

**Università degli Studi del Piemonte Orientale “Amedeo Avogadro”**



**Dottorato di Ricerca**  
**in**  
**Medicina Molecolare**  
*Ciclo XX*

**CROSS-TALK BETWEEN AUTOPHAGY AND APOPTOSIS  
AFTER RESVERATROL TREATMENT IN COLORECTAL  
CANCER**

*Responsabile Scientifico*  
**Prof. C. Isidoro**

*Coordinatore*  
**Prof. U. Dianzani**

**NICOL FRANCESCA TRINCHERI**

## **INDEX**

<b>INTRODUCTION</b>	<b>pag 3</b>
<b>TYPES OF CELL DEATH</b>	<b>pag 3</b>
<b>APOPTOSIS</b>	<b>pag10</b>
<b>AUTOPHAGIC CELL DEATH</b>	<b>pag17</b>
<b>PHYSIOLOGICAL FUNCTION OF AUTOPHAGY</b>	<b>pag24</b>
<b>AUTOPHAGY IN DISEASES</b>	<b>pag27</b>
<b>CANCER</b>	<b>pag32</b>
<b>APOPTOTIC CELL DEATH AND CARCINOGENESIS</b>	<b>pag37</b>
<b>ROLE OF AUTOPHAGY IN CANCER</b>	<b>pag48</b>
<b>LYSOSOMES IN CELL DEATH CONTROL</b>	<b>pag55</b>
<b>RESVERATROL</b>	<b>pag62</b>
<b>MECHANISM OF ACTION</b>	<b>pag64</b>
<b>CLINICAL SIGNIFICANCE OF RESVERATROL</b>	<b>pag70</b>
<b>FIRST WORK</b>	<b>pag73</b>
<b>SECOND WORK</b>	<b>pag109</b>
<b>OTHER WORKS</b>	<b>pag138</b>
<b>BIBLIOGRAPHY</b>	<b>pag139</b>



## **INTRODUCTION**

### **TYPES OF CELL DEATH**

The maintenance of cellular homeostasis is fundamental for tissue integrity in multicellular organisms.

Balance between cell division and cell death is of almost importance for the development and maintenance of multicellular organism. Disorders and either process have pathologic consequences and can lead to disturb embryogenesis, neurodegenerative disease or the development of cancer. Therefore, the equilibrium between life and death is tightly controlled and faulty elements can effectly be eliminated by a process called “programmed cell death” (PCD).

Three great categories of dynamic cellular activities that lead to cell death have been described in these years by researches: necrosis, apoptosis and autophagy.

This cell death classification is based on distinct biochemical and morphological characteristics present in the dying cell.

Two of these processes, apoptosis and autophagy, have been considered to be “programmed”, which refers to their strict genetic control (Broker et al., 2005; Danial and Korsmeyer, 2004).

Programmed cell death results in the disintegration of cellular components and their engulfment by surrounding cells.

Necrosis is generally considered passive respons to massive cellular insult.

	<b>APOPTOSIS</b>	<b>AUTOPHAGY</b>	<b>NECROSIS</b>
<b>CELL MEMBRANE</b>	Blebbing, membrane integrity maintained	Blebbing	Loss of membrane integrity
<b>NUCLEUS</b>	Chromatin condensation, DNA laddering, nuclear fragmentation	Partial chromatin condensation, no DNA	Random DNA degradation
<b>CYTOPLASM</b>	Condensed membrane-bound cellular fragments, depolymerization of cytoskeleton	Increased number of autophagic vesicles, degradation of Golgi, polyribosomes and ER	Swelling of cellular organelles

Fig.1: Scheme of cell death types

### **Apoptosis**

Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, by contrast, is a process in which cells play an active role in their own death (which is why apoptosis is often referred to as cell suicide).

The intracellular machinery responsible for apoptosis seems to be similar in all animal cells. This machinery depends on a family of proteases that have a cysteine at their active site and cleave their target proteins at specific aspartic acids that are called caspases.

These proteins breakdown or cleave key cellular components that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The

caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus.

