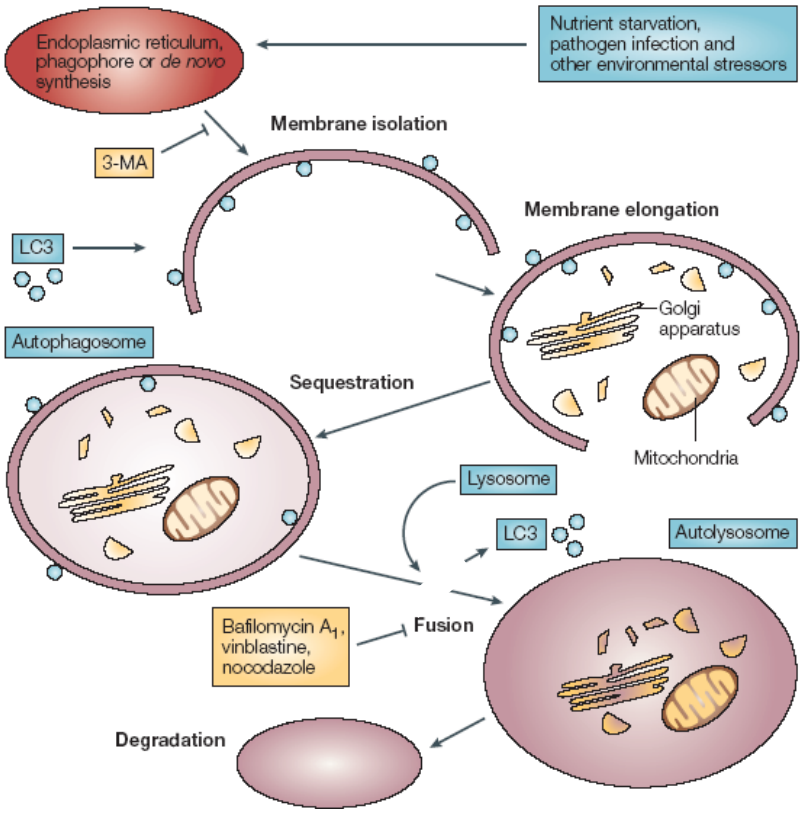


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Figure 3. The cellular process of autophagy.
Conditions such as nutrient starvation, pathogen infection and other environmental stressors, can induce autophagy. Autophagy begins with the isolation of double-membrane-bound structures inside an intact cell. The elongated double membranes form autophagosomes, which sequester cytoplasmic proteins and organelles such as mitochondria. The formation of the pre-autophagosomal structure can be inhibited by the phosphatidylinositol 3-phosphate kinase (PI3K) inhibitor 3-methyladenine (3-MA). The autophagosomes mature with acidification by the H+-ATPase and fuse with lysosomes to become autolysosomes (also known as the degradative autophagic vacuoles). Microtubules are important mediators of this fusion process. This process is inhibited by the H+-ATPase inhibitor bafilomycin A1, or by microtubule inhibitors such as vinblastine and nocodazole. Eventually, the sequestered contents are degraded by lysosomal hydrolases for recycling.

1.2.2 Molecular mechanisms of autophagy

Several molecules involved in the autophagic process have been identified, although the morphology of autophagy was first characterized in studies of mammalian cells, the molecular components of autophagy were initially elucidated in yeast. The study of autophagy in yeast have allowed the identification of the molecular machinery and their biological functions in higher eukaryotes, revealing a conservation of the autophagic mechanism (Mizushima et al. 2002). At least 25 specific yeast genes are exclusively involved in autophagy, and more than 40 additional yeast genes are also required for autophagy (Yang 2005). Recently, the autophagy-related genes and the products of these genes were named *ATG* and Atg, respectively (Klionsky et al. 2003). Despite the the resulst of previous studies have given a deep insight in the knowledge of autophagy, the physiological functions of many of these genes need to be further clarified. One of the most remarkable findings regarding the Atg proteins is the discovery of two ubiquitin-like conjugation systems, Atg12-Atg5 and Atg8-phosphatidylethanolamine (PE). Both Atg12-Atg5 and Atg8 conjugation systems are involved in autophagosomes formation. These genes are well conserved among eukaryotes, and are related to each other. Microtubule-associated protein 1 light chain 3 (LC3), the mammalian orthologue of Atg8, targets to the autophagosomal membranes in an Atg5-dependent manner. Thus LC3 is the only accepted marker of the autophagosome in mammalian cells. In wild-type cells, LC3 is detected in 2 forms: LC3-I (18 kDa) and LC3-II (16 kDa) (Kabeya et al. 2000). Twenty-two amino acids in the C-terminus of the newly synthesized LC3 are cleaved immediately by the mammalian orthologue of the yeast cysteine proteinase Atg4, autophagin, to produce an active cytosolic form, LC3-I. Then with the catalysis of Atg7 and Atg3, LC3-I undergoes a series of ubiquitination-like reactions, and is modified to LC3-II. LC3-I is located in the cytoplasm, while LC3-II is a tightly membrane

bound protein and is attached to PAS and autophagosomes. The relative amount of membrane-bound LC3-II reflects the abundance of autophagosomes, so the induction and inhibition of autophagy can be monitored through measuring total and free LC3-II levels (Kabeya et al. 2000). The conjugation systems involving Atg genes are summarysed in figure 4.

In cells grown in serum- and amino acid-free medium the increase in the amount of LC3-II is observed. Autophagy is induced under these conditions, and Kabeya Y et al. found a correlation between the rate of LC3-II increase and the rate of autophagosomes formation. Inhibitors of autophagosomes formation, i.e. 3-MA suppress the starvation-induced increase of LC3-II. Conversely drugs able to induce an accumulation of autophagosomes, such as vinblastine, chloroquine and bafilomycin A1, have a strong LC3-II- increasing effect.

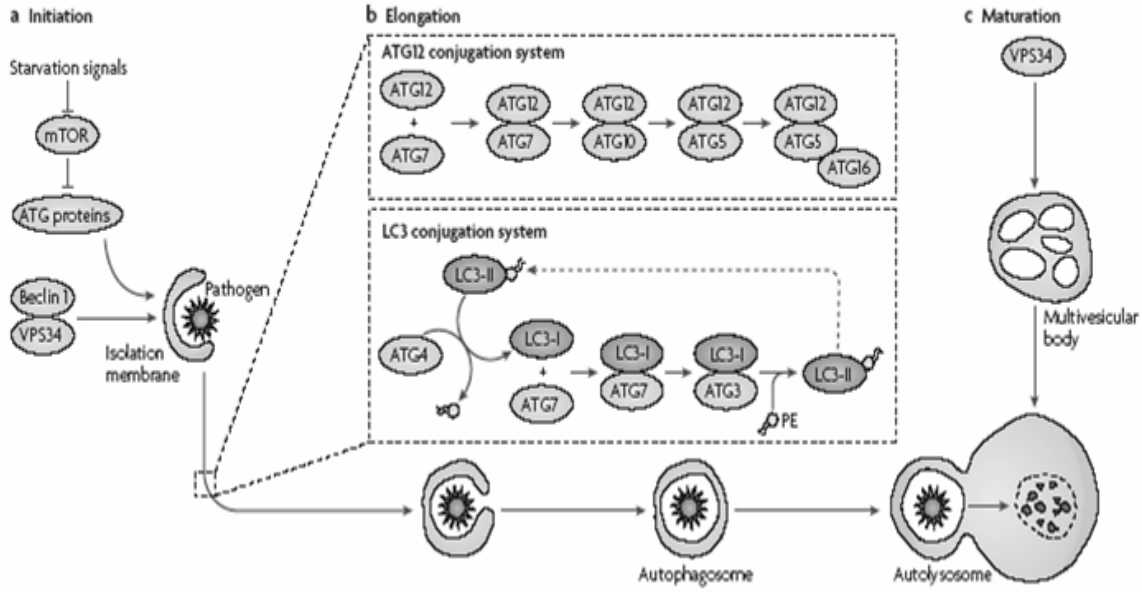

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Figure 4. Autophagy is regulated by a set of autophagy-related proteins (ATG proteins).
a) In the absence of amino acids or in response to other stimuli, ATG1 and a complex of the class III PI3K (phosphoinositide 3-kinase) VPS34 and beclin 1 lead to the activation of downstream ATG factors that are involved in the initiation (a), elongation (b) and maturation (c) of autophagy. b)The elongation and shape of the autophagosome are controlled by two protein (and lipid) conjugation systems, similar to the ubiquitylation systems: the ATG12 and LC3 (also known as ATG8)–phosphatidylethanolamine (PE) conjugation pathways, which include E1-activating and E2-conjugating enzymes. ATG12 is initially conjugated to ATG7 (an E1-activating enzyme) and then is transferred to the E2-like conjugating enzyme ATG10. This intermediate presents ATG12 for conjugation to an ATG5 lysine residue. The ATG5–ATG12 conjugate, stabilized non-

covalently by ATG16, triggers oligomerization on the outside membrane of the growing autophagosome, and enhances LC3 carboxy-terminal lipidation through the LC3 conjugation system. Upon autophagosome closure, ATG5–ATG12–ATG16 and LC3 (delipidated by ATG4) are recycled. C) LC3 associated with the lumenal membrane remains trapped in the autophagosome and is degraded during maturation into the autolysosome, which involves fusion of autophagosomes with late endosomes, including endosomal multivesicular bodies and lysosomal organelles, and dissolution of the internal membrane.

1.2.3 Regulation of autophagy

Autophagy is a multi-step process, and various signalling pathways have been implicated in its regulation (Meijer and Codogno 2004) (Fig. 5) .
One of most important pathways involved in the regulation of autophagy is the PI3K-Akt-mTOR signalling pathway.
Class I PI3K enzymes phosphorylate PtdIns4P and PtdIns(4,5)P2 to produce PtdIns(3,4)P2 and PtdIns(3,4,5)P3, which, via Pleckstrin Homology (PH) domains bind to protein kinase B (Akt/PKB) and its activator phosphoinositide-dependent kinase-1 (PDK1), that phosphorylates other kinases. It has been reported that the activation of this pathway either by receptors recruiting class I PI3K or by expressing a constitutive active form of PKB has an inhibitory effect on autophagy (Arico et al. 2001).
Upstream PI3K and Akt activation by growth factors activate mTOR, the mammalian target of rapamycin, a serine/threonine kinase belonging to the family of phosphatidylinositol kinase-related kinase. mTOR regulates translation and cell growth by its ability to phosphorylate both 4E-BP1 and p70s6k. p70s6 protein kinase of ribosomal 40S subunit S6 (p70s6) is the best candidate among potential mTOR substrates. Phosphorylation of S6 upregulates the translation of mRNAs containing 5' terminal oligopyrimidine tract (5' Top) that accounts for approximately 20% of all cell mRNAs. The major products of 5' Top mRNAs include ribosomal protein, elongation factor (EFla, EF2), and polyA binding protein. When nutrition is sufficient, TOR is turned on and the activity of the enzyme s6K increases.
The mammalian target of rapamycin (mTOR) kinase is also a key regulatory component that controls the induction of autophagy (Petiot et al 2002). Inhibition of mTOR (by nutrient-depletion, starvation or rapamycin) leads to cell cycle arrest, inhibition of cell proliferation, immunosuppression and induction of autophagy. Increased levels of the mTOR kinase are found to inhibit the autophagy process resulting in an increased in cell growth and tumor development. *Rapamycin* is a specific mTOR inhibitor, binding to a distinct region of mTOR upstream of the catalytic domain. It induces autophagy and inhibits the proliferation of a variety of cells (Takeuchi et al. 2005). The phosphatase PTEN,

which hydrolyzes PtdIns (3,4,5)P3, has a stimulatory effect on autophagy by relieving the class I PI3K/PKB inhibition (Arico et al. 2001).
Differently from class I PI3K, class III PI3K is a positive regulator of autophagy, promoting the sequestration of cytoplasmic material that occurs during autophagy (Petiot et al. 2000).
Beclin-1 is a 60KDa tumor suppressor protein that binds to class III P13K, forming a complex which promotes the trafficking of lysosomal enzymes to the lysosomes (Kihara et al. 2001). Reduced expression of Beclin-1 is associated with a reduced autophagic vacuole formation. Overexpression of Beclin-1 in MCF-7 human breast cancer cells is found to facilitate autophagy induced by serum and amino-acid deprivation, which indicates that Beclin-1 is a necessary regulator for autophagy (Liang 1999).
The mitogen-activated protein kinases are a family of serine-threonine kinases also involved in regulating autophagy. Infact, the extracellular signal-regulated kinases ERK1 and ERK2, when stimulated by the RAS–RAF1–mitogen-activated protein kinase kinase (MEK) signalling pathway, have been shown to induce autophagy in HT-29 colon cancer cells (Ogier-Denis et al. 2000).

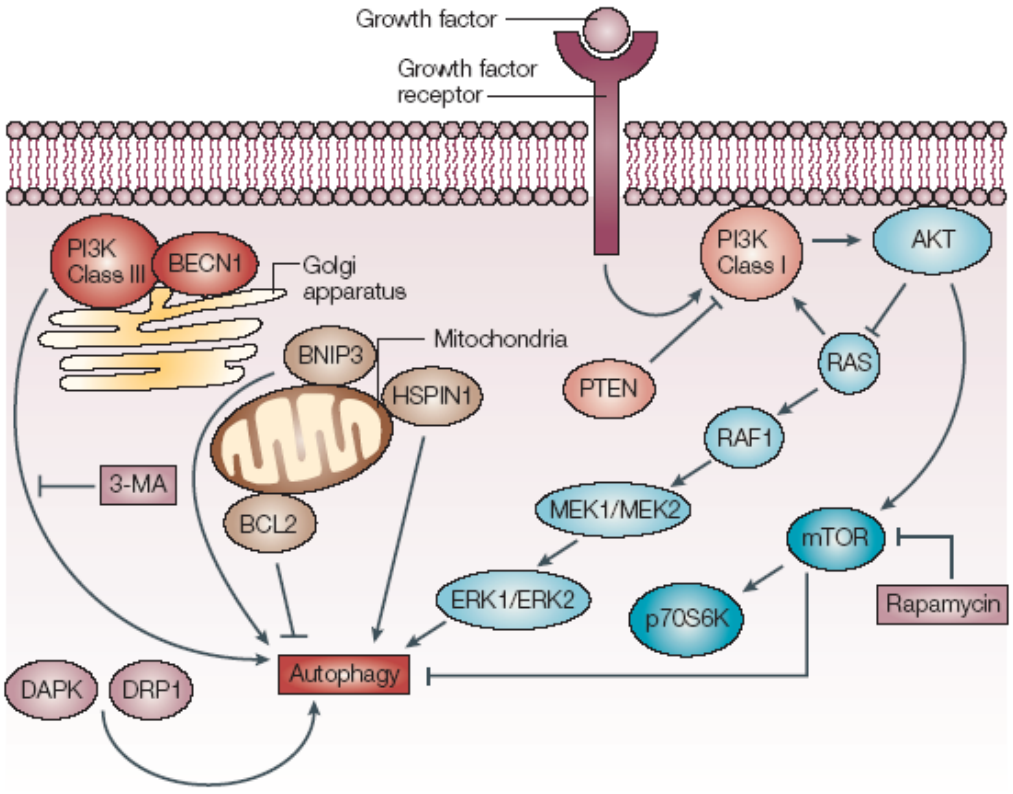

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Figure 5. The molecular regulation of autophagy.
In the presence of growth factors, growth factor receptor signalling activates class I phosphatidylinositol 3-phosphate kinase (PI3K) at the plasma membrane to keep cells from undergoing autophagy. PI3K activates the downstream target AKT, leading to activation of mammalian target of rapamycin (mTOR), which results in inhibition of autophagy. p70S6 kinase (p70S6K) might be a good candidate for the control of autophagy downstream of mTOR. Overexpression of the phosphatase and tensin homologue (PTEN) gene, by an inducible promoter, antagonizes class I PI3K47 to induce autophagy. When RAS activates the RAF1–mitogen-activated protein kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) cascade, autophagy is stimulated. Rapamycin, an inhibitor of mTOR, induces autophagy. A complex of class III PI3K and beclin 1 (BECN1) at the *trans*-Golgi network acts to induce autophagy. This pathway is inhibited by 3-methyladenine (3-MA). Downregulation of BCL2, or upregulation of BCL2–adenovirus E1B 19-kD-interacting protein 3 (BNIP3) or HSPIN1 at the mitochondria, also induces autophagy, indicating that BCL2 protects against autophagy. BNIP3 and HSPIN1 trigger autophagy. Autophagy is also induced by the cell death-associated protein kinase (DAPK) and the deathassociated related protein kinase 1 (DRP1).

1.2.4 Autophagy and cell survival

It has long been assumed that autophagy is a non specific process, in which cytoplasmic structures and macromolecules are randomly sequestered in order to generate the substrates and other molecules (e.g. amino acids) that are essential for cell survival when nutrients are scarce. However, we now know that, indeed, autophagy can also be very specific under certain conditions. Thus, the process can be involved in the elimination of damaged mitochondria (Rodriguez-Enriquez et al. 2004) or the selective removal of organelles that are functionally redundant (e.g. peroxisomes). Recent evidence suggests that the elimination of damaged mitochondria by autophagy may act as a rescue mechanism that the cell uses to escape from cell death, rather than as a mechanism producing cell death. A classical example is the mammalian liver, in which autophagy is switched on during starvation to produce amino acids which, after conversion into glucose, are used to meet the energy requirements of the brain and erythrocytes. Autophagy is also extremely important as a source of oxidizable substrates in the neonate, which is suddenly faced by a sudden interruption of the supply of nutrients via the placenta, but which has not yet received sufficient nutrients via the milk. Another situation in which autophagy is required to supply nutrients is that of cancer cells. Although suppression of autophagy may contribute to the initial rapid growth of tumors, in more advanced stages of cancer autophagy may be required to provide essential nutrients to the cells in the inner part of a solid tumor that do not have direct access to the circulation. Finally, autophagy can prevent cells from undergoing apoptosis by maintaining an adequate intracellular supply of substrates

despite nutrient depletion (Boya et al. 2005) or when the uptake of extracellular nutrients is inhibited by a lack of growth factor.

1.2.5 Autophagy and cell death

under some Autophagy can promote conditions cell death. Starvation-induced autophagy is a mechanism tightly controlled by amino acids and hormones. A more complex regulation of autophagy is observed during cell death since the signaling pathway overlap with those of the apoptotic signaling,
When involves autophagis cell death, is designated as type II PROGRAMMED CELL DEATH (PCD) in contrast to apoptosis, which is referred to as type I . The morphological and biochemical features of autophagic cell death and apoptosis are generally distinct. (Bursch et al. 2000). The main differences between apoptotic and autophagic cell death are summarysed in table 2.

Type of cell death	**Morphological changes**			**Biochemical features**
	Nucleus	***Cell membrane***	***Cytoplasm***	
Apoptosis	Chromatin condensation; Nuclear fragmentation; DNA laddering	Blebbing	fragmentation (formation of apoptotic bodies)	Caspase-dependent
Autophagy	Partial Chromatin condensation; No DNA laddering	Blebbing	increasing number of autophagic vescicles	Caspase-independent; Increased lysosomal activity

Table 2. Characteristics of apoptotic and autophagic cell death

Despite the different features of the two forms of programmed cell death, apoptosis and autophagy are not always separate and there can be crosstalk between the two pathways. In many cases, inhibition of apoptosis causes autophagy, and inhibition of autophagy triggers apoptosis (Kondo et al. 2005). When apoptosis was inhibited in mouse fibroblasts by a caspase-8 inhibitor, autophagic cell death was induced, and autophagy inhibitors decreased the amount of cell death (Yu et al. 2004). Conversely, the use of autophagy inhibitors such as

3-MA, or inhibition of autophagy by small interfering RNAs (siRNAs) targetted against autophagy-associated genes, induced apoptosis in HeLa cells (Boya et al. 2005).
Autophagy becomes apparent as a survival mechanism mostly in an apoptosis-defective background (Lum et al. 2005), thus tumor cells exhibiting signs of treatment-induced autophagy likely have inherent apoptotic defects and cannot undergo cell death characterized by classic signs of apoptosis. In this case, non-apoptotic cell death occurs via alternative death pathways, autophagy. The active role of autophagy as a cell death mechanism can be in principle validated by experiments documenting prolongation of cell survival upon autophagy downregulation (Chen and Karantza-Wadsworth 2009).
The mechanisms that regulate the mutually opposed survival-supporting and death-promoting roles for autophagy are still far from resolution. The most plausible explanation is that catabolism through autophagy is predominantly survival-supporting, but that an imbalance in cell metabolism, where autophagic cellular consumption exceeds the cellular capacity for synthesis, promotes cell death (Mathew et al. 2007).