## **Necrosis**

While apoptosis is increasingly well defined at the molecular level, necrosis has been lacking a molecular signature and has been referred to as form of cell death that is uncontrolled and pathological. Recent studies suggest that necrosis is a regulated event that may be involved in multiple developmental, physiological and pathological scenarios (Majno and Joris, 1995). The principal features of necrosis include cellular energy depletion, damage to membrane lipids and loss of function of homeostatic ion pumps/channels. Unlike apoptosis, in which the Bcl-2 family of proteins and caspases play key role, necrosis is induced by inhibition of cellular energy production, imbalance of intracellular calcium flux, generation of ROS and activation of nonapoptotic proteases. These events often potentiate each other and synergize to cause necrosis. It is a more chaotic way of dying, which results from circumstances outside the cell and is characterized by cytoplasmic swelling and disruption of the plasma membrane, leading to release of the cellular components. This release, in turn, results in massive inflammatory responses in the physiological environment around the dying cell (Scaffidi et al., 2002).

Moreover, it has been reported that necrotic cell death increases the probability of proto-oncogenic mutation. Necrotic cell death has been implicated in various pathological conditions, such as stroke, ischemia and several neurodegenerative diseases (Jiang et al., 2007; Stefani and Dobson, 2003).

Furthermore, it is often considered to be a causative factor of tumor promotion because excessive and prolonged inflammatory responses are

very closely related to the promotion of tumor growth, angiogenesis and invasion (Allavena et al., 2008) (Jiang et al., 2007).

As described above, one of the critical features of necrosis is early rupture of the plasma membrane, which is considered a result of ATP depletion since reduction in the function of the ATP-dependent ion pumps on the plasma membrana due to energy depletion may disturb the intracellular homeostasis. Several membrane receptors have been implicated in the triggering of both necrosis and apoptosis. For example, TRAIL induces necrotic-like cell death at a lower extracellular pH in human HT29 colon carcinoma and HepG2 hepatocarcinoma cell lines (Meurette et al., 2005). In addition, FADD deficiency sensitizes Jurkat T cells to TNF $\alpha$ -dependent necrosis during activation-induced cell death (Bertazza and Mocellin, 2008) and ligation of DR4 and DR5 causes necrosis (Bodmer et al., 2000).

Moreover, among the secondary messengers, it has been reported that Ca<sup>2+</sup> participates in receptor-mediated necrosis. Increased Ca<sup>2+</sup> influx induces necrosis through MEC-4(d) channel-activated Ca<sup>2+</sup> release from the endoplasmatic reticulum (ER) in neuron cells (Bianchi et al., 2004).

Another factor that triggers necrosis is the excessive production of ROS. In some cases, a specific type of ROS appears to be important for determination of the mode of cell death. For example, superoxide induces apoptosis, whereas hydrogen peroxide induces non-apoptotic cell death JNK-1mediated PARP-1 activation (Zhang et al., 2007).

Currently, although it is not clear why ROS generation leads to apoptosis in some cells but necrosis in other ones, the amounts or the types of ROS are believed to be a critical factor involved in determining the mode of cell death but other regulatory factors can not be excluded.

## **Autophagy**

Unlike apoptosis and necrosis, autophagy is not synonymous with cell death. Autophagy (from the Greek, “auto” oneself, “phagy” to eat) is evolutionarily conserved and occurs in all eukaryotic cells, from yeast to mammals (Levine and Klionsky, 2004).

Autophagy is activated in response to nutrient starvation, differentiation and developmental triggers. It is an adaptive process responding to metabolic stresses that results in degradation of intracellular proteins and organelles (Lum et al., 2005; Shintani and Klionsky, 2004).

During autophagy, portions of the cytoplasm are encapsulated in a double-membrane structure referred to as an autophagosome. Autophagosomes then fuse with lysosomes where the contents are delivered, resulting in their degradation by lysosomal hydrolases. Under normal physiological conditions, autophagy occurs at basal levels in most tissues, contributing to the routine turnover of cytoplasmic components. It can promote cell adaptation and survival during stresses such as starvation, but under some conditions cells undergo death by excessive autophagy.

In yeast, a cassette of autophagy-related genes have been identified that regulate autophagy induction, autophagosome formation and expansion, fusion with lysosomes and the recycling of autophagosome contents (Levine and Klionsky, 2004).

## **Cross-Talk between Apoptosis and Autophagy**

Several lines of evidence have found that a cross-talk exists between autophagic and apoptotic pathways. In general terms, it appears that similar stimuli can induce either apoptosis and autophagy. Whereas a mixed phenotype of autophagy and apoptosis can sometimes be detected in response to these common stimuli, in many other instances, autophagy



and apoptosis develop in a mutually exclusive manner, perhaps as a result of variable threshold for both processes, or as a result of a cellular “decision” between the two responses that may be linked to a mutual inhibition of the two phenomena.

Autophagy and apoptosis share many common inducers. The type of the initiating stimulus might determine which process will dominate. For example, during nutrient deprivation the default pathway would be autophagy, which creates a metabolic state with high (or increased) ATP that is anti-apoptotic (Katayama et al., 2007).

Successful removal of the damaged organelles followed by repair and adaption would allow for survival, while failure to restore homeostasis would result in delayed apoptosis. By contrast, the default pathway that is triggered by other signals such as DNA damage or death-receptor activation would be immediate apoptosis, which occurs in a rapid, self-amplifying process, precluding simultaneous autophagic responses.

Other examples, knockout or knockdown of ATG5 abolishes autophagy and reduces the incidence of apoptotic events in human cancer cells treated with staurosporin (Yousefi et al., 2006).

BAX and BAK are required for mitochondrial outer permeabilization. It has been shown that mouse embryonic fibroblasts (MEFs) from double-knockout  $Bax^{-/-} Bak^{-/-}$  mice are resistant to a range of apoptosis inducers. When treated with DNA-damaging agents such as etoposide,  $Bax^{-/-} Bak^{-/-}$  MEFs failed to undergo apoptosis and instead manifested massive autophagy followed by delayed cell death.

Beclin 1, the mammalian ortholog of yeast Atg6, was originally identified through its interaction with Bcl-2 (Shimizu et al., 2004).

Recent findings have shown that Bcl-2 and Bcl-xL expression can sensitize cells to autophagic death induced by etoposide (Shimizu et al., 2004), and that Bcl-2 inhibits Beclin 1-mediated autophagy in response to

starvation (Pattingre et al., 2005). These contradictory findings suggest that the outcome of the autophagocytic response may vary depending on the type of insult or cellular stress.

When HeLa or HCT116 cancer cells are cultured in the absence of nutrients, they rapidly induce autophagy in order to recycle essential metabolites, such as lipids and amino acids, for fuelling the bioenergetic machinery. In these circumstances, the inhibition of autophagy results in an accelerated cell death that manifests hallmarks of apoptosis including chromatin condensation, MOMP and activation of caspases (Boya et al., 2005). When autophagy is blocked at an early stage by depletion of Beclin-1, ATG5, ATG10, ATG12 or Vps34, no autophagic vacuoles are found in the cells, which undergo a typical type I cell death. By contrast, when the fusion of autophagosomes and lysosomes is blocked by the addition of lysosomal inhibitors (chloroquine or the vacuolar ATPase inhibitor bafilomycin A) or by depletion of the lysosomal protein LAMP2, autophagic vacuoles accumulate and the cells manifest a mixed type I-type II morphology before the cells succumb to death (Boya et al., 2005; Gonzalez-Polo et al., 2005).

## APOPTOSIS

Though the phenomenon had been described for almost a century, in 1972, Kerr, Wyllie and Currie first coined the term “apoptosis” in order to differentiate naturally occurring development cell death from necrotic cell death that results from acute tissue injury (Kerr et al., 1972).

They also noted that apoptosis was responsible for maintaining the equilibrium between cell proliferation and cell death. Morphologic characteristics of apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation and nucleosomal fragmentation. Under normal conditions, cells undergoing apoptosis are recognized by macrophages or neighboring cells that consume the cells’ fractionated carcasses. Apoptosis has been considered a major mechanism of chemotherapy-induced cell death and pathways regulating apoptosis are the focus of many preclinical drug discovery investigation.

The principle characters in the apoptotic cell death are the caspases. They are synthesized in the cell as inactive precursors, or pro-caspases, which are usually activated by cleavage at aspartic acids by other caspases. Once activated, caspases cleave, and thereby activate othe pro-caspases, resulting in an amplifying protelytic cascade (Earnshaw, 1999).