1.2.6 Modulation of autophagy for cancer treatment

Autophagy is believed to play an important role in tumour development. When baseline levels were compared, the amount of proteolysis or autophagic degradation in cancer cells was less than that of their normal counterparts (Gunn et al. 1977). Breast cancer cell lines frequently contain deletions of one allele of beclin 1 (*BECN1*), necessary to induce autophagy in response to nitrogen deprivation. Introduction of *BECN1* into MCF7 breast cancer cells induced autophagy and inhibited tumorigenicity (Liang et al.1999). The allelic deletion of chromosome 17q21, where *BECN1* is located, is common in breast, in ovarian and prostate tumours, so it is possible that deletion of *BECN1* are involved in the development of these tumours. A possible explanation could be that the early stages of tumour development require cancer cells to undergo a higher level of protein synthesis than protein degradation (Cuervo 2004). Therefore, inhibition of autophagy could maintain continuous tumour growth. Although autophagy is suppressed during the early stages of tumorigenesis, it seems to be upregulated during the later stages of tumour progression as a protective mechanism against stressful conditions (Ogier-Denis and Codogno 2003). Thus, suppression of autophagy may contribute to the initial rapid growth of tumors, but in more advanced stages of cancer, autophagy may be required.

Given this apparently quite complex role of autophagy in cancer, autophagy has become a very important target for cancer treatment. Some of the recent strategies for cancer treatment suggested include inducing autophagy in early developed cancers while inhibiting autophagy in advanced tumor cells with intact autophagy response to sensitize the cells to a variety of anticancer agents (Tan et al. 2009).

a) Treatment of autophagy-competent tumors.

Autophagy-competent tumors may activate autophagy as an adaptive response to anticancer agents, in which case autophagy may act as a treatment resistance mechanism. In this case, concurrent inhibition of autophagy is expected to enhance the efficacy of anticancer drugs. Given that apoptosis-defective cancer cells rely on autophagy for survival under metabolic stress, it is also expected that autophagy inhibition will likely be therapeutically more beneficial in the treatment of tumors with apoptosis defects, but functional autophagy. Autophagy inhibition as a means to sensitize cancer cells to treatment has been validated in several studies.

Inhibition of autophagy by chloroquine, a lysosomotropic agent that raises intralysosomal pH and interferes with autophagosome degradation within lysosomes, was shown to enhance the anticancer activity of the alkylating agent cyclophosphamide in a myc-induced lymphoma model (Amaravadi et al. 2007); both chloroquine and 3-methyladenine (3-MA), a class III PI3K inhibitor, synergistically augmented the proapoptotic effects and overall anticancer activity of the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) in chronic myelogenous leukemia (CML) cells (Carew et al. 2007). Inhibition of autophagy with the vacuolar-type H1-ATPase inhibitors bafilomycin A1 enhances imatinib-induced cytotoxicity in human malignant glioma cells through increasing apoptosis. (Shingu et al. 2009). Knockdown of autophagy, in combination with tamoxifen or 4-hydroxy-tamoxifen (4-OH-T), resulted in decreased cell viability of estrogen receptor-positive MCF-7 and T-47D cells (Qadir et al. 2008); inhibition of autophagy along with irradiation lead to enhanced cytotoxicity of radiotherapy in resistant cancer cells (Apel et al. 2008).

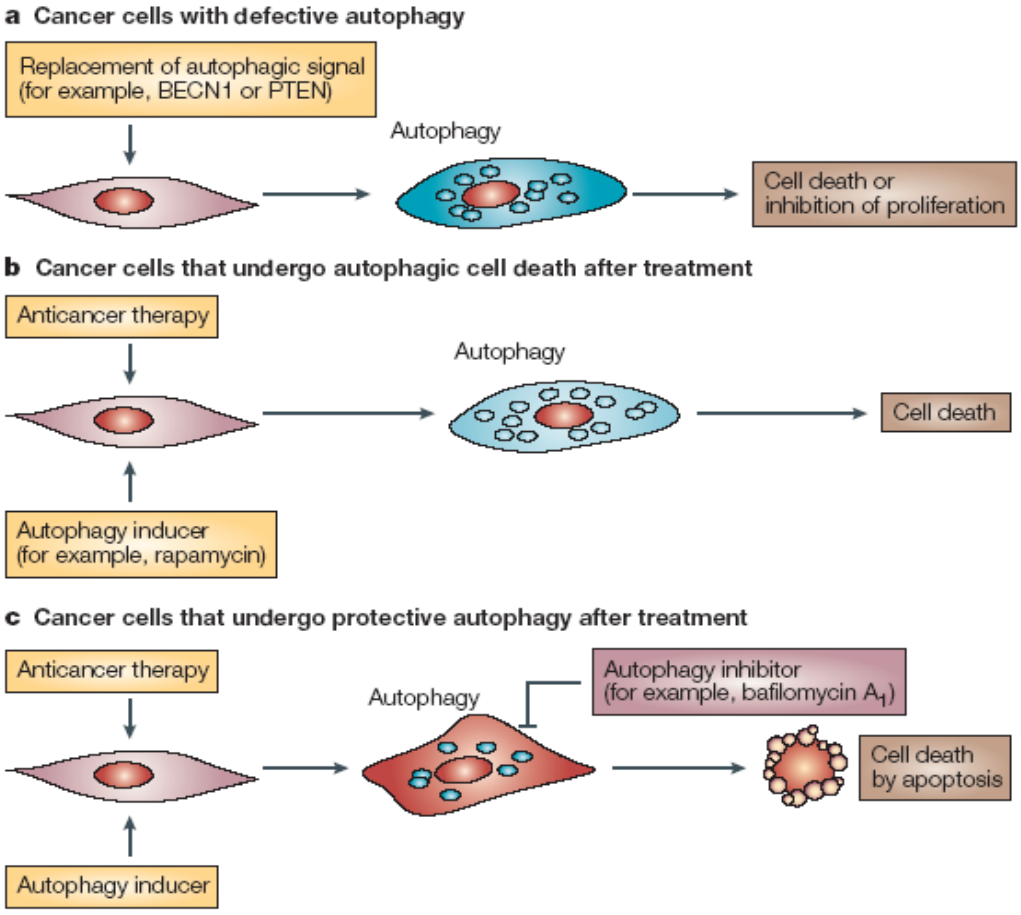
Clinical trial are currently in progress to validate this hypothesis (National Cancer Institute, NCI lists trials) (Chen and Karantza-Wadsworth 2009).

It has also been reported that autophagy-competent tumors may activate autophagy in response to anticancer agents, however tn these cases autophagy resulted in tumor cells elimination. These studies have shown that autophagy is involved in the cell death induced by therapeutic agents for glioma, such as temozolomide, rapamycin, irradiation, and oncolytic adenoviruses (Ito et al. 2006; Ito et al. 2005; Iwamaru et al. 2007; Jiang et al. 2007; Kanzawa et al. 2004; Yokoyama et al. 2008). Moreover, mTOR inhibitors combined with radiations or chemotherapeutic

agents increase anticancer effect in human gliomas, breast cancer and pancreatic cancer cells (Eshleman et al. 2002; Mondesire et al. 2004).
All these different evidences suggest that, upon autophagy activation, the choice between either survival or cell death programs depends on several factors, including the cell types, the duration, the concentration and the kind of stimuli.

b) Treatment of autophagy-deficient tumors.

Chronically autophagy-deficient tumors likely adjust to their autophagy-defective status over time and acquire compensatory cell survival mechanisms. Thus, cancer cells with autophagy defects are not expected to depend on autophagy for cytoprotection during chemotherapy and radiotherapy. Therefore Autophagy-defective tumors may be particularly sensitive to metabolic stress-inducing regimens, such as antiangiogenic drugs, growth factor receptor inhibitors and glucose deprivation, and to DNA damage-inducing agents. Defective autophagy may not only sensitize tumor cells to certain drugs, but it may also confer resistance to agents inducing gene amplification as a resistance mechanism..
Figure 6 summaryses the potential strategies for treating cancer by manipulating the autophagic process (Kondo et al. 2005).



Kondo et al. 2005

Figure 6. Potential strategies for treating cancer by manipulating the autophagic process.
a) Cancer cells that have defects in the autophagic pathway might be treated by replacing the autophagic signal through expression of beclin 1 (BECN1) or the phosphatase and tensin homologue (PTEN) tumour suppressor, resulting in induction of autophagy and cell death, or inhibition of proliferation. b) Cancer cells that are capable of undergoing autophagy in response to anticancer therapies might be treated with autophagy inducers, such as rapamycin, to promote autophagy-induced cell death. c) Cancer cells that undergo autophagy to protect themselves from the effects of anticancer therapies, might be treated with autophagy inhibitors, such as bafilomycin A1 or short interfering RNAs specific for the autophagy-related genes, to induce apoptosis.

2. AIM OF THE STUDY

Conditionally replicating adenoviruses (CRAds) are emerging as a promising tool in cancer therapy. Because of their capability to multiply, lyse infected tumor cells and spread to surrounding cells, CRAds may have better antitumor efficacy than that of non-replicating adenoviruses. Some adenoviral mutants have demonstrated the tumor-selective replication predicted by preclinical and clinical reports. Although safety and selectivity have been encouraging, durable objective responses with the virus as a single agent have been uncommon. The best chance for complete tumour eradication lies in a multimodal cancer therapy approach utilizing oncolytic viruses in conjunction with chemotherapy and radiotherapy. Therefore, is important to find new therapeutic strategies to potenciate the oncolytic viral activity. One of the aspects that can be investigated to enhance viral potency is better understanding the mechanisms of oncolytic virus-induced cell death. Infact, the exact mechanisms by which adenoviruses cause cell death remain uncertain, and the studies are often controversial.

Autophagy, a type of degradation system, has been shown to be activated in cells in response to viral infection and it has been demonstrated that some oncolytic adenoviruses induce autophagy in cancer cells. We have demonstrated that the oncolytic adenoviruses *dl*922-947 and AdΔΔ are active against glioma and prostate cancer cells; however, the mechanisms of virus-induced cell death are not clear. Glioma and prostate cancer cells have been demonstrated to activate autophagy in response to several stimuli and could represent an optimal experimental model to evaluate the activation of this cellular process. Therefore, I have decided to investigated the activation of autophagy in these cancer cells following the infection with *dl*922-947 and AdΔΔ adenoviruses.

Although it has been demonstrated that some oncolytic adenoviruses induce autophagy in cancer cells, little is known about the role of virus-induced autophagy: it may either be triggered in infected cancer cells as a defense mechanism to protect against intracellular pathogens, or represent a cell death modality induced by the viral replication complex. Thus, the induction or the inhibition of autophagy may have a therapeutic value. In order to enhance the adenoviral efficacy, I have decided to clarify the role of autophagy upon infection with oncolytic adenoviruses in glioma and prostate cancer cells, validating the autophagy as potential therapeutic target.

3. MATERIALS AND METHODS

3.1 Tumor cell lines

Human glioma cell lines U373MG and U87MG, human carcinoma cell lines from prostate PC3 and 22Rv1 and were purchased from American Type Culture Collection. All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 100 IU of penicillin/ml, 100 IU of streptomycin/ml and 2% L-glutamine in humified CO_2 incubator.

3.2 Preparation of adenoviruses

*dl*922-947 is a second generation adenoviral mutant that has a 24-bp deletion in E1A Conserved Region 2 (CR2) therefore, is unable to induce progression from G1 into S-phase of quiescent cells.

AdΔΔ carries two deletion, in E1A CR2 (ΔCR2) and in the E1B region that encodes the 19-KDa protein, with intact E3 region. AdGFP is a non replicating E1-deleted adenovirus encoding green fluorescent protein.

*dl*1520 (ONYX-015) is a chimaeric human group C adenovirus (Ad2 and Ad5) that has a deletion between nucleotides 2496 and 3323 in the E1B region that encodes the 55-kDa protein.

Ad5wt is a non-mutant adenovirus used as control adenovirus.

Viral stocks were expanded and titered in human embryonic kidney cell line HEK-293, which expresses the E1 region. Stocks were stored at -80°C after the addition of glycerol to a concentration of 50% vol/vol. Virus titer was determined by plaque-forming units (pfu) on the HEK-293 cells.

3.3 Viability assay

For the evaluation of the cytotoxic effects of *dl*922-947 and AdΔΔ viruses, cells were seeded in 96-well plates, and 24 h later increasing concentrations of viruses were added to the incubation medium. For the evaluation of the cytotoxic effects of the *dl*922-947 virus in combination with chloroquine or 3-MA, cells were seeded in 96-well plates, and 24 h later were treated with increasing concentration of viruses in combination or not with the drugs. Six days later, the media were fixed with 50% TCA and stained with 0.4% sulforhodamine B in 1% acetic acid. The bound dye was solubilized in 100 μl of 10 mM unbuffered Tris HCl solution and the optical density was determined at 540 nm in a microplate reader (Biorad).

The percent of survival rates of cells exposed to adenovirus vectors were calculated by assuming the survival rate of untreated cells to be 100%.

3.4 Cell cycle analysis

Adherent U373MG and U87MG cells detached with trypsin–EDTA were collected, fixed with 70% ethanol, and stained with a 10% propidium iodide solution (cellular DNA flow cytometric analysis reagent set; Roche) according to the manufacturer's instructions. DNA content was analyzed with a FACScan flow cytometer.

5. Quantifi cation of Acidic Vesicular Organelles With Acridine Orange

Autophagy is characterized by the development of acidic vesicular organelles. The cytoplasm and nucleoli of acridine orange–stained cells fluoresce bright green and dim red, respectively, whereas acidic compartments fluoresce bright red (Paglin et al 2001). Therefore, autophagy was assessed in U373-MG and U87-MG cells by the quantification of acidic vesicular organelles with supravital cell staining using acridine orange. To inhibit autophagy, 1.0 mM 3-MA (Sigma-Aldrich), or 10μM HCQ (Sigma-Aldrich), were added to cells the day after infection by *dl*922-947 or AdΔΔ. Cells that were detached with 0.05% trypsin–EDTA and stained with 1.0 μ g/mL acridine orange (Sigma-Aldrich) for 15 minutes at room temperature. Stained cells were then analyzed by flow cytometry using the FACScan cytometer (Becton Dickinson, San Jose, CA).

3.5 Western blot analysis

The methods are described in detail in the publication (Botta et al., pending revision) at the end of the references.

The primary antibody used are the following: polyclonal rabbit antibody against LC3-I/II (Santa Cruz) 1:100, mouse antibody against caspase-3 (Abcam) 1:500, rabbit antibody p-ERK1/2 (Cell Signalling) 1:1000, rabbit polyclonal antibody against p-Akt (Cell Signalling) 1:1000, rabbit polyclonal antibody against p-p70s6k (Cell Signalling), 1:500, or with the goat antibody against actin. (Santa Cruz) 1:2000 .

4. RESULTS AND DISCUSSION

4.1 Cytotoxic effects of *dl*922-947 in glioma cells

I have previously demonstrated that glioma cells are sensitive to the effect of the selective replicating oncolytic adenovirus *dl*922-947, although displaying different sensitivity (Botta et al. 2009). In this study, the sensitivity of U87MG and U373MG glioma cells to *dl*922-947 was evaluated. Cells were seeded in 96-well plates and infected with different multiplicity of infection (MOIs) of *dl*922-947, expressed as *plaque forming unit* (pfu/cell) and cell survival was evaluated after seven days. The results are shown in Fig.7. In the diagrams the percentage of cell survival is represented as a function of pfu/cell. Both U87MG and U373MG glioma cell lines are sensitive to *dl*922-947. However the two cell lines display a different sensitivity to the virus.
U373MG cell line displayed higher sensitivity to *dl*922/947 with an IC_{50} of MOI 0.0001 (pfu/cell), whereas for U87MG IC_{50} was observed at a MOI of 19,59 (pfu/cell).

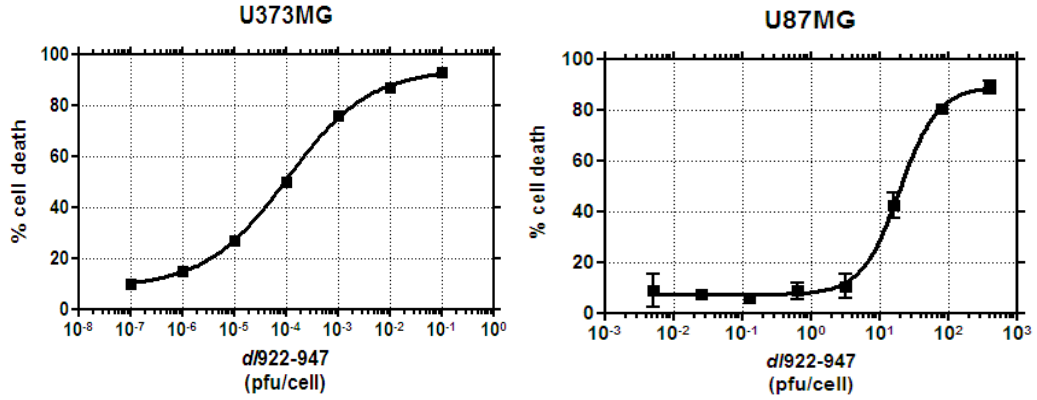
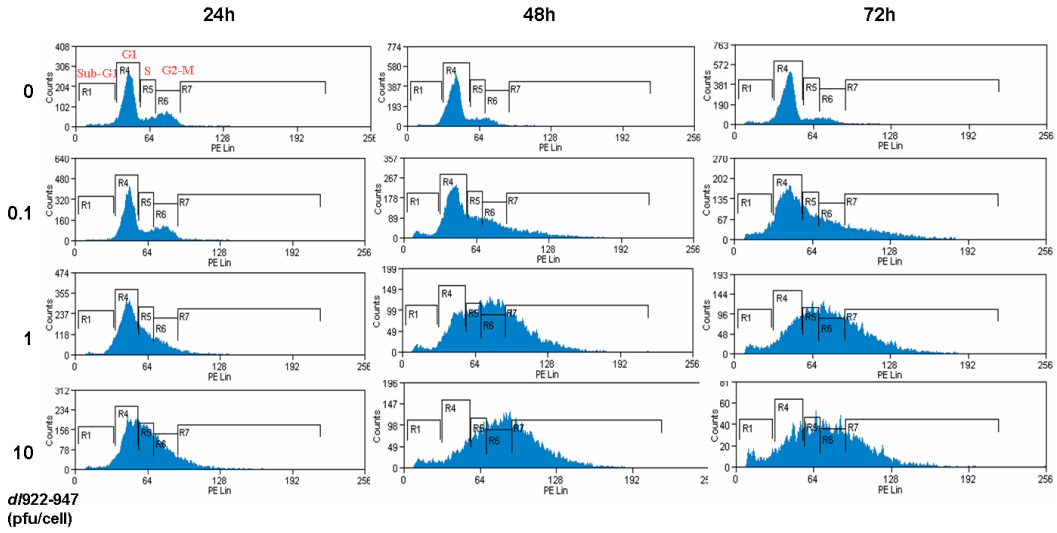


Figure 7. Sensitivity of human glioma cells to the oncolytic adenovirus *dl*922-947
U373MG and U87MG cells were infected with increasing concentration of *dl*922-947 for seven days and analysed for viability assay. U373MG cells display higher sensitivity to the cytophatic effect of the virus compared to U87MG cells.

Next, I investigated whether *dl*922-947 could induce apoptosis in glioma cells. To evaluate whether viral infection induces the appearance of a sub-G1 fraction, suggestive of cell death either by apoptosis or by necrosis, a cell cycle analysis was performed. Cells were treated with different concentrations of *dl*922-947 (0-0.1-1-10 pfu/cell), and cell cycle profile analysed 24, 48 and 72 hours post infection (hpi). For all times and concentrations used, *dl*922-947 infection

increased the percentage of cells in S and G2-M phase, but no increase of sub-G1 fraction, compared to untreated cells, was observed (Fig.8a).
Caspase-3 analysis by western blotting did not show any activation of pro-caspase3 after infection in U373MG cells, while U87MG cells showed a slightly activation of caspase-3 only after infection with the highest concentration of the virus (Fig 8b).
The lack of caspase-3 activation and of a sub-G1 fraction increase clearly indicate that *dl*922-947-induced cell death in glioma cells is not apoptosis-mediated.

a)



b)

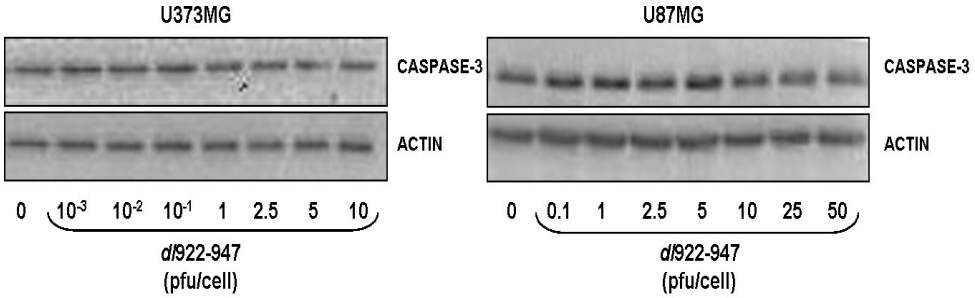


Figure 8. Cell cycle profiles and caspase-3 levels in *dl*922-947-infected glioma cells
a) U373MG cells were infected with different concentrations of *dl*922-947 (0.1, 1, 10 pfu/cell) and stained with PI 24, 48 and 72 hpi. Cell cycle distribution was quantified by flowcytometry. First peak indicates the cells in G_0/G_1 phase, second peak indicates G_2/M phase and in-between is S phase. b) U373MG and U87MG cells show no significant cleavage of procaspase-3. Equal amounts of protein lysates (50µg) were loaded.

4.2 Induction of autophagy in malignant glioma cells by *dl*922-947

Previous studies have shown that cancer cells can undergo autophagy in response to radiation or chemotherapy (Paglin et al. 2001; Kanzawa et al. 2004). Recently it has been demonstrated that also oncolytic adenoviruses are able to activate the autophagic process in cancer cells (Yokoyama et al.2008; Baird et al. 2007).
Therefore, I evaluated the activation of autophagy in glioma cells infected with *dl*922-947. The development of acidic vescicular organelles (AVOs) is a peculiar feature of autophagic process, thus I examined AVOs development upon infection with *dl*922-947. For detecting of the acidic compartment, I used the lysosomotropic agent acridine orange, a weak base that moves freely across biological membranes when uncharged. Its protonated form accumulates in acidic compartments, where it forms aggregates that fluoresce bright red. The intensity of the red fluorescence is proportional to the degree of acidity and/or the volume of the cellular acidic compartment. Therefore, by comparing the mean red:green fluorescence ratio within different cell populations, I could measure a change in the degree of acidity and/or the fractional volume of their cellular acidic compartment (Paglin et al. 2001). U373MG and U87MG cells were infected with different MOIs of *dl*922-947 and vital staining of infected cells was performed with acridine orange 72 hours later. Flow cytometry using acridine orange revealed that the infection with *dl*922-947 increased the strength of the bright red fluorescence of cells, indicating an increase of the development of AVOs in a MOI-dependent manner (up to 20% in U373MG cells and 23% in U87MG cell), whereas not infected cells exhibited mainly green fluorescence with minimal red fluorescence (Fig. 9a, b). Thus, *dl*922-947 induces AVOs accumulation in glioma cells, suggesting the activation of the autophagic process.

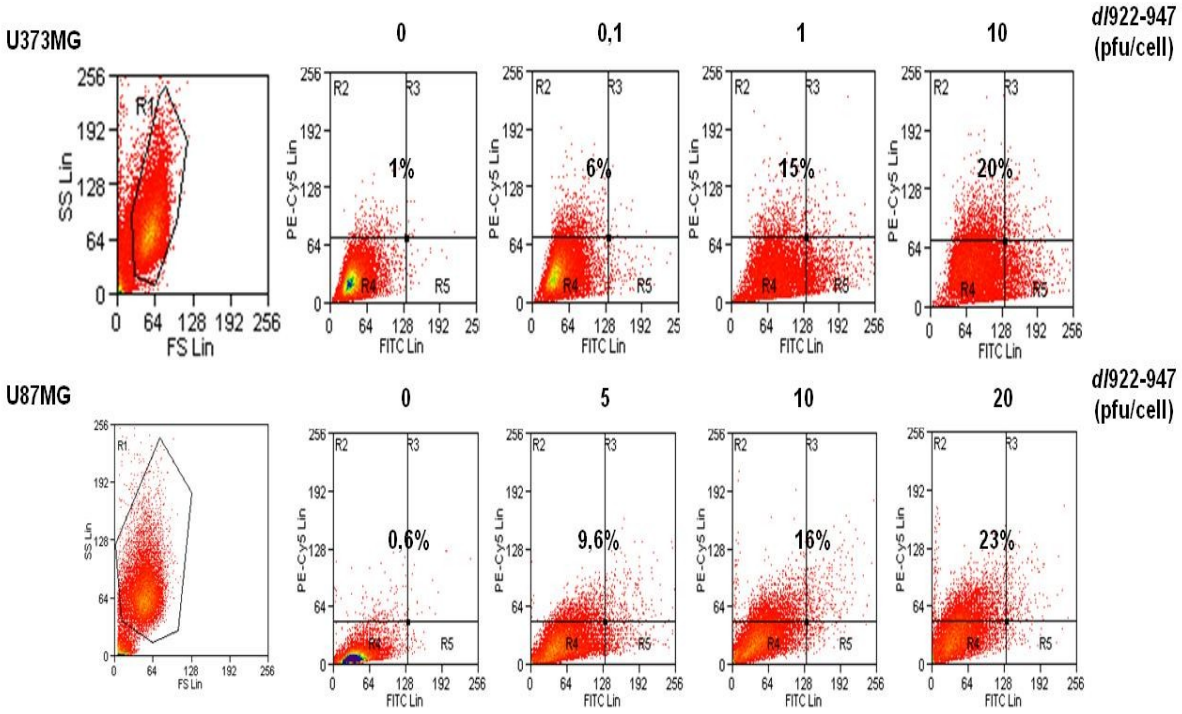


Figure 9. Quantification of acidic vescicular organelles (AVOs) by acridine orange staining in *dl*922-947-infected glioma cells

U373MG and U87MG cells were infected with different concentrations of *dl*922-947 (0.1, 1, 10 pfu/cell for U373MG and 5, 10, 20 pfu/cell for U87MG) for 72 h and subjected to acridine orange staining. Percentage of cells with enhanced red fluorescence was quantified using flow cytometry and was indicated in the upper quadrants. AVOs development increases in *dl*922-947-infected glioma cells in a dose-dependent manner.

In amino acid starvation-induced autophagy, the microtubule-associated protein 1 light chain 3 (LC3) is localized in autophagosome membranes. LC3 protein exists in two cellular forms: LC3-I and LC3-II. LC3-I, the cytoplasmic form, is processed by enzymatic cleavage into LC3-II, which is associated with the autophagosome membrane. Therefore, an increase in the ratio of LC3-II to LC3-I is closely correlated with the autophagosome formation. Thus, I analyzed the accumulation of LC3-I and LC3-II in *dl*922-947–infected cells. U373MG and U87MG cells were infected with different MOIs of *dl*922-947 and LC3 content was measured 72 hpi by western blotting (Fig. 10). In both cell lines, the amount of LC3-II and the ratio of LC3-II to LC3-I was clearly increased by infection with *dl*922-947 in a dose-dependent manner, whereas it was unchanged in not infected cells (Fig. 10a, b). These results, together with AVOs formation, demonstrate that *dl*922-947 infection induces autophagy in malignant glioma cells.

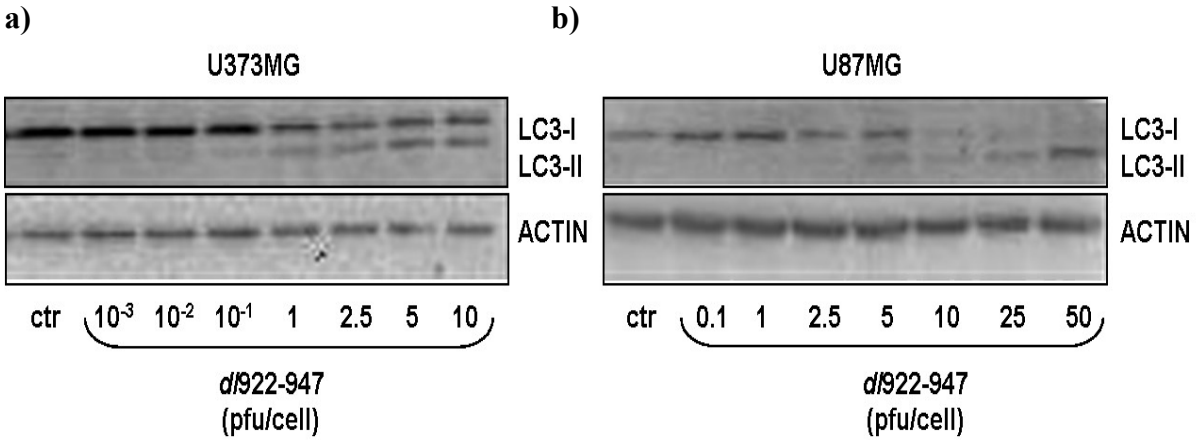


Figure 10. Western blot analysis of LC3-I and LC3-II in *dl*922-947-infected glioma cells
U373MG and U87MG cells were infected with the indicated concentrations of *dl*922-947 (pfu/cell) for 72 h and subjected to western blotting using polyclonal rabbit anti-microtubule-associated protein 1 light chain 3 (LC3)-I and –II antibody, that detects LC3-I and LC3-II at a molecular mass of approximately 16 and 14 KDa respectively (Mizushima and Yoshimori, 2007). Equal amounts of protein lysates (50μg) were loaded. Anti β-actin antibody was used as a loading control. LC3-I/II conversion increases in U373MG (a) and in U87MG cells (b) in dose-dependent manner.

Next, I infected glioma cells with other mutant adenoviruses: *dl*1520, carrying a deletion in E1B region and the newly generated AdΔΔ oncolytic adenoviruses carrying two deletion, in E1A CR2 (ΔCR2) and in the E1B region; the non replicating virus encoding green fluorescent protein (AdGFP) and the wilde type virus (Ad5) were used as controls. Both cell lines were infected with 0.1 and 1 pfu/cell of each virus and LC3 levels were analysed 72 hpi. AdGFP, a non replicating adenoviruse did not modify LC3 cleavage (Fig. 11a, b). In U373MG cells *dl*922-947 induced a marked decrease of LC3-I levels compared to the other replicating viruses, indicating that *dl*922-947 activates autophagic pathway more efficiently with respect to the other viruses (Fig. 11c). This observation suggest that the induction of autophagy in glioma cells is a specific feature of *dl*922-947 infection.

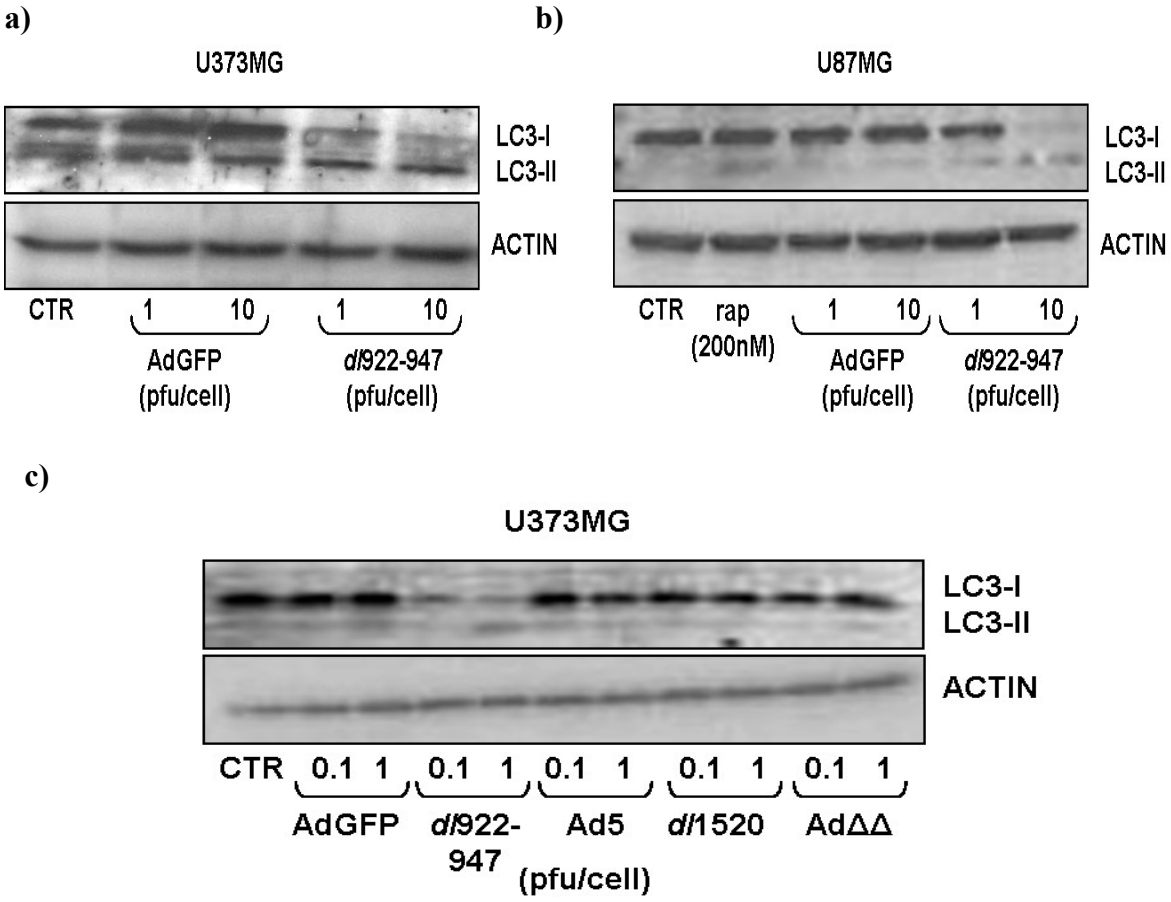


Figure 11. Effect of different oncolytic adenoviruses on LC3 cleavage
U373MG cells were infected with 0, 0.1, 1, 10 pfu/cell of different adenoviruses, and LC3 levels were analysed 72 h later by western blotting. Equal amount of protein were loaded in each lane (50 μg). The infection with the non replicating adenovirus AdGFP does not increase LC3-II levels compared to *dl*922-947 in U373MG (a) and in U87MG cells (b).
c) the wilde type virus (Ad5), the oncolytic adenoviruses *dl*1520 and AdΔΔ do not modify LC3 cleavage compared to *dl*922-947.

4.3 Effect of Inhibition of *dl*922-947–induced autophagy on malignant glioma cells

The role of autophagy is still controversial. According to some studies, treatment–induced autophagy in cancer cells represents a protective reaction, whereas in other studies autophagy has been shown to represent a cell death mechanism (Kondo et al. 2005). To assess the role of *dl*922-947–induced autophagy in glioma

cells, I decided to block virus-induced autophagy using pharmacological inhibitors. Several autophagy inhibitors have been developed (Kondo et al. 2005). Among those, two autophagy inhibitors, acting at different autophagic steps, were chosen. 3-methyladenine (3-MA), a class-III-PI3K inhibitor blocks early stages of autophagy process by inhibiting pre-autophagosome formation, thus reducing the acidic compartment. Hydroxychloroquine (HCQ) is a lysosomotropic agent that inhibits later stages of autophagy by preventing the fusion of autophagosomes and lysosomes, blocking the formation of autolysosomes where LC3-II should be degraded, and leading to accumulation of the acidic vescicular organelles. Both drugs have been reported to have different effects in cancer cells in combination with other treatments, (depending on the autophagy initiator, the type of inhibitors used and the extent of cellular damage) (Kondo et al. 2005).
Therefore, I tested the effect of these drugs on the development of acidic vesicular organelles.
U373-MG cells were infected with *dl*922-947 at an MOI of 0.1 and 1.0 pfu/cell in combination with HCQ (5μM) or 3-MA (1.0 mM) for 72 hours and then stained with acridine orange. As shown in Fig. 12, treatment with HCQ increased the percentage of red-positive cells (Fig.12a), whereas 3-MA partially reverted the induction of acidic vesicular organelles in U373-MG cells infected with *dl*922-947 (Fig. 12b).
In Fig. 13 is shown that the increase in LC3-II induced by *dl*922-947 was strongly augmented by 10μM HCQ (Fig. 13a), in agreement with inhibitory activity of HCQ. In contrast to HCQ, 1 mM 3-MA partially reverted the *dl*922-947-induced increase in LC3-II (Fig. 13b), indicating inhibition of autophagy upstream to conversion of LC3-I.

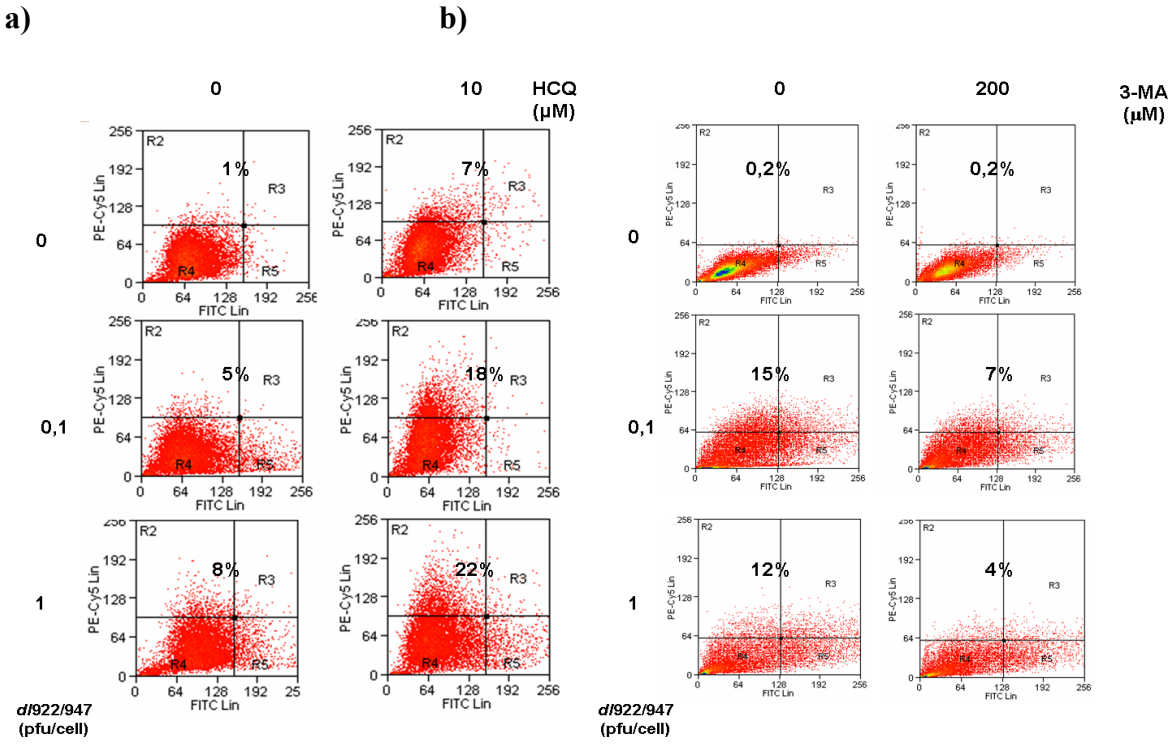


Figure 12. Effect of autophagy inhibitors on *dl*922-947-mediated AVOs development in glioma cells

U373MG cells were infected with *dl*922-947 (0, 0.1, 1 pfu/cell) in presence of 10μM of chloroquine (HCQ) (a) or 200μM of 3-methyladenine (3-MA) (b) for 72 h and stained with acridine orange. The indicated percentages represent the amount of cells red fluorescence-positive.

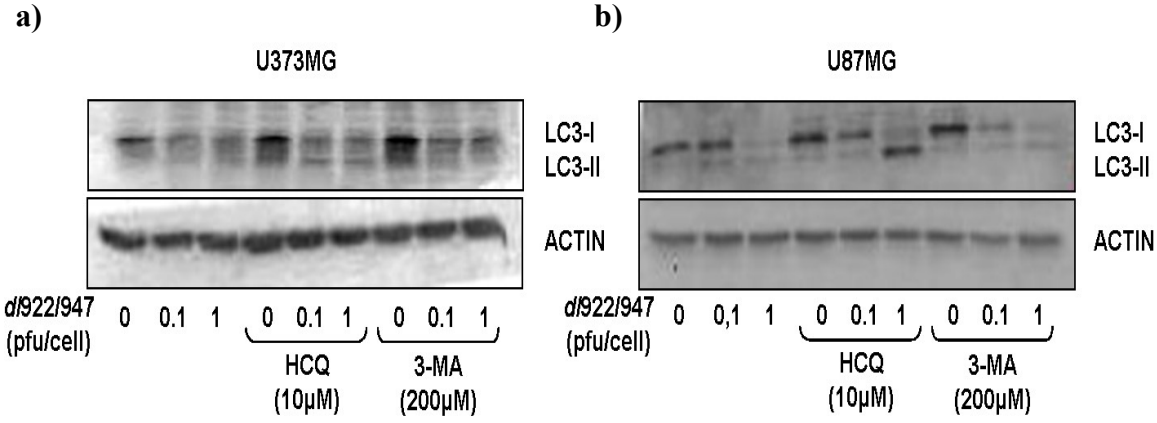


Figure 13. Effect of autophagy inhibitors on *dl*922-947-mediated LC3 cleavage in glioma cells

U373MG and U87MG cells were infected with *dl*922-947 (0, 0.1, 1 pfu/cell) in presence of chloroquine (HCQ) (10μM) or 3-methyladenine (3-MA) (200μM) for 72 h and subjected to western

blotting using polyclonal rabbit anti-microtubule-associated protein 1 light chain 3 (LC3)-I and –II antibody. Anti β-actin antibody was used as a loading control. The autophagy inhibitors have similar effect on LC3 cleavage in U373MG (a) and in U87MG cells (b).