In mammals, a wide array of external signals may triggers two major apoptotic cell death:

-the *intrinsic* or mitochondria-mediated pathway

-the *extrinsic* or extracellular activated pathway (death receptor pathway)

The *intrinsic pathway* is usually activated in response to intracellular stress signal, which include DNA damage and high levels of reactive oxygen species (ROS). As well as by viral infection and activation of oncogenes. The *extrinsic pathway* is triggered by the binding of an

extracellular ligand to a receptor on the plasma membrane. The endosomal pathway that involves the lysosomal cathepsins. These pathways activate proteolytic enzymes called caspase that mediate the rapid dismantling of cellular organelles and architecture.

### **The intrinsic pathway**

The most important turning point in the course of the intrinsic apoptotic process occurs in the mitochondria. A pivotal event in the mitochondrial pathway is mitochondrial outer membrane permeabilization (MOMP), that is mainly mediated and controlled by Bcl-2 family members.

Bcl-2 family members act by regulating the efflux of apoptogenic proteins from mitochondria. They have four BH domain. The number and the combination of the BH domains dictate if the proteins are pro- or anti-apoptotic.

Antiapoptotic Bcl-2 members contain all four BH domain and include Bcl-2, Bcl-xL, Mcl-1, Bcl-w and Bfl-1/A1.

Instead proapoptotic members lack the BH4 domain and are divided into two groups, the BH3-only members and the multidomain BH1-3 proapoptotic members Bax and Bak. In mouse cells, deletion of these two protein is sufficient to prevent mitochondrial outer membrane permeabilization induced by upstream apoptotic events (Lindsten et al., 2000; Wei et al., 2001).

Bax and Bak normally exist as inactive monomers. Bax resides in the cytosol or loosely attached to intracellular membranes (Suzuki et al., 2000), and Bak is bound by Mcl-1, Bcl-xL or voltage-dependent anion channel protein 2 (VDAC-2) in the mitochondrial anion membrane (Scorrano and Korsmeyer, 2003; Scorrano et al., 2003; Willis et al., 2005).

The generalized scheme of intrinsic pathway activation is the oligomerization of Bax and Bak in the mitochondrial outer membrane causing the permeabilization of it and the release of apoptogenic factors such as cytochrome c, second mitochondria-derived (Smac-DIABLO) and Omi stress-regulated endoprotease/high temperature requirement protein A2 (Omi/HtrA2).

Once released cytochrome c binds apoptotic protease-activating factor 1 (Apaf-1), which recruits pro-caspase 9, promoting its self-activation. Activated caspase-9 cleaves the downstream effectors caspase-3 and caspase-7, which rapidly cleave intracellular substrates. Protein of the IAP family, including X-linked IAP (XIAP), c-IAP1 and c-IAP2 can bind and inhibit the active sites of caspase-3 and caspase-7 and caspase-9. When released from mitochondria, Smac/DIABLO and Omim/HtrA2 can bind these IAPs and prevent their inhibition of the activated caspases (Du et al., 2000) (Suzuki et al., 2001) (Verhagen et al., 2000).

Antiapoptotic Bcl-2 proteins block oligomerization of bax and bak preventing the mitochondrial membrane permeabilization. However it has been shown that Bax oligomerization on mitochondrial and lysosomal membranes is cathepsin D-dependent (Castino et al., 2008).

Members of the BH3-only family include Bid, Bad, Bim, Puma, Noxa, Bmf and several others. Their activity appears to be kept in check by either transcriptional events. For example, cellular stresses, such as ionizing radiation (IR) or chemotherapy activate a DNA damage response that stabilizes the p53 tumor suppressor protein. p53 acts to either arrest the cell division cycle by transcriptionally activating the cyclin-dependent kinase inhibitor p21, giving the cell time to repair the damage or else it helps to mediate apoptotic cell death. p53 also activates proapoptotic genes, including those encoding Bax and the BH3-only protein Puma, Noxa and Bid (El-Deiry, 2003).

## **The extrinsic pathway**

It is activated by apoptotic stimuli comprising extrinsic signals such as the binding of death inducing ligands to cell surface receptors.

It is activated by members of two protein families, the tumor necrosis factor (TNF) and the receptors for these ligands (TNFR) (Locksley et al., 2001).

Most TNF family members bind receptors that activate signals involved in proinflammatory responses and do not signal cell death. The TNF ligands that can induce apoptosis are TNF- $\alpha$ , FasL (also known as CD95L) and TNF receptor apoptosis-inducing ligand (TRAIL, also known as Apo2L) (LeBlanc and Ashkenazi, 2003; Peter and Krammer, 2003).