Next, I examined the effect of the two inhibitors on *dl*922-947-induced cytotoxicity, by measuring cell survival after treating cells with different concentrations of *dl*922-947, in the presence of HCQ or 3-MA. *dl*922-947–induced cytotoxicity was significantly augmented by both HCQ (Fig. 14a) or 3-MA (Fig. 14b), with synergistic lethal effect, whereas treatment alone did not induce cell death. Similar results were observed in U87MG cells.
These results suggest that *dl*922-947–induced autophagy is a protective response to *dl*922-947 infection and contrasts the antitumoral activity of the virus. Therefore, these data indicate that pharmacological inhibition of autophagy can sensitize glioma cells to the oncolytic effect of *dl*922-947.

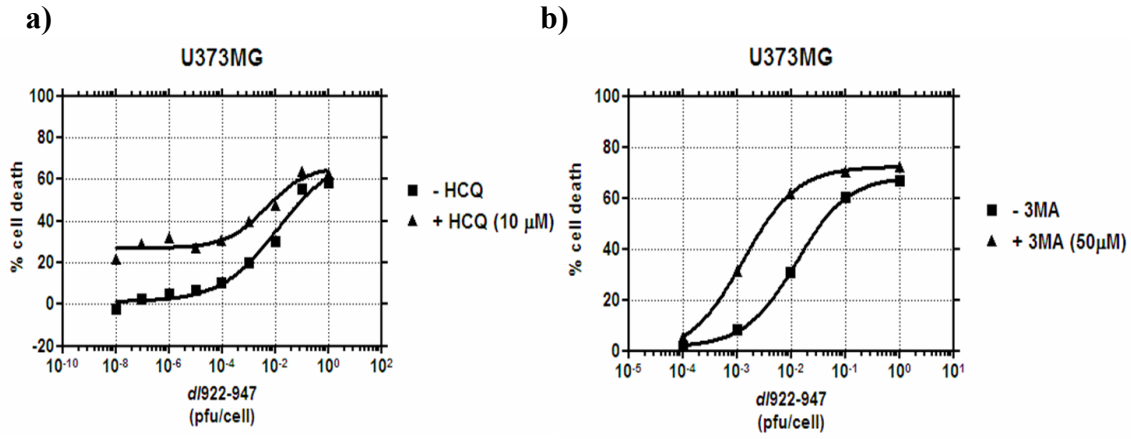


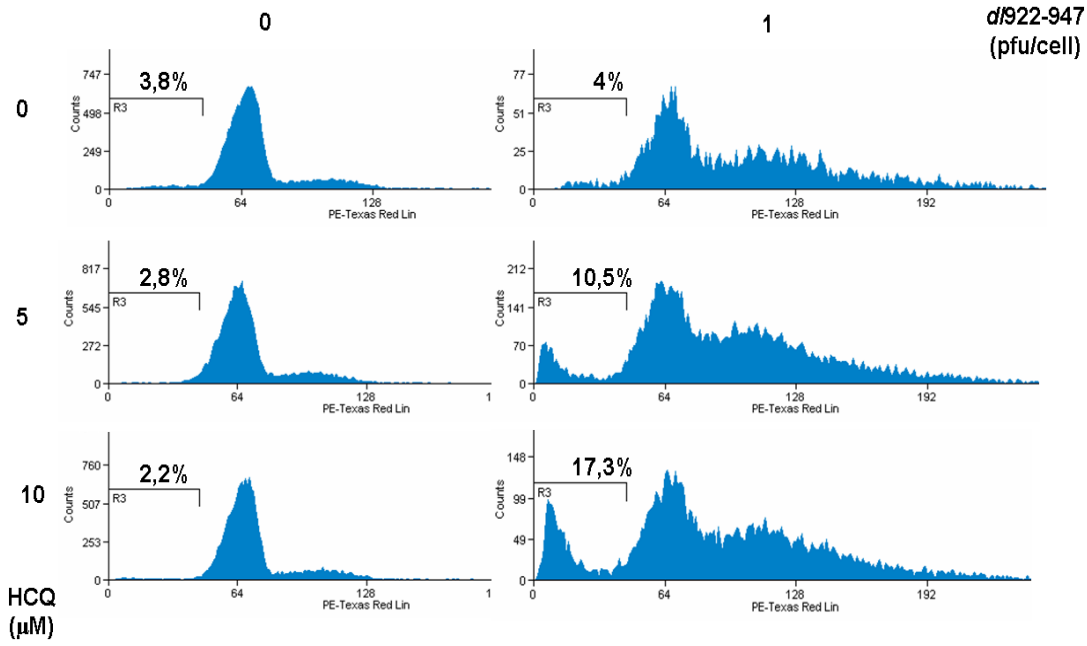
Figure 14. Autophagy inhibition enhances *dl*922-947-mediated cytotoxicity in glioma cells
U373MG cells were infected with increasing concentration of *dl*922-947 in combination with 10µM of chloroquine (HCQ) (a) or 200µM of 3-methyladenine (3-MA) (b) for seven days and analysed for viability assay. Both HCQ and 3-MA enhance the cytotoxic effect of *dl*922-947.

4.4 Pharmacological inhibition of autophagy activates apoptotic pathway in *dl*922-947 –infected glioma cells

Recent evidences have suggested the existence of a double switch between the two main lethal signaling pathways, type 1 (apoptotic) and type 2 (autophagic) cell death. Inhibition of apoptosis can lead to a chronic degenerative autophagic cell death (Xue et al. 2001). On the other hand, all autophagy inhibitors have been

reported to induce nuclear apoptosis and caspase-3 activation. (Boya et al. 2005). Moreover, inhibition of autophagy increased apoptotic cell death in various cancer cells irradiated or treated with chemotherapeutic agents (Kanzawa et al. 2004; Paglin et al. 2001). Since *dl*922-947-induced cytotoxicity in glioma cells was significantly augmented by HCQ and 3-MA, I investigated whether apoptosis contributes to the enhancement of cell death. U373MG cells were treated with *dl*922-947 (1 pfu/cell) in combination with HCQ (1, 5 µM), and cell cycle profile analysed 72 hours post infection (hpi). In presence of HCQ, *dl*922-947 infection increased the percentage of sub-G1 population compared to single treatments. No changes were detected in cells treated with HCQ alone (Fig. 15a).
An immunoblot in Fig. 15b, c confirmed a stronger reduction of pro-caspase-3, and subsequently a clear activation of caspase-3 in cells undergoing the combined treatment, compared to untreated cells. Similar results were observed in U87MG cells. Taken together, inhibitors of autophagy at both early (3-MA) or late (HCQ) stages augment *dl*922-947–induced cell death, probably stimulating signals triggering caspase-3 activation and, subsequently, apoptotic cell death.

a)



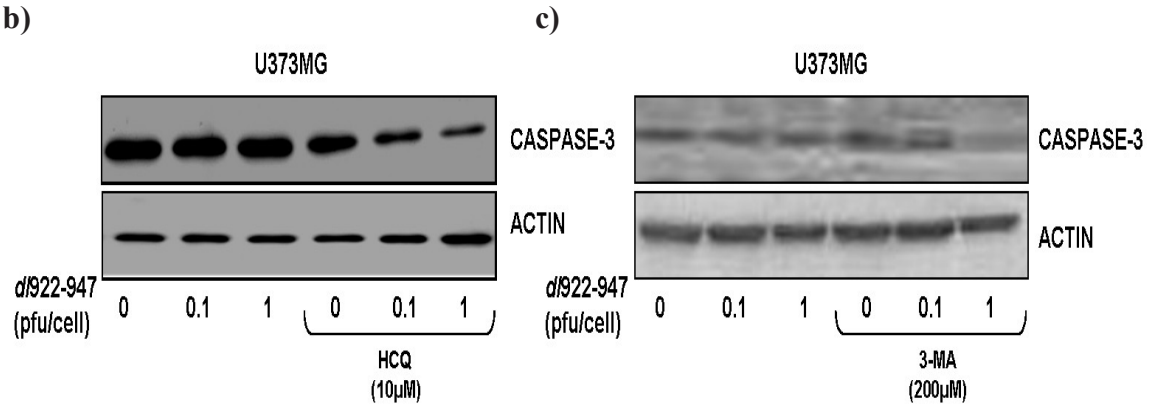


Figure 15. Autophagy inhibition induces apoptosis in *dl*922-947-infected glioma cells
a) U373MG cells were infected with *dl*922-947 (1 pfu/cell) in combination with 5 and 10μM of chloroquine (HCQ) and stained with PI 72 hpi. Cell cycle distribution was quantified by flowcytometry and the indicated percentage represent sub-G1 cell population. Combination with HCQ increases the percentage of cells in sub-G1 phase.
In *dl*922-947-infected cells procaspase-3 was activated after combination either with 10μM of HCQ (b) or 200 μM of 3-MA (c).

4.5 Effect of *dl*922-947 on autophagy signalling pathways in glioma cells

Since I have shown that *dl*922-947 infection of glioma cells elicits the activation of the autophagy as a cell survival mechanism, I have decided to analyse the involvment of two crucial pathways controlling autophagy activation or inhibition. Akt/mTOR/p70s6k pathway is the main pathway involved in the negative regulation of autophagy (Arico et al. 2001), whereas the ERK1/2 pathway is involved in positively regulating autophagy in cancer cells (Ogier-Denis et al. 2000). I examined the effect of the infection with *dl*922-947 on the two pathways. U373MG cells were infected with *dl*922-947 in a time-course and in a dose-response experiments. Phosphorilation of Akt and p70s6k increased in a time- (Fig.16a) and dose-dependent manner (Fig.16b) up to 12 hours after exposure to *dl*922-947. The expression level remained high 48 hours after infection. Conversely, ERK1/2 phosphorilation upon infection with *dl*922-947 was reduced in a time- (Fig. 16a) and in a dose-dependent manner (fig.16b).
These results demonstrated that *dl*922-947 infection activates Akt/mTOR/p70s6k pathway and inhibits ERK pathway.
Both changes in Akt/mTOR/p70s6k and ERK pathways could represent the attempt of the virus to suppress the survival response of glioma cells, being conceivable that glioma cells activate autophagy in response to the infection as a defence mechanism, maybe in order to degrade viral proteins.

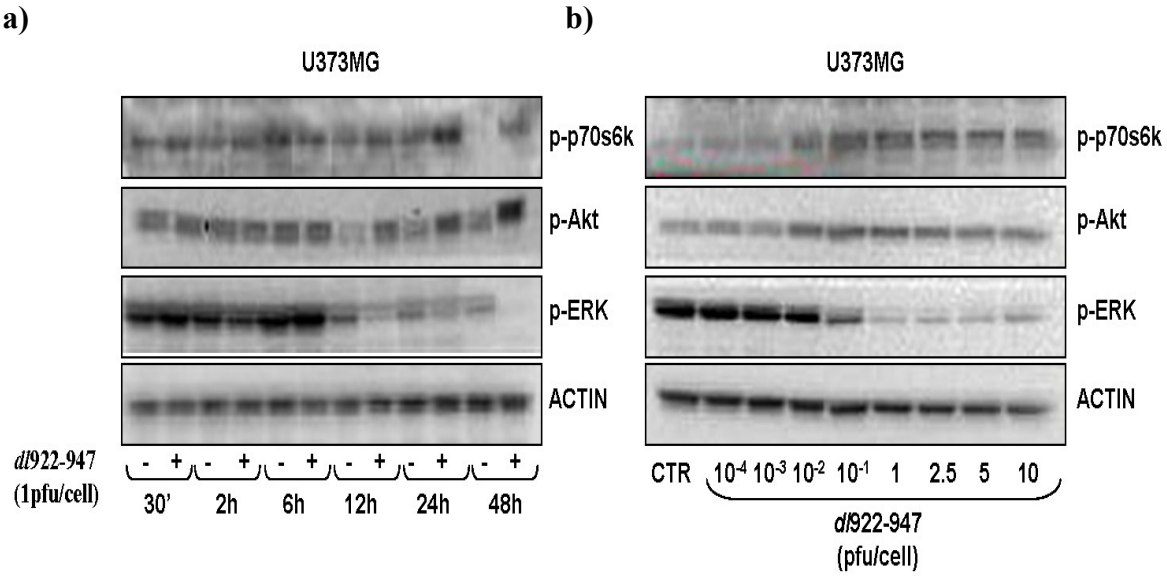


Figure 16. *dl*922-947 activates the Akt/mTOR/p70S6K pathway and inhibits the ERK pathway in glioma cells

a) U373MG cells were treated with 1 pfu/cell of *dl*922-947 and relative levels of phosphorylated ERK (p-ERK), Akt (p-Akt) and p70s6k (p-p70s6k) were analysed by western blotting at different times (ranging from 30 min up to 48 h). b) U373MG cells were infected with different concentration of *dl*922-947 (ranging from 10^{-4} up to 10 pfu/cell) and subjected to western blot after 72 h. Equal amounts of protein lysates (50μg) were loaded. Anti β-actin antibody was used as a loading control. *dl*922-947 infection induces increased p-Akt and p- p70s6k, whereas reduced p-ERK in time- and dose-dependent manner.

I have observed that *dl*922-947, but not other oncolytic adenoviruses, induce LC3 cleavage and autophagy activation. Thus, the effects of these oncolytic adenoviruses on Akt/mTOR/p70s6k and ERK1/2 signalling pathways were analysed. Both cell lines were infected and phosphorilation levels analysed 72 hpi. The blot in figure 17 shows that the oncolytic adenoviruses *dl*1520 and AdΔΔ did not change Akt, p70s6k and ERK1/2 phosphorilation levels as efficiently as *dl*922-947, paralleling LC3 cleavage (shown in Fig. 11c above). These results clearly indicate a strong correlation between the modulation of Akt/mTOR/p70s6k and ERK1/2 signalling pathways and autophagy induction in glioma cells infected with *dl*922-947. Moreover, the modulation of these two pathways is a specific feature of *dl*922-947 infection in glioma cells.

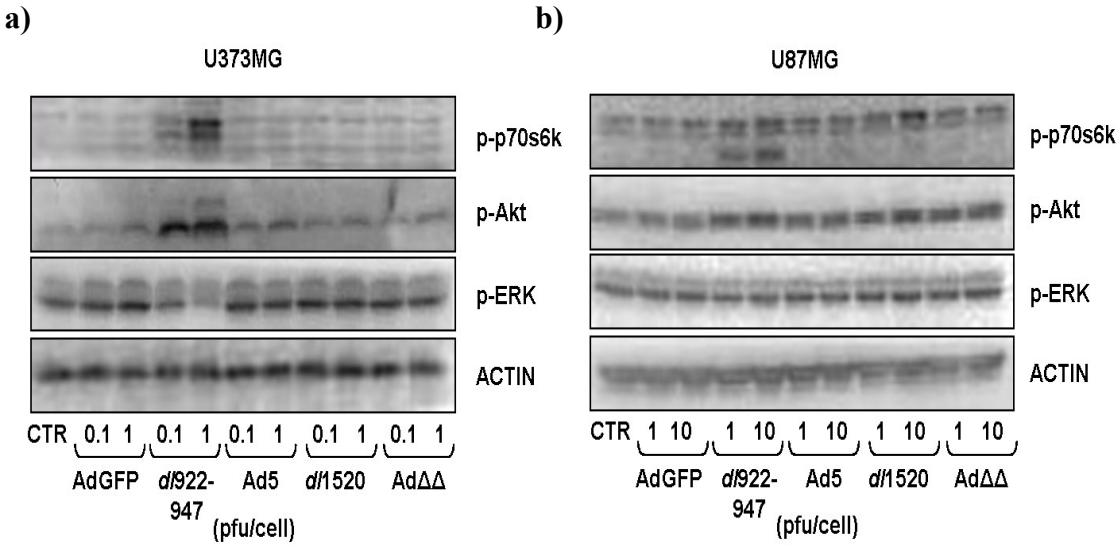


Figure 17. Effect of different oncolytic adenoviruses on Akt/mTOR/p70S6K and ERK pathways

U373MG (a) and U87MG (b) cells were infected with the indicated concentrations of adenoviruses for 48 h. Equal amount of extracted protein in each sample (50 μg) was subjected to immunoblotting using anti-phosphorylated (p-) Akt, p70s6k, or ERK1/2. Anti β-actin antibody was used as a loading control.

4.6 Inhibition of ERK pathway inhibits *dl*922-947-induced autophagy and induces apoptosis

It has been demonstrated that ERK1/2 pathway is involved in the positive regulation of autophagy; however, the role of this pathway in autophagy in response to anticancer therapy is not clear yet.

Since *dl*922-947 strongly reduced ERK1/2 activation, I speculated that inhibitors of ERK signalling might increase the antineoplastic effects of this virus against glioma cells. To test this hypothesis, I used a MEK inhibitor, PD98059. U373MG and U87MG cells were infected with *dl*922-947 (pfu/cell) for 48 hours in presence of PD98059 (50 μM) and the inhibitory effect of PD98059 on *dl*922-947-induced autophagy was analysed by evaluating LC3 cleavage. Expression of LC3-II was decreased in infected cells in presence of PD98059 for 48 hours (Fig. 18a, b).

Next, I examined the effect of this inhibitor on *dl*922-947-induced cytotoxicity, by measuring cell survival after treating U373MG cells with different concentrations of *dl*922-947 in the presence of PD98059 (5, 10, 25 μM). *dl*922-947–induced cytotoxicity was significantly augmented by PD98059, with synergistic lethal effect (Fig. 18c).

The ERK pathway is known to exert an antiapoptotic action, and it represents one of the overlaps of signalling worknets found between autophagy and apoptosis. Therefore, I investigated whether inhibition of this pathway induces apoptosis in *dl*922-947-infected glioma cells. Caspase-3 was clearly cleaved in U373MG cells treated with *dl*922-947 and PD98059, compared to untreated cells or single treatment (Fig. 18d).
Taken together, these results indicate that ERK pathway is involved in *dl*922-947-induced autophagy. It is also possible to conceive that the disruption of ERK pathway could enhance the efficacy of *dl*922-947 infection in U373MG and U87MG cells.