After extracellular ligand binding, the cytoplasmic end of the TNFR recruits initiating caspases. TRAIL binding to its death-inducing receptors acts in a manner similar to FasL, while TNF-mediated signalling is more complex. The ligand-bound Fas or TRAIL death receptors (DR4 and DR5) recruit the adapter protein Fas-associating death domain-containing protein (FADD) (Chinnaiyan et al., 1995).

Bound FADD recruits initiator caspase-8 and caspase-10 and this assembly of protein (receptor, FADD, and caspases) is termed the death-inducing signalling complex (DISC) (Kischkel et al., 1995).

Recruitment of caspase-8/10 to the DISC leads to their autoproteolytic cleavage (Boatright and Salvesen, 2003; Donepudi et al., 2003).

Caspase-8/10 activity can be blocked by a protein with which they share high homology, FLIP (FADD-like interleukin-1 $\beta$  -converting enzyme inhibitory protein). FLIP can oligomerize with caspase-8/10 but lacks critical residues in its caspase domain, including the catalytic cysteine, suggesting it to be a dominant-negative inhibitor.

The BH3-only protein Bid connects the extrinsic pathway to mitochondria. Infact, Bid is cleaved by caspase-8, resulting in its myristoylization of a newly-exposed glycine residue to form tBid. tBid is then targeted to membranes where it promotes Bax and Bak oligomerization (Li H et al. 1998, Luo X et al. 1998).

The extrinsic pathway is involved in maintaining immune homeostasis and preventing the emergence of lymphomas or the development of autoimmunity (Bidere N et al. 2006). Cell derived from the hematopoietic progenitor (CD8<sup>+</sup> T cells, natural killer cells and dendritic cells) have the capacity to mediate cell death through the use of extrinsic pathway. For example, anigen stimulation of T cells causes the induction of FasL, TNF and TRAIL (Brunner T et al. 1995, Zheng L et al. 1995, Janssen EM et al. 2005).

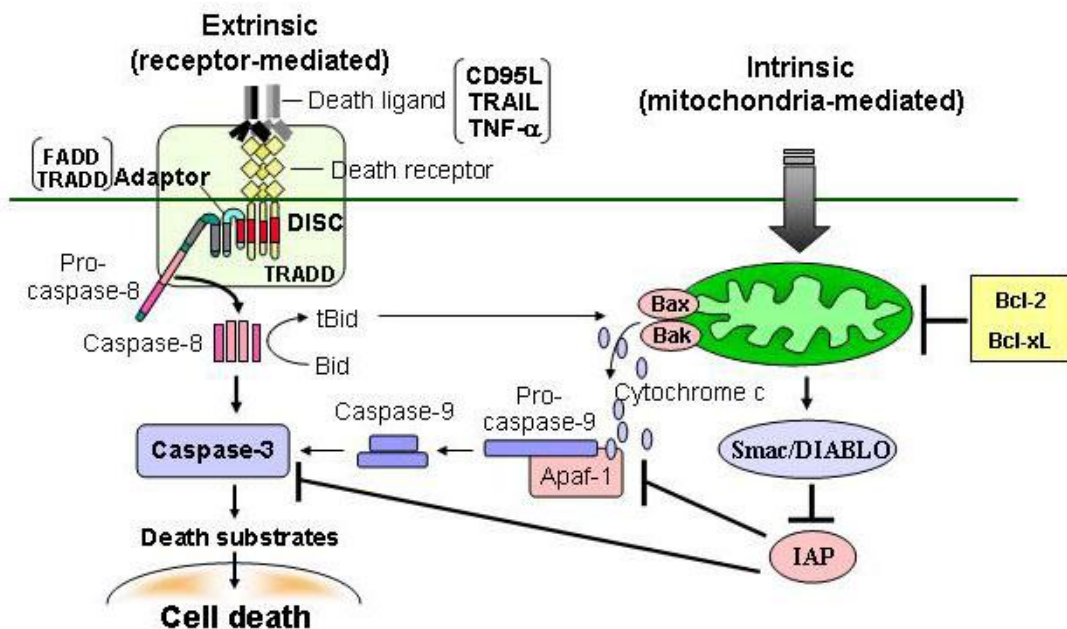


Fig 2: Scheme for the extrinsic and intrinsic pathway

## **The endosomal-lysosomal pathway**

Although caspases are known to play a central role in apoptotic cell death, an emerging body of evidence has also implicated non-caspase proteases, including cathepsin proteases. The cathepsin proteases reside primarily in endosomes and lysosomes (Kirshke H et al. 1987, Chapman HA et al. 1997). Cathepsins can be divided into three categories based on their catalytic amino acid:

the aspartate proteases (cathepsins D and E)

the cysteine proteases (cathepsins B, C, F, H, K, L, O, S, T, V, W, and X),

the serine proteases (cathepsins A and G)

It has long been thought that the primary function of cathepsins is the terminal degradation of proteins in the lysosomal compartment.

Several studies demonstrated that the release of some idrolitic enzymes in the cytosol, after a cellular damage, and the activation of apoptotic pathway (Li et al. 2000, Brunk and Svensson 1999)

However, recent studies have determined that cathepsins B and D relocate to the cytoplasm in response to certain apoptotic stimuli, and participate in the execution of apoptosis (Guicciardi ME et al. 2004, Johnson DE 2000, Demoz et al. 2002).

Early studies showed that inhibition of cathepsin B with CA-074-Me abrogated bile salt-induced apoptosis in hepatocytes (Roberts LR et al. 1997). Subsequently, cathepsin B was shown to be released into the cytosol following treatment of WEHI-S fibrosarcoma cells with tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) (Foghsgaard L et al. 2001) or treatment of non-small cell lung cancer cells with microtubule stabilizing agents (Broker LE et al. 2004). Inhibition of cathepsin B with pharmacologic inhibitors or antisense directed against cathepsin B mRNA-attenuated microtubule stabilizing agent- and TNF $\alpha$ -mediated apoptosis.



Moreover, Guicciardi et al. have shown that hepatocytes from cathepsin B-deficient mice are markedly resistant to TNF $\alpha$ .

Cathepsin D is the major intracellular aspartate protease and is expressed in all human tissues. The cathepsin D enzyme is synthesized as a 52 kDa precursor, which undergoes processing to active single chain (48 kDa) and double chain (31 and 14 kDa subunits) forms (Faust PL et al. 1985, Guicciardi ME et al. 2001).

Mice that are deficient in cathepsin D die on postnatal day 26 due to atrophy of the intestinal mucosa and consequent anorexia (Saftig P et al. 1995). Cathepsin D has been shown to be relocalized to the cytoplasm following treatment of cells with hydrogen peroxide (Yin L et al. 2005, Castino R et al. 2007), oxidized low-density lipoprotein (Yuan XM et al. 2000) and the protein kinase C inhibitor staurosporine (Johansson AC et al. 2003) and here it can induce the alteration of lysosomal and mitochondrial membrane integrity.

Reports demonstrating participation of cathepsin D in apoptosis execution have relied heavily on the pharmacologic inhibitor pepstatin A. Unfortunately, pepstatin is not entirely specific, and is also known to inhibit cathepsin E, pepsin, and renin.