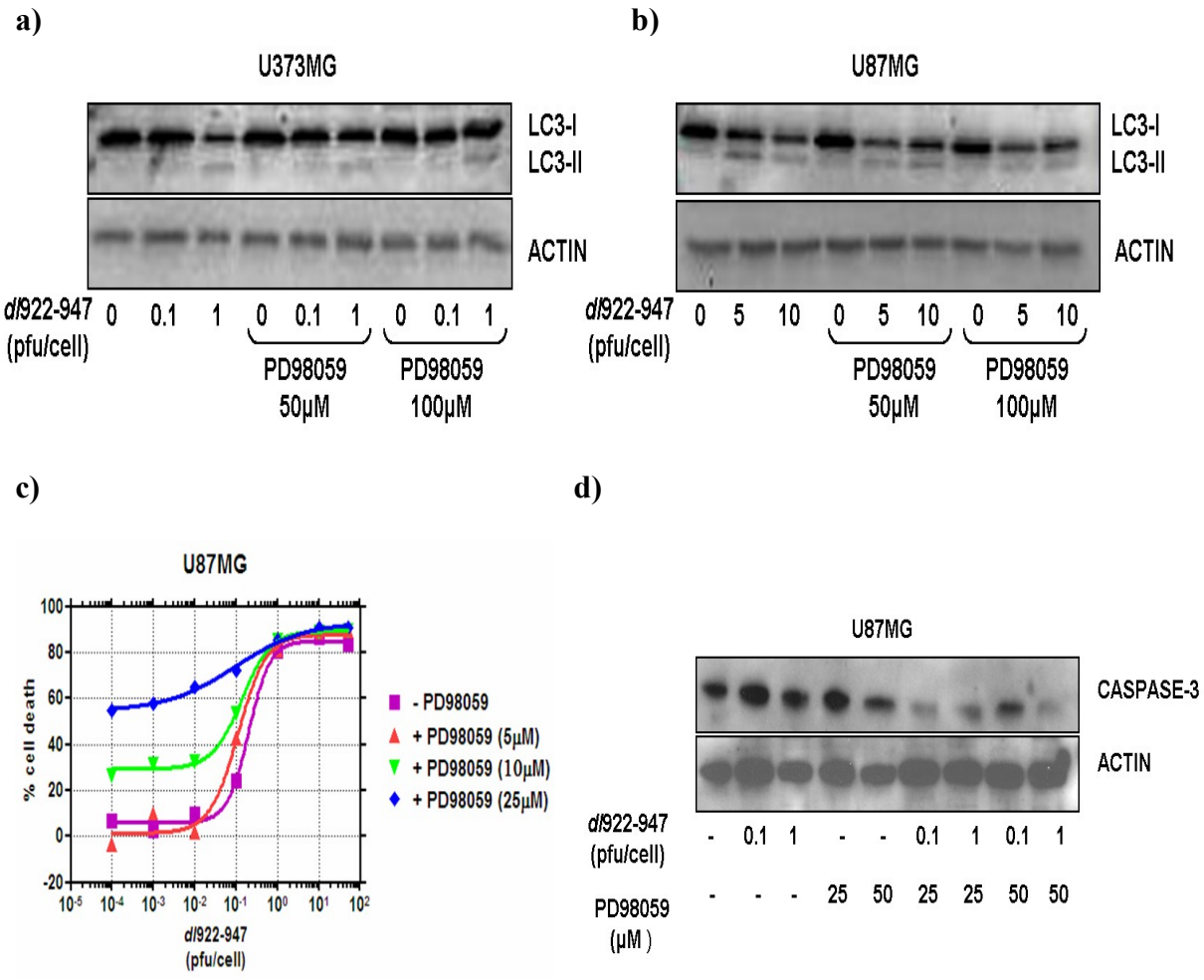


Figure 18. inhibition of ERK pathway reduces *dl*922-947-induced autophagy and enhances *dl*922-947 cytotoxicity

U373MG (a) and U87MG (b) cells were infected with the indicated pfu/cell of *dl*922-947 in presence or not of the MEK inhibitor PD98059 (50, 100 μM) for 72 h and equal amount of extracted protein in each sample (50 μg) was subjected to immunoblotting using polyclonal rabbit anti-microtubule-associated protein 1 light chain 3 (LC3)-I and –II antibody. Anti β-actin antibody was used as a loading control. Treatment with PD98059 partially reverted the *dl*922-947-induced LC3 cleavage in both cell lines. c) U87MG cells were infected with increasing concentration of *dl*922-947 in combination with PD98059 (5, 10, 25 μM) for seven days and analysed for viability assayd. d) In *dl*922-947-infected U87MG cells procaspase-3 was activated after combination either with 25μM or 50μM of PD98059 for 72 h.

4.7 The novel adenoviral mutant AdΔΔ

In the frame of a collaboration with Dr Gunnel Hallden, I have spent three months in the Centre for Molecular Oncology, John Vane Science Centre (London). In this laboratory has been recently generated a new oncolytic adenovirus mutant, carrying two deletions, in E1A CR2 and in the E1B region that encodes the 19-KDa protein, with intact E3 region (AdΔΔ). This virus targets both altered pRb (ΔCR2) and apoptotic pathways (ΔE1B19K). An improved efficacy and selectively of this virus, both as single agents and in combination with therapeutic agents, have been reported. Oberg et al. have demonstrated AdΔΔ to be effective against prostate, pancreatic and lung carcinoma cells and to have a cell killing potency either superior or similar to wild type virus. They also found higher viral activity in vivo (human prostate cancer xenograft) (Oberg 2009).
Since it has been demonstrated that the combination of the clinically used cytotoxic drugs docetaxel and mitoxantrone could significantly increase prostate cancer cell killing when combined with Ad5, during these three months, I have decided to test the effect of these drugs on the AdΔΔ-mediated cytotoxicity in prostate cancer cells and the cell death pathways activated upon infection.

4.8 Cytotoxic effects of AdΔΔ in prostate cancer cells

First, I evaluated the AdΔΔ cell killing effect on two prostate cancer cell lines, PC3 and 22Rv1. Cells were seeded in 96-well plates and infected with different MOIs of AdΔΔ, espressed as particle per cell (ppc). Cell survival was evaluated after seven days. The results are shown in Fig. 19. In the diagrams the percentage of cell survival is represented as a function of used ppc. Both PC3 and 22Rv1 prostate cell lines are sensitive to AdΔΔ, although displaying a different sensitivity. 22Rv1 cell line displayed higher sensitivity to AdΔΔ with an IC_{50} of MOI 20.8 ppc, whereas for PC3 IC_{50} was observed at a MOI of 12428 ppc (Fig.19).
E1B19K gene product normally exerts an antiapoptotic function. Therefore, AdΔΔ virus is expected to induce apoptosis in cancer cells. Accordingly, I analysed caspase-3 activation in infected cells. Caspase-3 was clearly activated in AdΔΔ-infected cells and this activation was greater than that observed in cell infected with a non replicating virus (dl312) and the wilde type virus (Ad5) (Fig. 19c, d).
Thus, I confirmed that AdΔΔ is effective against prostate cancer cells and induces caspase-3 activation.

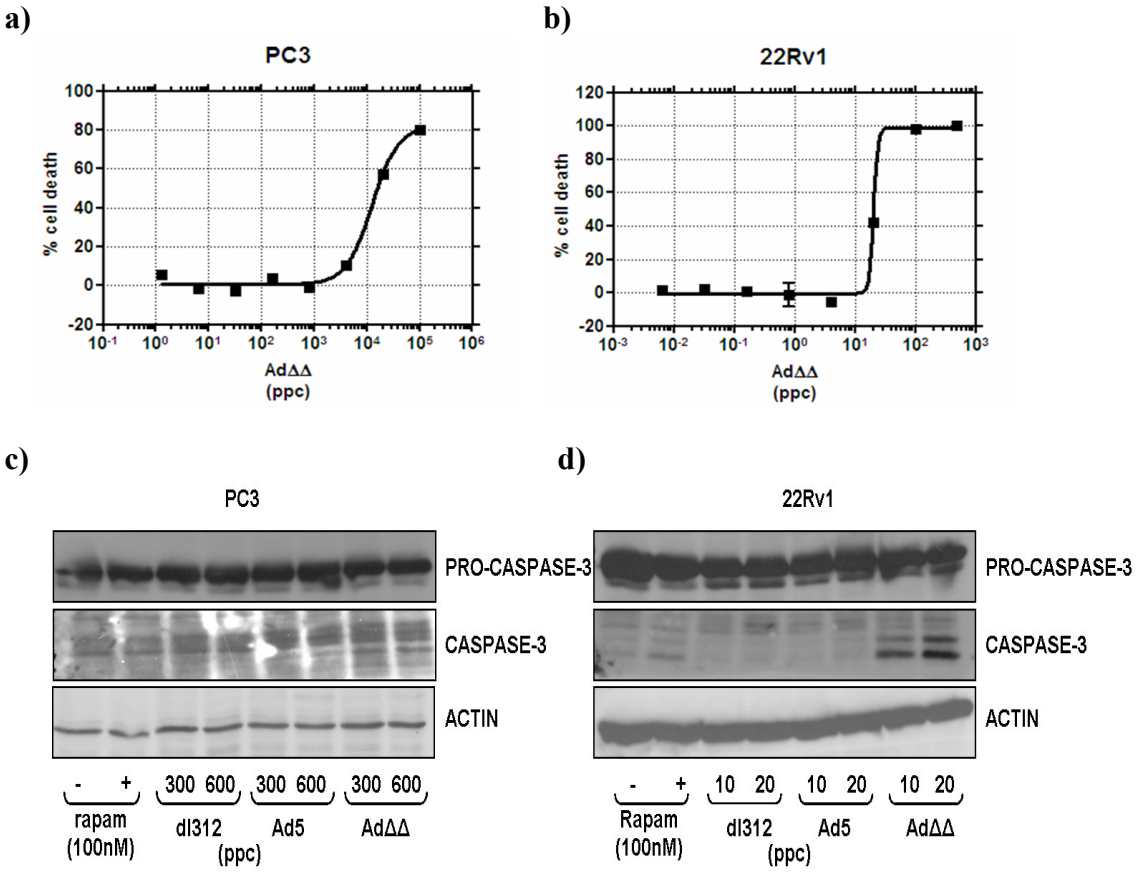


Figure 19. Sensitivity of human prostate cancer cells to the oncolytic adenovirus AdΔΔ
PC3 (a) and 22Rv1 (b) cells were infected with increasing concentration of AdΔΔ for seven days and analysed for viability assay. 22Rv1 cells display higher sensitivity to the cytophatic effect of the virus compared to PC3 cells. PC3 (c) and 22Rv1 (d) cells were infected with indicated particle per cell of AdΔΔ (ppc) for 72 h and equal amount of extracted protein in each sample (50 µg) was subjected to immunoblotting using anti-caspase-3 antibody, which detects pro- and caspase-3 levels. AdΔΔ infection induces caspase-3 activation in both cell lines.

4.9 Combination treatments

Next, potency of the combination treatment with docetaxel or mitoxantrone was evaluated in PC3 and 22Rv1 cells. Cells were infected with AdΔΔ in combination with docetaxel (2nM for 22Rv1 and 10 nM for PC3 cells), or mitoxantrone (50 nM for 22Rv1 and 100 nM for PC3 cells) and then assayed for viability assay 7 days after seeding. Results are shown in figure 20. Both drugs caused synergistic death in combination with AdΔΔ in both cell lines (Fig. 20a, b).

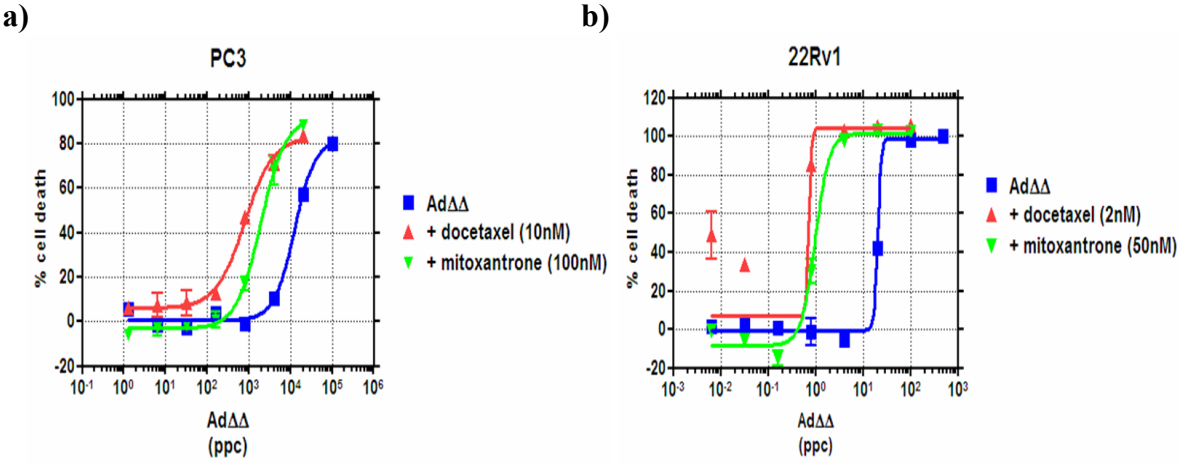


Figure 20. Combination treatment with docetaxel or mitoxantrone enhances AdΔΔ-induced cell death in prostate cancer cells
PC3 (a) and 22Rv (b) cells were infected with increasing concentration of AdΔΔ (ppc) in combination with the indicated concentration of docetaxel or mitoxantrone for six days and analysed for viability assay. Both docetaxel and mitoxantrone enhance the cytotoxic effect of *dl*922-947.

4.10 Suppression of autophagy by AdΔΔ in prostate cancer cells via Akt pathway

Prostate cancer cells have been demonstrated to undergo autophagy following several stimuli (DiPaola RS et al. 2008). Moreover, the oncolytic adenovirus regulated by the human telomerase reverse transcriptase promoter (hTERT-Ad, OBP-301) induced tumor-specific autophagic cell death in PC3 prostate cancer cells (Ito et al. 2006). The results of these previous studies indicate that prostate cancer cells thus represent a good experimental model to evaluate the activation of autophagy.
I measured the content of LC3-I and LC3-II by western blotting after infection of prostate cancer cells. PC3 and 22Rv1 cells were infected with AdΔΔ, using the wilde type adenovirus (Ad5) and the non replicating adenovirus (dl312) as controls. Rapamycin (100nM) was also used as positive control. LC3 levels were analysed 72 hpi. Surpringly, AdΔΔ decreased LC3-II levels in both PC3 (Fig. 21a) and 22Rv1 (Fig. 21b) cells. dl312 did not affect LC3 levels compared to untreated cells, wherease Ad5 virus had similar effects to that of AdΔΔ (Fig. 21a, b).
In agreement with these results, the acridine orange staining revealed no development of acidic vescicular organelles in both cell lines upon infection with AdΔΔ compared to untreated cells (data not shown). Taken togheter, all these data

demonstrate that AdΔΔ does not activate the autophagic pathway in prostate cancer cells. This observation is also in agreement with the results obtained infecting glioma cells with AdΔΔ (Fig. 11c).
Moreover, since AdΔΔ induced caspase-3 activation, the inhibition of LC3 cleavage by AdΔΔ confirmed that in AdΔΔ-infected prostate cancer cells an inverse correlation between apoptosis and autophagy exists.

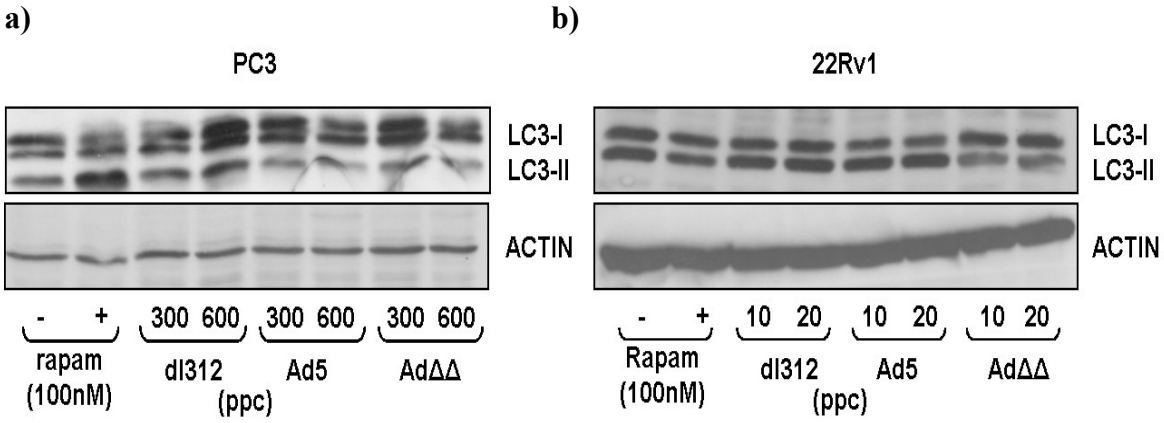


Figure 21. Effect of AdΔΔ on LC3 cleavage
PC3 (a) and 22Rv (b) cells were infected with AdΔΔ (PC3: 300, 600 ppc; 22Rv1: 10, 20) for 72 h and equal amount of extracted protein in each sample (50 μg) was subjected to immunoblotting using polyclonal rabbit anti-microtubule-associated protein 1 light chain 3 (LC3)-I and –II antibody. Rapamycin was used as positive control. Anti β-actin antibody was used as a loading control. AdΔΔ reduced LC3-II levels in both cell lines.

In order to investigate the molecular mechanisms by which AdΔΔ inhibits autophagy in prostate cancer cells, we investigated the involvement of the Akt/mTOR pathway, the main pathway that negatively regulates autophagy.
PC3 cells were infected with different MOIs of AdΔΔ and phosphorilation levels of Akt and p70s6k were analysed 72 hpi. Western blotting in figure 22 shows an increase in Akt and p70s6k phosphorilation after infection in a dose-dependent manner (Fig. 22a, b). This observations indicate that AdΔΔ suppresses autophagy in prostate cancer cells by modulating the Akt/mTOR autophagic pathway.

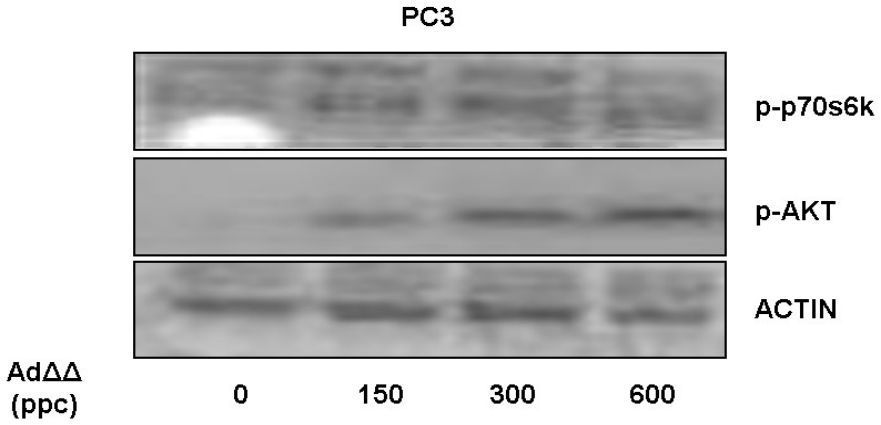


Figure 22. AdΔΔ activates the Akt/mTOR/p70S6K pathway in prostate cancer cells
PC3 (a) cells were infected with the indicated particle per cell (ppc) of AdΔΔ for 72 h and equal amount of extracted protein in each sample (50 μg) was subjected to immunoblotting using anti-phosphorylated (p-) Akt and p70s6k. Anti β-actin antibody was used as a loading control. AdΔΔ activates Akt/mTOR/p70s6k pathway.

To confirm that AdΔΔ inhibits autophagy, I tested whether AdΔΔ is able to revert the effect of rapamycin, a known inducer of autophagy.
Rapamycin and its analogues (such as CCI-779, RAD001, and AP23573) inhibit mTOR, the kinase that suppresses autophagy and is active when nutrients are abundant (Bjornsti and Houghton 2004). I verified whether AdΔΔ modified the rapamycin-induced AVOs accumulation. To this aim, I used different MOIs of AdΔΔ or dl312 to infect PC3 and 22Rv1 cells in presence of rapamycin (100nM) and 72 hpi I analysed the development of acidic vescicular organelles by flow cytometry after staining cells with acridine orange. The diagrams in Fig. 23 reports the percentage of red-positive cells, representing cells with increased acidic compartment. Rapamycin increased the percentage of red-positive cells in 22Rv1 (from 12% to 18%), and PC3 (from 18% to 65%), indicating abundant cytoplasmic AVOs formation. AdΔΔ clearly inhibited the AVOs development in rapamycin-treated cells (fig. 23a, b); conversely, the non replicating virus dl312 was not able to reverte the rapamycin effect (data not shown).
Next, I examined the expression of LC3-I and LC3-II. In accordance with literature, LC3-II/I ratio increased in PC3 and 22Rv1 cells treated with rapamycin (100nM). In cells infected with AdΔΔ the rapamycin-induced increase in LC3-II/I ratio was partially inhibited (Fig. 23c, d). These results indicate that AdΔΔ suppresses autophagy in prostate cancer cells by reducing LC3-II levels, partially reverting LC3-II increase mediated by rapamycin.

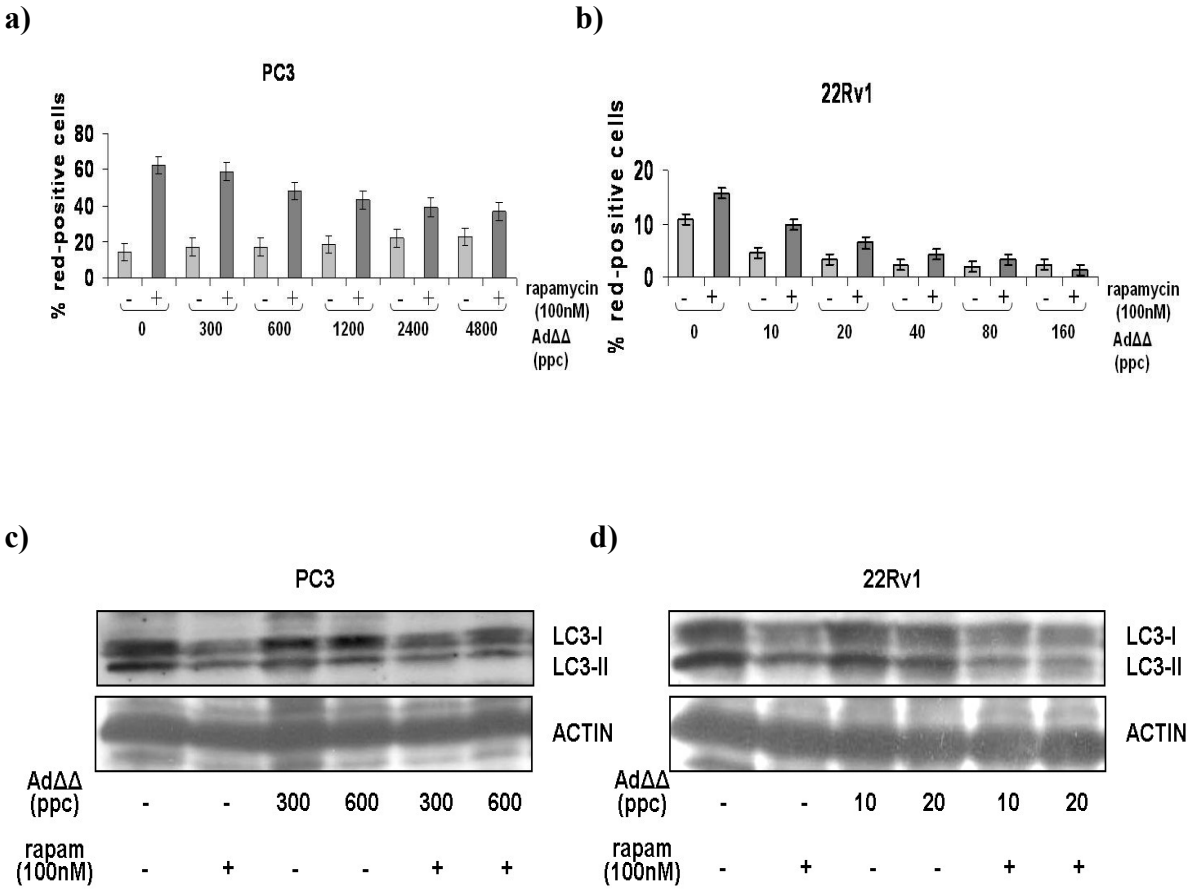


Figure 23. AdΔΔ reduces the rapamycin-induced autophagy in prostate cancer cells
PC3 (a) and 22Rv1 (b) cells were infected with different particle per cell (ppc) of AdΔΔ, in presence or not of 100nM of rapamycin for 72 h and stained with acridine orange. The percentages of red fluorescence-positive cells was calculated by flow cytometry and shown in the diagrams.
c,d) Cells were treated with different concentration of AdΔΔ for 72 h in presence or not of rapamycin (100nM) and equal amount of extracted protein in each sample (50 μg) was subjected to immunoblotting using polyclonal rabbit anti-microtubule-associated protein 1 light chain 3 (LC3)-I and –II antibody. Anti β-actin antibody was used as a loading control. AdΔΔ reduced rapamycin-induced AVOs accumulation and LC3 cleavage in both cell lines.

4.11 Effect of 3-methyladenine on AdΔΔ-induced cytotoxicity in prostate cancer cells

In prostate cancer cells, autophagy is clearly reduced by AdΔΔ. To investigate the role of this pathway (as cell survival or cell death mechanism), I decided to

analyse the effect of autophagy inhibition, by using 3-MA. PC3 and 22Rv1 cells were infected with different concentrations of AdΔΔ in the presence of 3-MA (1mM), and cell survival was analysed seven days later. AdΔΔ–induced cytotoxicity was slightly increased by 3-MA in both cell lines (Fig. 24a, b), demonstrating that autophagic process in prostate cancer cells acts as a protective response to the infection with AdΔΔ.
These results also confirm that pharmacological autophagy inhibition can enhance the effect of oncolytic viruses in prostate cancer cells.

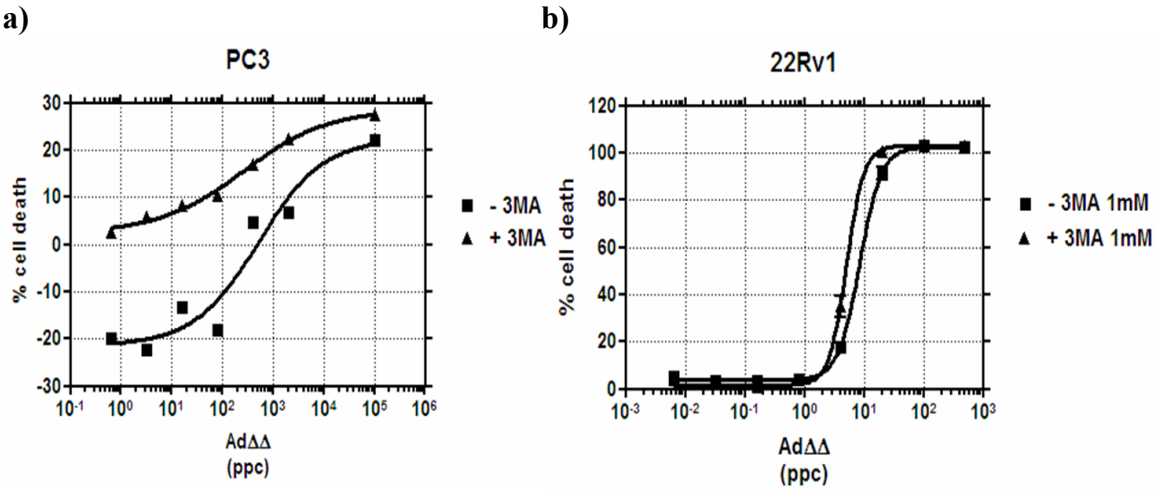
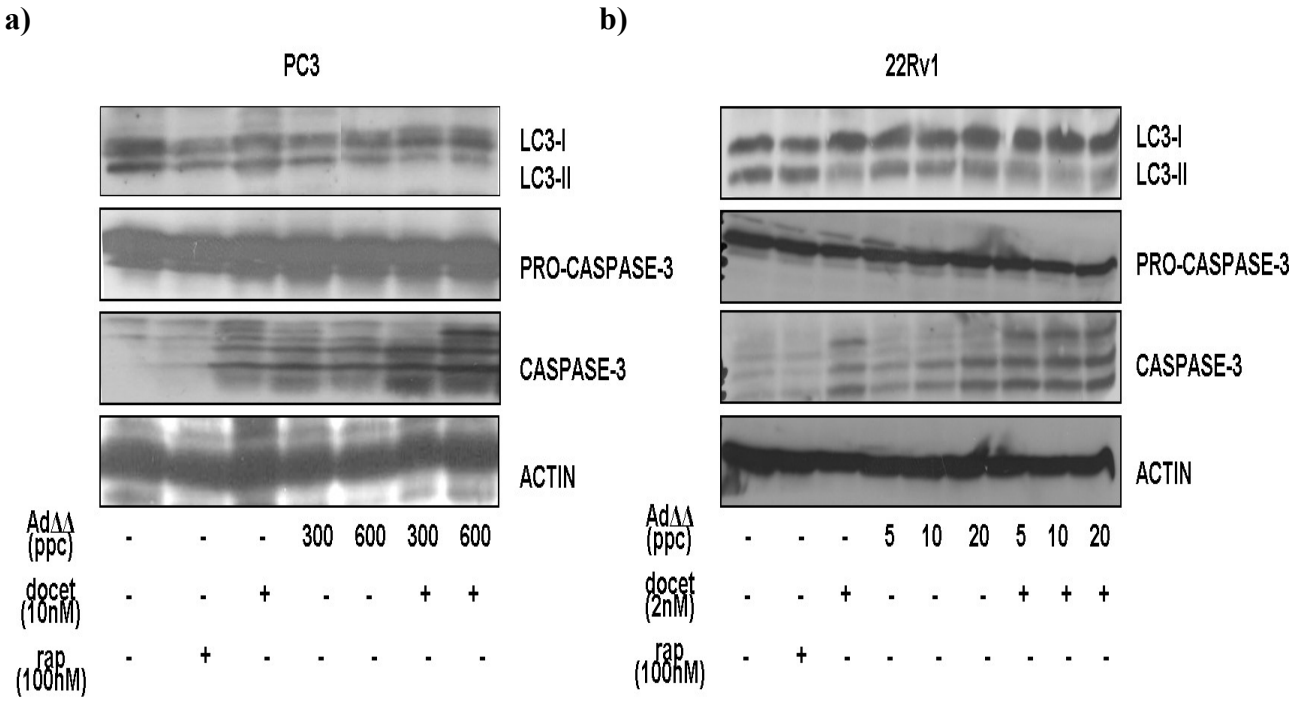


Figure 24. Autophagy inhibition enhances AdΔΔ-mediated cytotoxicity in prostate cancer cells
PC3 (a) and 22Rv1 (b) cells were infected with increasing concentration of AdΔΔ in combination with 1mM of 3-methyladenine (3-MA) for six days and analysed for viability assay. Combination with 3-MA enhances the cytotoxic effect of AdΔΔ in both cell lines.

4.12 AdΔΔ mutant synergistically enhances docetaxel/mitoxantrone-induced cell killing by inhibiting autophagy

Oncolytic effects of AdΔΔ in prostate cancer cells were potenciated by the combination with docetaxel or mitoxantrone. Since AdΔΔ inhibited autophagy and induced apoptosis in PC3 and 22Rv1 cells, I decided to examine whether the autophagic and/or the apoptotic process could be involved in the synergistic effect of this virus with the drugs.
To this aim, I analysed whether the combination treatment affects the LC3 cleavage and caspase-3 activation. 22Rv1 and PC3 cells were treated with AdΔΔ in presence of docetaxel or mitoxantrone. LC3 levels were analysed 72 hours later

(Fig. 25). AdΔΔ or docetaxel, as single treatment, caused a decrease in LC3-II levels. In the combination treatment, a further decrease in LC3-II levels was observed (Fig.25a, b). Rapamycin (100nM) was used as positive control. Conversly, mitoxantrone treatment activated autophagy, by increasing LC3-II levels in both cell lines, but this effect was reduced by AdΔΔ (Fig. 25c, d).
Surpringly, among these two drugs, docetaxel but not mitoxantrone can activate caspase-3, wherease a clear caspase-3 activation was observed when AdΔΔ was combined with both docetaxel or mitoxantrone (Fig. 25).
In conclusion, in prostate cancer cells, the oncolytic adenovirus AdΔΔ inhibits autophagy and its oncolytic effects are enhanced by the pharmacological inhibition of autophagy. Docetaxel and mitoxantrone have opposite effects on autophagy: docetaxel inhibits it by reducing LC3-II levels, whereas mitoxantrone induces LC3 cleavage increase. However, the combination treatment with AdΔΔ led to a further inhibition of autophagy and to a strong apoptosis induction in both cases.
These findings indicate that also in prostate cancer cells a direct correlation between autophagy inhibition and apoptosis induction exists. Therefore, it is possible to conceive that adequate combination treatments could enhance the efficacy of AdΔΔ infection in PC3 and 22Rv1 cells, by modulating the autophagic pathway.



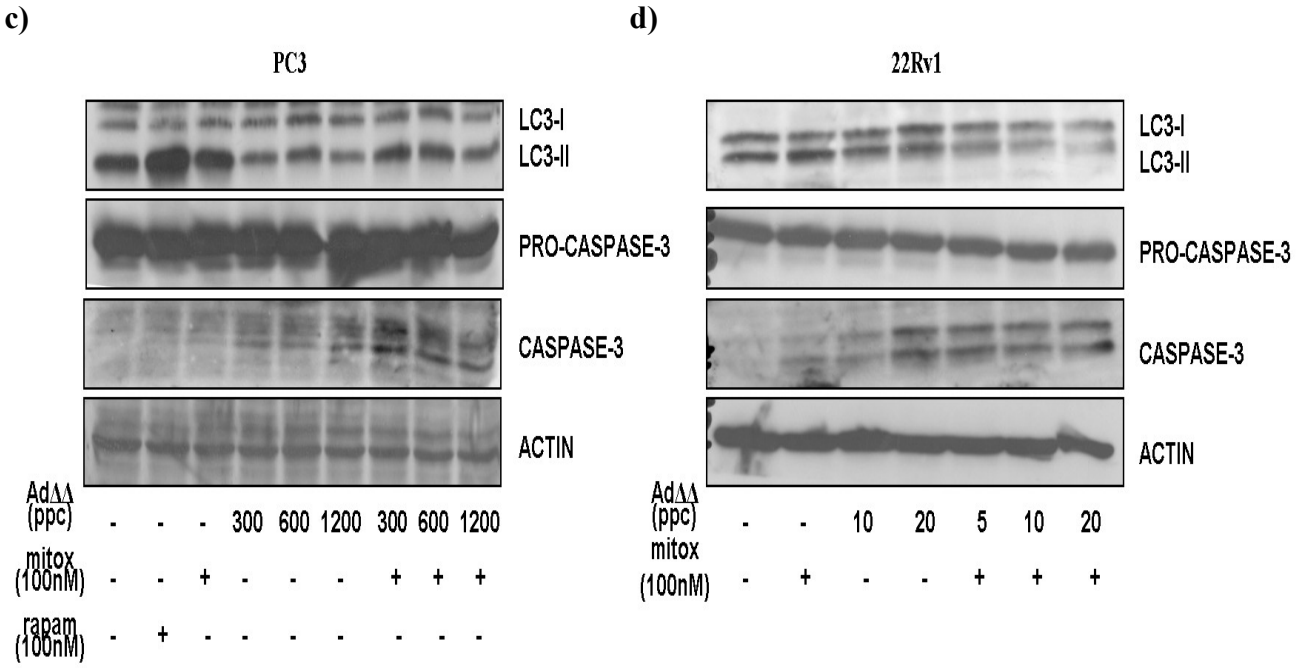


Figure 25. Combination treatment with docetaxel or mitoxantrone enhances AdΔΔ-induced autophagy inhibition and apoptosis induction

PC3 and 22Rv1 cells were infected with different concentrations of AdΔΔ (ppc) in combination with the indicated concentrations of docetaxel (a,b) or mitoxantrone (c,d) for 72 h and equal amount of extracted protein in each sample (50 μg) was subjected to immunoblotting using polyclonal rabbit anti-microtubule-associated protein 1 light chain 3 (LC3)-I and –II and caspase-3 antibodies. Anti β-actin antibody was used as a loading control. Rapamycin (100 nM) was used as positive control.

5. CONCLUSIONS

In this study I demonstrated the efficacy of the oncolytic adenoviruses *dl*922-947 and AdΔΔ for treating malignant glioma cells and prostate cancer cells, respectively. In order to find new therapeutic strategies to potenciate the oncolytic viral activity, I have investigated the mechanisms of oncolytic virus-induced cell death. I showed that in *dl*922-947-infected U373MG and U87MG glioma cells, autophagy, but not apoptosis, occurs. Autophagy is a type of protein degradation system, prominently observed in cells undergoing environmental stressors, such as amino acid starvation, but also viral and bacterial infection. I showed that the infection of glioma cells with *dl*922-947 led to the development of acidic vescicular organelles and to the increase of LC3-II levels, that are typical autophagic features. Autophagy is a survival process, allowing cells to survive in unfavourable conditions; however, it is also a type of programmed cell death alternative to apoptosis. Infact, studying the role of autophagy in treated cells may have a therapeutic value. The data presented in my study indicate that autophagy represent a survival mechanism, occurring in both glioma and prostate cancer cells infected with the oncolytic adenoviruses. Indeed, blocking of autophagy with pharmacological inhibitors enhanced the oncolytic viral effects triggering caspase-3 activation and apoptotic cell death.

It is well known that the Akt/mTOR/p70s6k pathway is the main pathway involved in the negative regulation of autophagy, whereas the ERK pathway is a positive regulator. Surpringly, this study clearly showed that both *dl*922-947 and AdΔΔ adenoviruses activate Akt/mTOR/p70s6k pathway, resulting in autophagy inhibition. Moreover, *dl*922-947 clearly inhibited ERK pathway and the inhibition of ERK pathway by using PD98059 enhanced *dl*922-947-induced cytotoxicity by activating apoptosis.

In this study I also showed that AdΔΔ clearly inhibited autophagy in prostate cancer cells and is also able to reverte the rapamycin-induced autophagy. Moreover, the combination with docetaxel or mitoxantrone increased autophagy inhibition and apoptosis induction.

Given these observations, the activation of autophagy in glioma and prostate cancer cells is a survival mechanism in response to the infection and oncolytic adenoviruses try to suppress this process modulating important autophagic signalling pathways.

In conclusion, the results described in this study encourage the use of *dl*922-947 and AdΔΔ oncolytic adenoviruses for the treatment of glioma and prostate cancer, using new therapeutic protocols. In particular, I have proposed that a novel therapeutic approach based on the combination between viruses and anti-autophagic drugs could represent a better option for virotherapy against these so aggressive human tumors.

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PED/PEA-15 MODULATES COXSACKIE AND ADENOVIRUS RECEPTOR (CAR) EXPRESSION AND ADENOVIRAL INFECTIVITY VIA ERK-MEDIATED SIGNALS IN GLIOMA CELLS

Ginevra Botta[1]*, Giuseppe Perruolo[1,2]*, Silvana Libertini[1], Angela Cassese[1,2], Antonella Abagnale[1], Francesco Beguinot[1], Pietro Formisano[1] and Giuseppe Portella[1,3]

[1]Dipartimento di Biologia e Patologia Cellulare e Molecolare Università Federico II, Napoli-Italy. [2] Istituto di Endocrinologia e Oncologia sperimentale –C.N.R. Napoli and [3]Cattedra di Patologia Clinica Università Federico II, Napoli-Italy.
(*equal contribution)

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§ Corresponding author: Giuseppe Portella
Dipartimento di Biologia e Patologia Cellulare e Molecolare, Facoltà di Medicina e Chirurgia, Università di Napoli Federico II,
via S. Pansini 5, 80131 Napoli, Italy.
Tel: ++39 081 746 3052; Fax: 39 081 7463308; e-mail: Portella@unina.it

Abstract

Aims: Glioblastomas multiforme (GBM) is the most aggressive human brain tumour, and is highly resistant to chemo and radiotherapy. Therefore novel treatments are required. Selective replicating oncolytic viruses represent a novel approach for the treatment of neoplastic diseases. Coxsackie and Adenovirus Receptor (CAR) is the primary receptor for adenoviruses, and loss or reduction of CAR greatly decreases adenoviral entry. The understanding of the mechanisms regulating CAR expression and localisation will contribute to increase the efficacy of oncolytic adenoviruses.

Results. Two glioma cell lines (U343MG and U373MG) were infected with the oncolytic adenovirus *dl*922-947. U373MG cells were more susceptible to cell death following viral infection, compared to U343MG cells. The enhanced sensitivity was paralleled by increased adenoviral entry and CAR mRNA and protein levels in U373MG cells. In addition, U373MG cells displayed decreased ERK1/2 nuclear/cytosolic ratio, compared to U343MG cells. Intracellular content of PED/PEA-15, an ERK1/2 interacting protein, was also augumented in these cells. Both ERK2 overexpression and genetic silencing of PED/PEA-15 by antisense oligonucleotides increased ERK nuclear accumulation and reduced CAR expression and adenoviral entry.

Conclusions: Our data indicate that *dl*922-947 could represent an useful tool for the treatment of GBM and that PED/PEA-15 modulates CAR expression and adenoviral entry, by sequestering ERK1/2.

Introduction

Malignant glioma of astrocytic origin or glioblastoma multiforme (GBM) is the most common primary brain tumour in adults and the most aggressive human brain tumour.

Primary GBM develops *de novo* from glial cells, typically has a clinical history of <6 months, and is most frequent in older patients (Brandes *et al*., 2008). Secondary GBM develops from preexisting low-grade astrocytomas and affects younger patients. GBM is an anaplastic, highly cellular tumour with poorly differentiated, round or pleomorphic cells (Furnari *et al*., 2007; Brandes *et al*., 2008).

Treatment normally includes tumour resection, radiation and chemotherapy, however GBM cells are highly infiltrative, preventing the complete tumour resection, and largely resistant to chemo and radiotherapy. Consequently, only a small minority of GBM patients achieves long-term survival (Furnari *et al*., 2007; Brandes *et al*., 2008). Novel treatment strategies are therefore required in order to increase the therapeutic options.

Selective replicating oncolytic viruses represent a novel platform for the treatment of neoplastic diseases and several studies have been performed showing the feasibility of this therapeutic strategy in glioblastoma patients (Haseley *et al.*, 2009).

Adenoviruses and other viruses have been engineered for selective replication within neoplastic cells. The most common approach is the deletion of viral gene whose product is necessary for replication in normal cells but expendable in cancer cells (Vähä-Koskela *et al*., 2007).

The first replication-competent-virus described is *dl*1520 (Onyx-015), an adenoviral mutant containing a deletion of E1B-55K gene, that abolishes its capability to bind and inhibit p53. Being unable to avoid the subsequent p53 induced apoptosis, it has been hypothesised that *dl*1520 can only replicate in cells lacking functional p53 pathway (Bischoff *et al.*, 1996). E1B-55K also mediates

late-viral RNA transport and the loss of E1B-55K restricts the viral replication to tumour cells capable of taking over the RNA export function of the viral gene product (O'Shea *et al.*, 2005). Although active against tumoural cells and well tolerated in several clinical trials, objective responses with *dl*1520 are limited to date, highlighting the need of new oncolytic adenoviruses with higher replication efficiency (Kirn, 2001).

*dl*922-947 is a second generation adenoviral mutant bearing a 24-bp deletion in E1A-Conserved Region 2 (CR2), necessary for binding and inactivation of pRb family of proteins (Heise *et al*., 2000); *dl*922-947 mutant is unable to induce progression from G1 into S-phase of normal cells, but replicates with high efficiency in cells with an abnormal G1-S checkpoint.

The G1-S checkpoint is critical for cell growth progression (Sherr, 2000) and is abnormal in GBM (Solomon *et al.*, 2008), therefore mutant E1A adenoviruses have been proposed for the therapy of gliomas and are now in preclinical development as antiglioma therapy (Giacomo *et al*., 2003).

The efficacy of adenoviral vectors as therapeutic agents depends on the ability of neoplastic cells to bind and internalise adenoviruses. Adenoviral infection involves two distinct virus-cell interactions. First cell surface attachment is mediated by binding of the viral fiber protein to the cellular Coxsackie and Adenovirus Receptor (CAR) (Bergelson *et al*., 1997; Tomko *et al*., 2000). Internalization, via receptor-mediated endocytosis, involves interactions between the viral penton protein and cellular integrins, such as $\alpha_v\beta_3$, $\alpha_v\beta_5$ $\alpha_3\beta_1$ that act as coreceptors (Nemerow, 2000).Low or absent expression of CAR is seen in many primary tumour tissue (Rein *et al.*, 2006) and a low expression of CAR receptor has been observed in grade IV gliomas (Fuxe *et al.*, 2003). The analysis of CAR expression has been proposed to identify and select cancer patients, that could be successfully treated with adenoviral mutants.

However, the molecular mechanisms by which CAR expression is regulated have been only partially elucidated. Interestingly, the inhibition of ERK (extracellular-signal-regulated kinase)/MAPK (mitogen-activated protein kinase) pathway has been reported to up-regulate CAR expression (Anders *et al.*, 2003).

Here we show that, in glioblastoma cell lines (Hao *et al*., 2001; Xiao *et al.*, 2002), the expression of PED/PEA (phosphoprotein enriched in diabetes/ phosphoprotein enriched in astrocytes)-15, a protein which binds ERK and prevents its nuclear accumulation (Formstecher *et al*., 2001; Hill et al., 2002; Renault *et al.*, 2003; Whitehurst *et al*., 2004; Renganathan et al., 2005), correlates with CAR mRNA levels as well as with the sensitivity to adenoviral infection and *dl*922-947 killing activity. Indeed, silencing of PED/PEA-15 promotes ERK nuclear translocation and simultaneously reduces CAR expression and adenoviral entry into glioblastoma cells.

Material and methods

Cell lines, plasmids and transfections

Glioma cell lines U343MG, and U373MG were purchased from American Type Culture Collection. All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 100 IU of penicillin/ml, 100 IU of streptomycin/ml and 2% L-glutamine in humified CO_2 incubator.

Sequences of scramble and antisense oligonucleotides are as follows:

AS-PED/PEA-15 human 5'-tGACGCCTCTGGAGCTGAGA,

Scr-PED/PEA-15 human 5'-gGCAATTTCGAGCGGCACGT (Sigma).

The plasmid pcDNA3-HA containing ERK2 cDNA (pcDNA3-HA-pMAPK2) was kindly provided by dr. Mario Chiariello.

Transfection of PED antisense and scramble oligonucleotides, or pcDNA3-HA containing ERK2 cDNA, was accomplished using the lipofectamine (Invitrogen) method as described previously (Condorelli *et al*., 1998).

Preparation of adenoviruses, infection and viability assay

*dl*922-947 is a second generation adenoviral mutant that has a 24-bp deletion in E1A Conserved Region 2 (CR2). AdGFP is a non replicating E1A-deleted adenovirus encoding green fluorescent protein. Viral stocks were expanded in the human embryonic kidney cell line HEK-293, and purified, as previously reported (Portella *et al*., 2002).

Stocks were stored at -70°C after the addition of glycerol to a concentration of 50% vol/vol. Virus titre was determined by plaque-forming units (pfu) on the HEK-293 cells.

For the evaluation of the cytoxic effects of the *dl* 922-947 virus, 1 x10^3 cells were seeded in 96-well plates, and 24 hours later cells infected with at different Molteplicity Of Infection (MOIs). After ten days cells were fixed with 10% TCA and stained with 0.4 % sulforhodamine B in 1% acetic acid (Skehan *et al*., 1990). The bound dye was solubilised in 200 □ l of 10 mM unbuffered Tris solution and the optical density was determined at 490 nm in a microplate reader (Biorad). The percent of survival rates of treated cells were calculated by assuming the survival rate of untreated cells to be 100%.

For the evaluation of infectivity cells were detached, counted, and plated in 6 well plate at 70% cell density. After 24 hours cells were infected with AdGFP diluted in growth medium at different MOIs, medium was replaced after 2 hours. Cells were washed 24 hours post infection (hpi), then trypsinized and analyzed for GFP expression on a FACS cytometer (Dako Cytomation, U.S.A.) and Summit V4.3 software (Dako).

Quantitative PCR of dl 922-947

To quantify the amount of *dl*922-947 virus genome, cells were infected with *dl*922-947 at different MOIs (0.1, 1 and 10 pfu/cell). At 48 hpi, cell supernatant was collected and viral DNA extracted using a QIAamp DNA mini kit (Valencia, CA, USA), then quantified by real-time PCR using assay-specific primer and probe. A Real time-based assay was developed using the following primers: 5'-GCCACCGAGACGTACTTCAGCCTG-3' (Upstream primer) and 5'-TTG TAC GAG TAC GC G GTA TCCT-3' (Downstream primer) for the amplification of 143 bp sequence of the viral hexon gene (from bp 99 to 242 bp). For quantification, a standard curve was constructed by assaying serial dilutions of *dl*922-947 virus ranging from 0.1 to 100 pfu/cell to quantify the input dose.

Detection of cell-surface CAR receptor and mRNA quantification

Cells were grown in 6-well plates. After 48 hours cells were detached in PBS-EDTA 10mM, washed with PBS and then incubated with a mouse anti-CAR monoclonal antibody RmcB (Hsu et al., 1988), a secondary antibody (polyclonal rabbit anti-mouse antibody conjugated to fluorescein isothiocyanate [FITC]; Sigma) and analyzed for CAR expression on a FACS cytometer (Dako Cytomation, U.S.A.) and Summit V4.3 software (Dako).

To block CAR receptor, cells were pretreated with increasing concentrations of the mouse anti-CAR monoclonal antibody RmcB (1:100, 1:250, 1:500) , for 1 hour at room temperature before addition of virus. Cells were harvested and analysed as previously described.

To analyse CAR mRNA levels cells were harvested and the total RNA was isolated and DNase digested using the RNeasy minikit (QIAGEN) according to the manufacturer's recommendations. One microgram of tissue or cell RNA from each sample was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). PCR products were analyzed using Sybr green mix (Invitrogen).

Reactions were performed using Platinum Sybr green qPCR Super-UDG using an iCycler IQ multicolour real-time PCR detection system (Bio-Rad). All reactions were performed in triplicate, and β-actin was used as an internal standard.

The primer sequences were:

Cxadr Forward, 5'-ATGAAAAGGAAGTTCATCAACGTA-3',

Cxadr Reverse, 5'-AATGATTACTGCCGATGTAGCTT-3', generating an amplicon of 93 nucleotides scattered among sixth and seventh exon;

β-actin Forward, 5'-GCGTGACATCAAAGAGAAG-3';

β-actin Reverse, 5'-ACTGTGTTGGCATAGAGG-3'.

The conditions used for PCR were 10 min at 95 C and then 45 cycles of 20 sec at 95 C and 1 min at 60 C. To calculate the relative expression levels, we used the $2^{-[\Delta][\Delta]Ct}$ method, where $[\Delta][\Delta]Ct = [\Delta]Ct_{,sample} - [\Delta]Ct_{,reference.}$

Protein extraction, cell subfractionation and western blot analysis

In all experiments, 70% confluent cells were used. Subcellular fractionation was performed by a previously described method (Ruvolo *et al.*, 1998). Briefly, cells were broken in ice-cold hypotonic Hepes buffer (10 mM HEPES, pH 7.4, 5 mM MgCl2, 40 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 10 g/ml aprotinin, 10 g/ml leupeptin). Broken cells were centrifuged at 200 x *g* to pellet nuclei. The resulting supernatants were centrifuged at 10,000 x *g* to pellet the heavy membrane fraction. The last supernatant represented the cytosolic fraction. The nuclear membranes were isolated by centrifugation of the nuclei through a 2M sucrose cushion at 150,000 x *g*. For protein extraction cells were homogenised directly into lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1%Triton-X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 0.5 mM sodium orthovanadate, 20 mM sodium pyrophosphate). The lysates were clarified by 20 minutes centrifugation at 14,000g. Protein concentrations were estimated by a Bio-Rad assay (Bio-Rad, München, Germany),

and then proteins were boiled in Laemmli buffer for 5 min before electrophoresis. Proteins were subjected to SDS-PAGE (10% polyacrylamide) under reducing condition. After electrophoresis, proteins were transferred to nitrocellulose membranes (Immobilon Millipore Corporation). After blocking with TBS-BSA, the membrane was incubated with the primary antibody: polyclonal rabbit antibody against CAR receptor SC-15-405 (Santa Cruz) 1:250, Rabbit anti PED serum (1:2000), previously described (25), rabbit antibody ERK 1 and 2 (1:1000) or with the rabbit antibody against actin (1:2000)(Santa Cruz) for an overnight incubation.

Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:2000) for 45 min (at room temperature) and the reaction was detected with an ECL system (Amersham Life Science, UK).

Results

U373MG and U343MG cells show a different sensitivity to adenoviral infection

First, we evaluated the antineoplastic activity of the selective replicating oncolytic adenovirus *dl*922/947 against U373MG and U343MG glioma cell lines (Fig 1 A). Cells were infected at different MOIs of *dl*922/947 and cell survival was evaluated after seven days. U373MG cell line displayed higher sensitivity to *dl*922/947 with an IC_{50} of MOI 0.0001 (pfu/cell), whereas for U343MG IC_{50} was observed at a MOI of 0.1 (Fig 1 A). Genome equivalent copies analysis showed that both cell lines can sustain viral replication. However, a higher copy number of *dl*922/947 was detected in U373MG compared to U343MG (Fig. 1B).

Our data indicate that both glioma cell lines are sensitive to *dl*922-947, although displaying a different sensitivity to its oncolytic activity.

To study whether this difference could be due to unlike viral entry, we used a non replicating adenovirus encoding the green fluorescent protein (AdGFP). Cells were infected with different MOIs of AdGFP and 24 hpi the amount of GFP-positive cells was quantified by FACS analysis. U373MG cells were readily infected by AdGFP, showing 80% of positive cells at 1 pfu/cell of virus, whilst at the same MOI only 40% of U343MG cells were GFP positive (Fig 1C).

Adenoviral entry is CAR-mediated in U343MG and U373MG cells

It has been reported that CAR (Coxsackie And Adenovirus Receptor) is the main mediator of the adenoviral entry (Bergelson *et al*., 1997; Nemerow et Tomko *et al*., 2000; Arnberg, 2009). Therefore, we have analysed CAR expression by western blot and cytofluorimetric analysis in U343MG and U373MG cell lines. U373MG cells displayed higher total (Fig 2 A) and membrane levels (Fig 2 B), compared to U343MG cells; this observation parallels the higher viral entry and sensitivity to the oncolytic activity of *dl*922-947. To evaluate whether CAR may play a direct role in adenoviral entry, U343MG and U373MG cells were pretreated 1 h with increasing amount of the blocking anti-CAR monoclonal antibody RmcB and infected with 25 pfu/cell of AdGFP.

Significant decreases of GFP emission were observed in U373MG ($p<0.005$) and in U343MG cells ($p<0.001$), upon treatment with RmcB (Fig 2 C). These data confirm that CAR plays a crucial role in adenoviral entry in both cell lines.

ERK modulates U373MG cells infectivity by regulating CAR expression

It has been described that ERK signalling regulates CAR levels in cancer cell lines (Anders *et al.*, 2003). Therefore, we have evaluated ERK1/2 phosphorylation and subcellular localisation in U373MG and U343MG cells. No difference of ERK1/2 phosphorylation was detected in the two cell lines (Fig 3A left). However, in U373MG cells, ERK1/2 localization was mostly cytosolic. Conversely, in

U343MG it was mostly localised in the nuclei (Fig 3 A right), suggesting that ERK1/2 nuclear accumulation, might down-regulate CAR expression.

Next, a plasmid containing ERK2 cDNA (pcDNA3-HA pERK2) was transiently transfected into U373MG cells, in order to force ERK into the nucleus. Transfection efficiency was evaluated by western blot (Fig 3 B). ERK2 overexpression was paralleled by increased detection of ERK2 in the nuclei and by a reduction of CAR total and membrane levels (Fig 3 B). CAR mRNA levels, evaluated by RT Real Time PCR, also showed a reduction of about 80%(Fig 3 C), thus suggesting that ERK nuclear shift down-regulates CAR gene expression. Accordingly, a significant reduction in GFP emission was observed in ERK2-transfected U373MG cells, following infection with AdGFP (Fig 3 D).

Down regulation of PED/PEA-15 decreases CAR levels and adenoviral infectivity in U373MG cells

PED/PEA-15 is a death effector domain-containing protein, which is involved in the regulation of apoptotic cell death (Hao *et al*., 2001; Xiao *et al*., 2002). PED/PEA-15 is highly expressed in cells of glial origin (Hao *et al*., 2001; *Xiao et al*., 2002; Sharif *et al*., 2004). Moreover, it has been reported that PED/PEA-15 inhibits nuclear translocation and activity of ERK1/2 (Formstecher *et al*., 2001; Hill *et al*., 2002; Renault *et al*., 2003; Whitehurst *et al*., 2004; Renganathan *et al*., 2005).

As also previously reported (Hao *et al.*, 2001) PED/PEA-15 levels were higher in U373MG than in U343MG cells (Fig. 4 A). To assess whether PED/PEA-15 may control ERK localization, a PED/PEA-15 antisense oligonucleotide (PED-As) was transfected into U373MG cells, using a scrambled oligonucleotide as control (PED-Scr). PED/PEA-15 levels in transfected cells were evaluated by western blot (Fig. 4 B).

Upon PED-As transfection, nuclear/cytosolic ratio of ERK distribution was shifted toward nucleus (Fig 4 C).

Down-regulation of PED/PEA-15 was accompained by a reduction in total (Fig. 4 B) and membrane (Fig. 5 A) CAR levels respectively.

Moreover, Real Time RT PCR experiments showed about 50% reduction of CAR mRNA levels (Fig 5 B). Finally, upon infection with 25 MOIs of AdGFP for 24 hours, PED-As transfected cells showed a significant decrease in GFP emission (Fig 5 C), thus suggesting that PED/PEA-15 is involved in CAR expression and in the control of adenoviral infectivity in glioma cells.

Discussion

GBM is surgically incurable in the vast majority of patients (Brandes *et al*., 2008), with median survival duration of about 9-15 months (Furnari *et al*., 2007). The recently developed protocol of adjuvant therapy, radiation followed by chemotherapy, administered after surgery, had only demonstrated a moderate increase in survival (Andreas *et al*., 2009). Therefore novel therapeutic approaches are required. The availability of novel prognostic and/or predictive markers could be also beneficial to tailor the treatment of GBM patients.

Genetically engineered, conditionally replicating viruses are promising therapeutic agents for cancer. Indeed, radio and chemotherapy treatments are often potentiated by oncolytic adenoviruses in experimental and in clinical settings (Chu *et al*., 2004; Vähä-Koskela *et al*., 2007) and therefore mutant adenoviruses could be used to for the development of combined treatments.

Several oncolytic viruses have been already tested in preclinical or clinical studies for the treatment of gliomas. Herpes simplex oncolytic type 1 (HSV-1) has been tested in a phase I clinical study, showing that can be safely administered into human brains (Todo, 2008). Replication competent adenoviruses also hold a promise for the treatment of GBM. Delta 24, an adenovirus able to target Rb pathway and bearing a mutation similar to *dl*922/947, has been shown to be effective against GBM cells (Jiang *et al*., 2007).

In the present study, we have observed that *dl*922/947 is active against two glioblastoma cell lines, U343MG and U373MG, reinforcing the concept that the therapy of glioblastoma could benefit of the use of oncolytic viruses. However, a differential sensitivity to the oncolytic activity of *dl*922/947 was evidenced in the two cell lines, being U343MG cells more resistant to the virus.

This difference could be potentially due to factors affecting viral life cycle (such as attachment, entry, viral gene expression, etc). It is generally accepted that poor adenoviral entry in neoplastic cells represents the most important obstacle for an

effective therapy based on replicating oncolytic adenoviruses (Vähä-Koskela *et al.*, 2007).

Coxsackie and Adenovirus Receptor (CAR) is the primary receptor for adenoviruses, and loss or reduction of CAR greatly decreases adenoviral entry (Bergelson *et al*., 1997; Nemerow and Tomko *et al.*, 2000; Rein et al., 2006). Higher levels of CAR expression were observed in U373MG, as compared to U343MG and this was paralleled by increased viral entry. However, a significant reduction was observed in both cell lines upon blocking of the receptor with an anti CAR antibody.

Although a complete abrogation of adenoviral entry was not obtained, possibly due to residual entry via alternative pathways (Arnberg *et al*., 2009) or sub-total blockade with the antibody, our data are consistent with the hypothesis that CAR-mediated internalization plays a major role in both cell lines.

It has been demonstrated that disruption of signaling through the Raf-MEK-ERK pathway by MEK inhibitors (U0126 and PD184352) up-regulates CAR expression (Anders *et al*., 2003) Interestingly, U373MG cells displayed a higher ERK1/2 cytosolic localisation, compared to U343MG cells, where ERK1/2 was mostly nuclear. Since nuclear translocation is a crucial step for ERK-mediated regulation of gene expression, we hypothesized that ERK nuclear activity could control CAR expression. Indeed, overexpression of ERK2 into U373MG cells was accompanied by a forced nuclear localization and decreased CAR mRNA and protein levels, finally leading to reduced viral entry.

PED/PEA-15 is a death effector domain-containing protein involved in the regulation of apoptotic cell death and highly expressed in cells of glial origin (Hao *et al*., 2001; Xiao *et al*., 2002). PED/PEA-15 regulates the ERK/MAPK pathway by binding ERK1/2 and preventing its nuclear accumulation and activity (Formstecher et al., 2001; Hill et al., 2002; Renault et al., 2003; Whitehurst et al., 2004; Renganathan et al., 2005). Moreover, abrogation of ERK1/2 binding as a

result of point mutations in PED/PEA-15 restores normal ERK1/2 function (Whitehurst *et al.*, 2004). We have therefore hypothesised that PED/PEA-15 could be involved in CAR regulation and adenoviral infectivity by controlling ERK subcellular distribution. Indeed, PED/PEA-15 levels are higher in U373MG than in U343MG and positively correlate with the relative ERK cytosolic abundance, CAR levels, adenoviral infectivity and *dl*922/947 killing capacity.

To further support this hypothesis, genetic silencing of PED/PEA-15 with a specific antisense oligonucleotide enhanced ERK1/2 nuclear distribution and led to a reduction of CAR mRNA and protein levels as well as of viral entry. Thus, our data indicated that PED/PEA-15 levels correlate with infectivity and sensitivity to oncolytic adenoviruses and suggested that, in association with CAR, the evaluation of PED/PEA-15 levels could represent an useful tool to address glioblastoma patients toward specific therapeutic options.

Indeed, it is important to note that PED/PEA-15 is involved in the regulation of apoptotic cell death (Hao *et al*., 2001; Xiao *et al.* and Condorelli *et al.*, 2002) and it has been demonstrated that, in glioma cell lines, the apoptotic cascade activated by TRAIL is negatively regulated by PED/PEA15 (Hao *et al.*, 2001; Xiao *et al*., 2002; Song *et al*., 2006). Moreover, overexpression of PED/PEA-15 induces a marked resistance against glucose deprivation-induced apoptosis in glioma cells (Eckert *et al.*, 2008). Glioma cells overexpressing PED/PEA-15 also show a marked resistance to radiotherapy (Perruolo et al., unpublished data) and it has been reported that PED/PEA-15 may contribute to the resistance to chemotherapeutic agents in breast cancer cells (Stassi *et al.*, 2005), B-cell chronic lymphocytic leukaemia cells cancer cells (Garofano *et al*., 2007) and in human non-small cell lung cancer (Zanca *et al*., 2008).

All together these findings support the hypothesis that PED/PEA 15 increases the resistance to chemotherapeutic agents, radiations and to cytokines-based therapies (i.e TRAIL) and indicate that PED/PEA-15 expression could predict resistance to

apoptosis. Our data suggest a dual role of PED/PEA-15 as a predictive biomarker in glioblastoma patients: i) biomarker of resistance to chemo and radiotherapy for its antiapoptotic activity, ii) biomarker of sensitivity to adenoviral-based therapies, for its role on CAR expression. Further studies are required to assess this latter point.

In conclusion, our data show that adenoviral infectivity is mostly CAR-mediated in glioblastoma cells and that PED/PEA-15 up-regulates CAR expression, by preventing ERK nuclear translocation. These data also suggest the use of PED/PEA-15 as potential predictive marker, to select the patients likely to advantage of therapies based on selective replicating oncolytic viruses.

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Figure legends

Figure 1

Comparison of the cell killing activity, replication of *dl*922-947 and infectivity in U343MG and U373MG glioblastoma cell lines

A. Cytotoxic effects of the oncolytic adenovirus *dl*922-947 were evaluated on U343MG and U373MG glioma cells. The percent of survival rates of cells exposed to adenovirus were calculated by assuming the survival rate of untreated cells to be 100% . *dl*922-947-infected U373MG cells showed significant or highly significant differences in viral sensitivity compared to U343MG cells. A highly significant difference *($p<0.001$)* in cell survival was observed at all points with respect to the control for both cell lines; * indicates significance compared to equally infected U343MG cells. The differences observed were at least significant (* $p<0.05$; ** $p<0.001$).

The standard deviation was calculated (bar). Points, mean percentage of the untreated cells and SD from three different experiments.

B. Replication was assessed by Real-Time PCR genome equivalent analysis. Cells were infected with *dl*922-947 at different MOIs (0.1, 1 and 10 pfu/cell). At 48 hpi, cell medium was collected and viral DNA extracted and quantified. At a MOI of 0.1 and 1 pfu/cell the difference of *dl*922-947 replication between U343MG and U373MG levels were highly significant (** $p<0.001$),whereas at a MOI of 10 the difference was significant (* $p<0.05$).

C. Cells were seeded in 6-well plates and infected with AdGFP at different MOIs (1, 10, 25, 50, 100 pfu/cell). At 24 hpi, cells were collected and the percentage of GFP-positive cells was quantified by FACS analysis. The data are the mean of three different experiments.

Figure 2

CAR receptor in U343MG and U373MG cells

A. Western blot analysis of CAR expression in glioma cells. β-actin was used as loading control. U373MG cells displayed higher levels of total CAR expression.

B. Cytofluorimetric analysis of CAR expression on the membrane of glioma cell lines. U343MG and U373MG cells were harvested and incubated with an anti-CAR (RmcB) monoclonal antibody or fluorescein isothiocyanate (FITC)-labeled-mouse alone. In all experiments, 70% confluent cells were used.

C. U343MG and U373MG cells were pretreated 1 hour with increasing concentrations of the mouse anti-CAR monoclonal antibody RmcB (1:100, 1:250, 1:500), and then infected with AdGFP (25 pfu/cell). At 24 hpi GFP emission was analyzed by citofluorimetric analysis. GFP emission decreased in a dose-dependent manner (>50%) after pre-incubation with RmcB, in both cell lines.

Figure 3

ERK pathway regulates CAR expression in glioma cells

A. U343MG and U373MG cells were analyzed for the expression of p-ERK1/2 by western blot, using ERK1/2 total levels as loading control (left). Subcellular fractionation (nuclear and cytoplasmatic) of U343MG and U373MG cells was performed, proteins extracted and ERK expression analysed by western blot in both fractions. The results were quantified by laser densitometry and the ERK1/2 nucleus/cytosol ratio was determined (right). U373MG cells show sustained

ERK1/2 cytosolic amounts compared to U343MG cells. The data shown are representative of three independent experiments.

B. U373MG cells were transiently transfected with the plasmid pcDNA3-HA containing ERK2 cDNA (pcDNA3-HA-pMAPK2) or pcDNA3-HA plasmid as a control. Total lysates, membrane enrichments and nuclear fractions were obtained 48 hours after transfection. □-Actin , IGF1R and H1 histon levels were used as loading control and as markers for total, membrane and nuclear lysates, respectively.

C. CAR mRNA levels were quantified in U373MG cells by RT-Real-time PCR 24 hours after transfection with ERK2 plasmid or pcDNA3-HA control plasmid. Expression levels were normalized to the expression of β-actin. The data are the mean of three experiments. ERK2 overexpression greatly reduced CAR mRNA levels.

D. Effect of ERK2 transfection on infectivity of glioma cells. U373MG cells were transfected with ERK2 plasmid or pcDNA3-HA control plasmid, and 48 hours post-transfection were infected with AdGFP (25 and 50 pfu/cell). GFP expression was analyzed by citofluorimetric analysis. The data are the mean of three experiments.

Figure 4

PED/PEA-15 antisense (PED As) transfection

A. Differential expression of PED/PEA-15 in glioma cell lines. U343MG and U373MG cells were analyzed for the expression of the antiapoptotic protein PED/PEA-15 on western blot, using β-actin levels as loading control.

B. Effect of PED/PEA-15 downregulation on CAR. U373MG cells were transiently transfected with PED/PEA-15 antisense oligonucleotides (PED As) and PED/PEA-15 scramble oligonucleotides (PED Scr). PED/PEA-15 and CAR total levels were analysed 48h after transfection.

C. Effect of PED/PEA-15 down regulation on ERK1/2 localisation. U373MG cells were transiently transfected with PED/PEA-15 antisense (PED As), or with a scrambled oligonucleotide as control (PED Scr). Subcellular fractionation (nuclear and cytoplasmatic) of PED As-transfected U373MG cells was performed, and ERK1/2 expression analysed in both fractions. □-actin and H1 histon were used as a loading control for cytosolic and nuclear fractions, respectively.

Figure 5

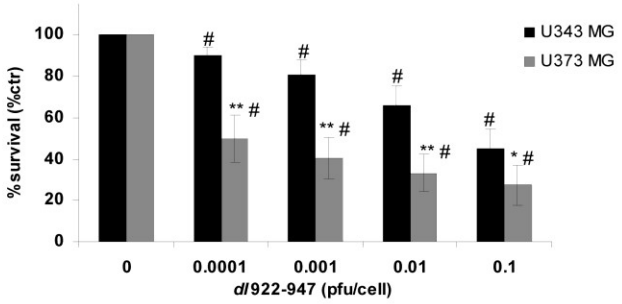
Transfection of PED/PEA-15 As reduces CAR levels and infectivity in U373MG cells.

A. U373MG cells transfected with PED As or PED Scr. oligonucleotides were analysed for expression of CAR receptor by surface labeling. 48h after transfection, cells were harvested and incubated with anti-CAR or secondary FITC-labeled anti-mouse antibody alone. Labeled cells were analyzed for CAR expression by cytofluorimetric analysis. The data are the mean of three different experiments. A strong reduction in CAR membrane levels was observed in PED As-transfected cells.

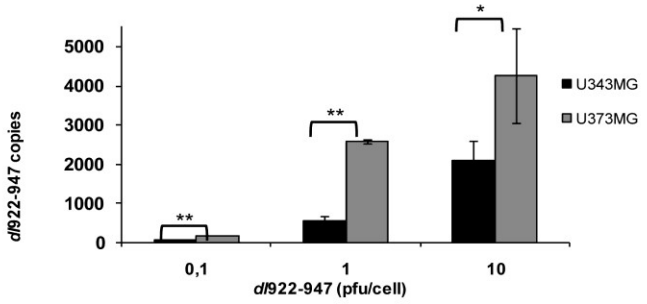
B. CAR mRNA levels were quantified on U373MG cells by Real-time PCR 24 hours after transfection. Expression levels were normalized to the expression of β-actin. PED As-transfected cells displayed lower CAR mRNA levels.

C. U373MG cells were transfected with PED/PEA-15 Antisense or Scrambled sequence and 48 hours post-transfection were infected with AdGFP (25 pfu/cell). GFP expression was analyzed by citofluorimetric analysis; PED As transfection reduced GFP emission.

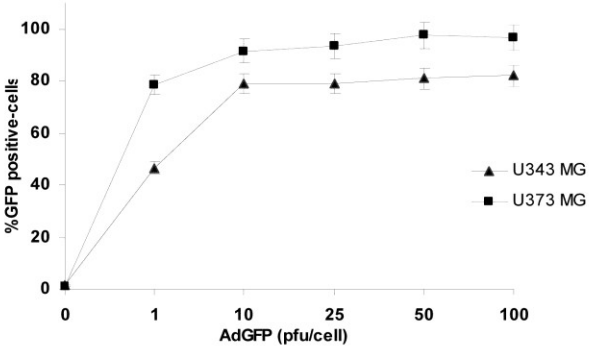
A

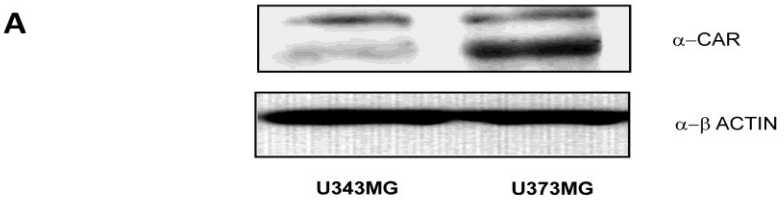

100
80
60
40
20
0
% survival (%ctr)
U343 MG
U373 MG
#
** #
* #
0
0.0001
0.001
0.01
0.1
d/922-947 (pfu/cell)

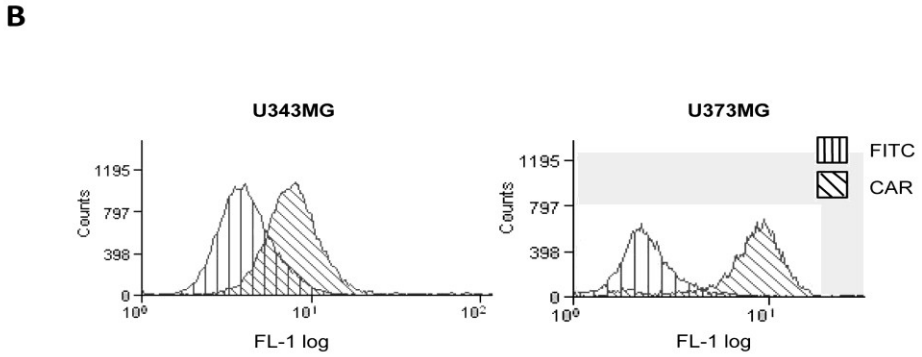
B

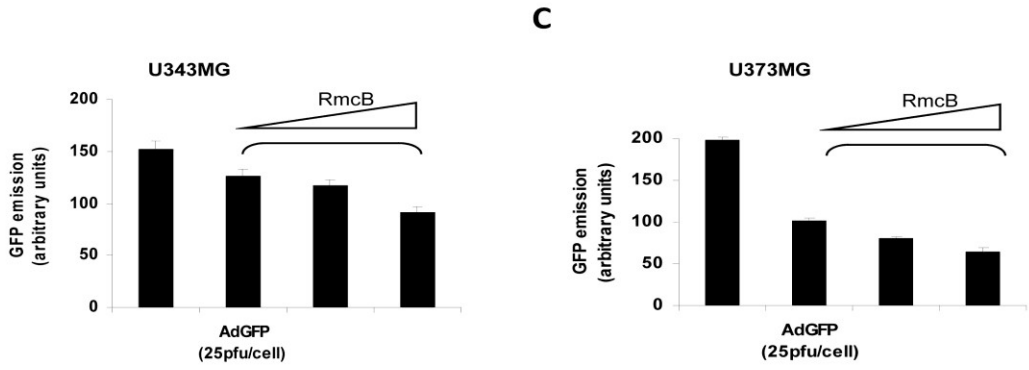

5000
4000
3000
2000
1000
0
d/922-947 copies
U343MG
U373MG
**
*
0,1
1
10
d/922-947 (pfu/cell)

C


100
80
60
40
20
0
%GFP positive-cells
U343 MG
U373 MG
0
1
10
25
50
100
AdGFP (pfu/cell)


A
α–CAR
α–β ACTIN
U343MG
U373MG


B
U343MG
U373MG
FITC
CAR
Counts
1195
797
398
0
10⁰
10¹
10²
FL-1 log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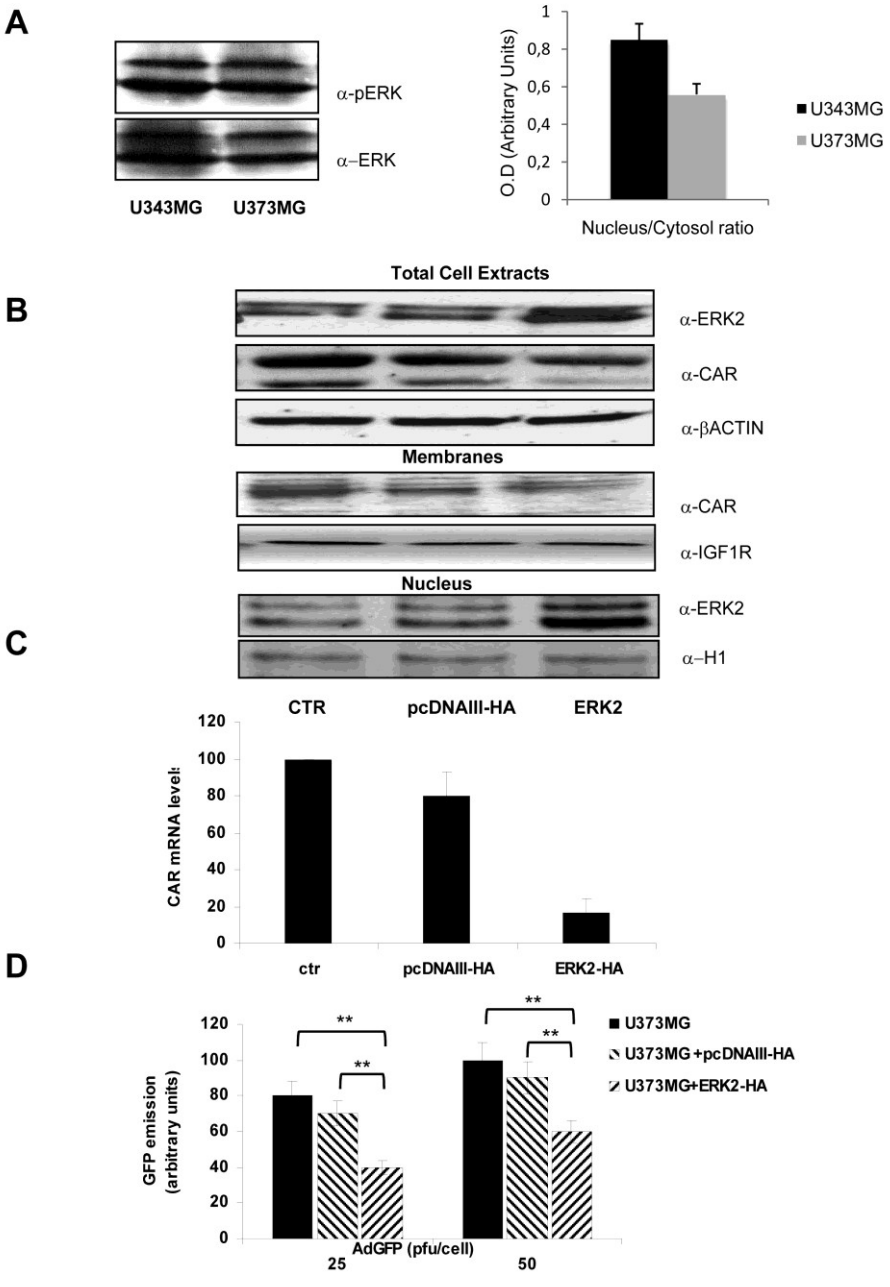
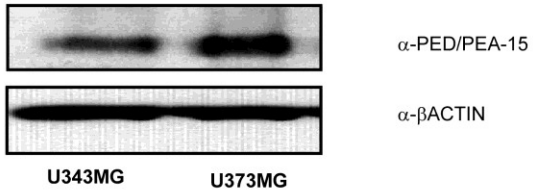
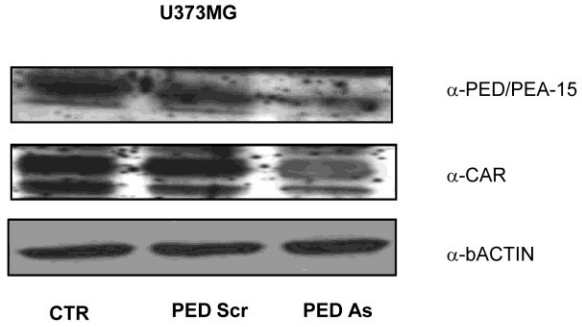
U343MG
U373MG
RmcB
GFP emission (arbitrary units)
200
150
100
50
0
AdGFP (25pfu/cell)


A
α-pERK
α–ERK
U343MG
U373MG
O.D (Arbitrary Units)
1
0,8
0,6
0,4
0,2
0
Nucleus/Cytosol ratio
U343MG
U373MG
B
Total Cell Extracts
α-ERK2
α-CAR
α-βACTIN
Membranes
α-CAR
α-IGF1R
Nucleus
α-ERK2
α–H1
C
CTR
pcDNAIII-HA
ERK2
CAR mRNA level
120
100
80
60
40
20
0
ctr
pcDNAIII-HA
ERK2-HA
D
GFP emission (arbitrary units)
120
100
80
60
40
20
0
**
**
**
**
U373MG
U373MG +pcDNAIII-HA
U373MG+ERK2-HA
AdGFP (pfu/cell)
25
50

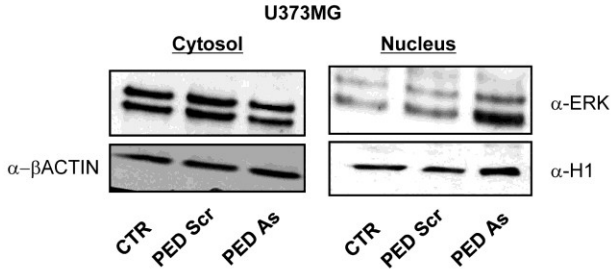
A


α-PED/PEA-15
α-βACTIN
U343MG
U373MG

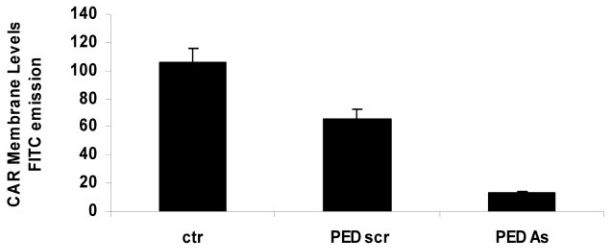
B


U373MG
α-PED/PEA-15
α-CAR
α-bACTIN
CTR
PED Scr
PED As

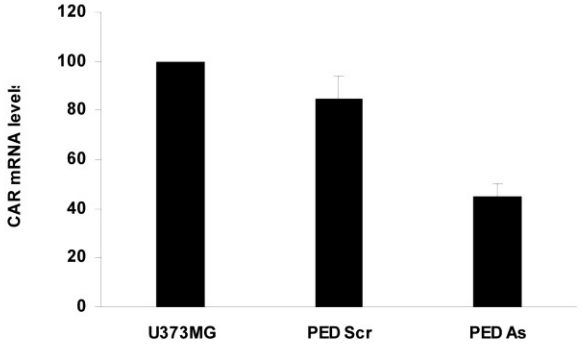
C


U373MG
Cytosol
Nucleus
α-ERK
α–βACTIN
α-H1
CTR
PED Scr
PED As
CTR
PED Scr
PED As

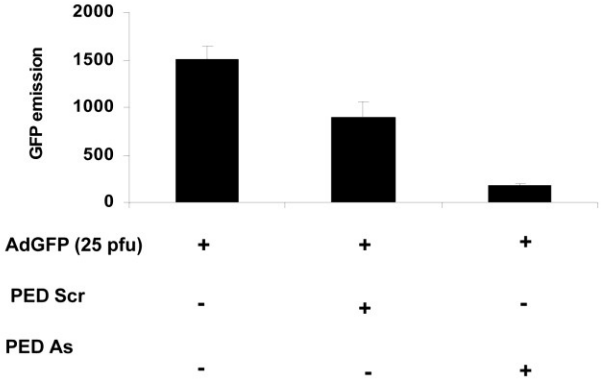
A


CAR Membrane Levels
FITC emission
140
120
100
80
60
40
20
0
ctr
PED scr
PED As

B


CAR mRNA level
120
100
80
60
40
20
0
U373MG
PED Scr
PED As

C


GFP emission
2000
1500
1000
500
0
AdGFP (25 pfu) + + +
PED Scr - + -
PED As - - +