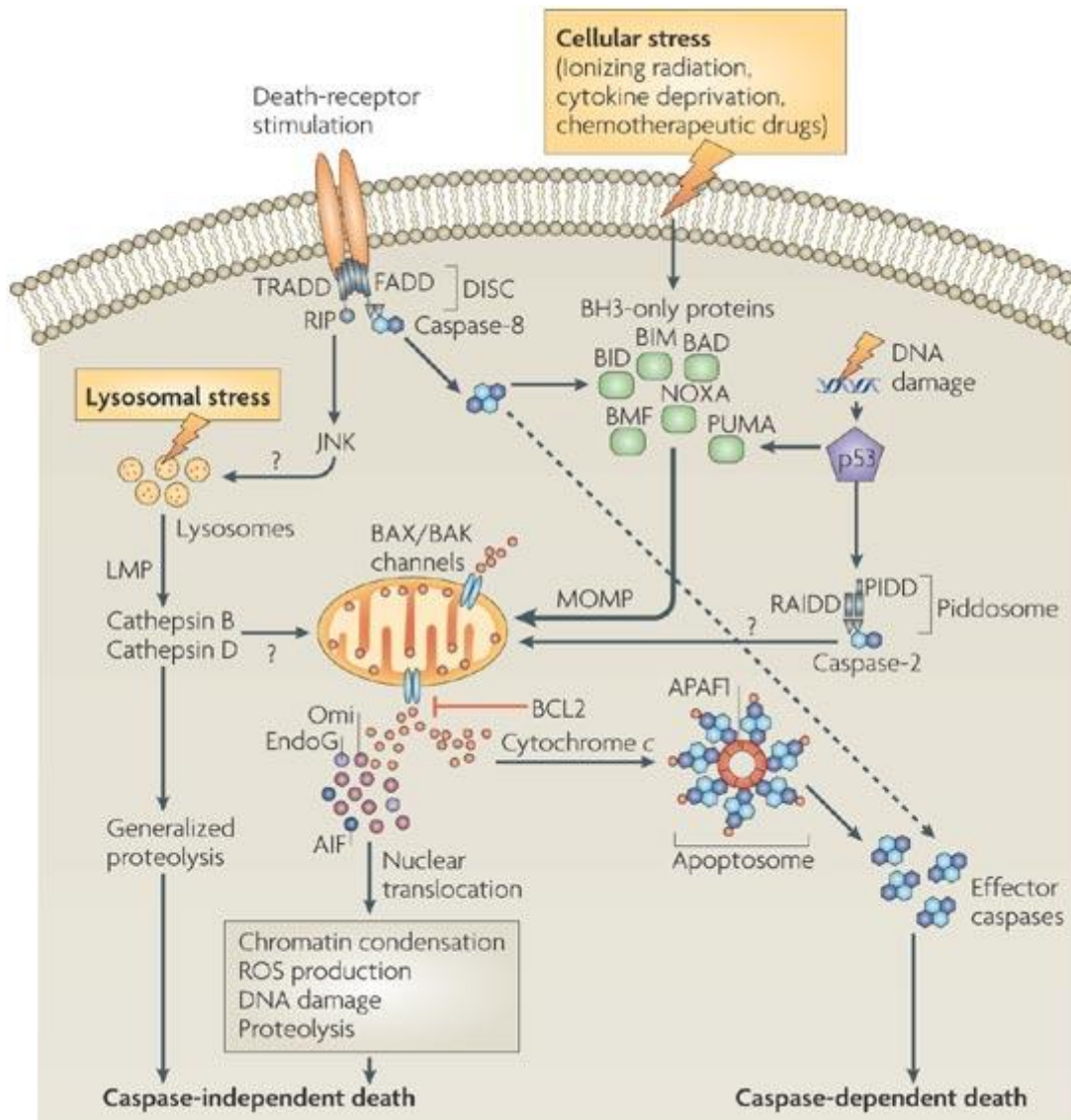
Moreover it has been shown that there is an involvement of Cathepsin D in the TNF- $\alpha$  -apoptosis induction (Demoz M et al. 2002).

However, antisense-mediated inhibition of cathepsin D has served to verify a role for this enzyme in IFN-g- and Fasmediated apoptosis (Deiss LP et al. 1996). Furthermore, Bidere et al. have shown that down-regulation of cathepsin D using small interfering RNA (siRNA) inhibits early apoptotic events associated with staurosporine-induced apoptosis in human T lymphocytes.

Although previous reports have indicated the importance of cathepsin D during apoptosis caused by the agents and stimuli described above, less is

known about the involvement of this enzyme in chemotherapy-induced apoptosis.

Intriguing findings were provided by Wu and collaborators, who showed that fibroblasts derived from cathepsin D knockout mice are more resistant to Adriamycin and etoposide (VP-16).



### Endosomal-lysosomal pathway

Fig 3: The endosomal-lysosomal pathway scheme

## AUTOPHAGIC CELL DEATH

### **The Autophagic pathway**

Autophagy is generally defined as a lysosome-dependent mechanism of intracellular degradation that is used for the turnover of cytoplasm.

During these years, three forms of programmed cell death of kind II have been identified:

-chaperone-mediated autophagy

-microautophagy

-macroautophagy

They differ with respect to their physiological functions and the mode of cargo delivery to the lysosome.

The chaperone-mediated autophagy (CMA) involves selectively delivering soluble cytosolic proteins that contain a chaperone-mediated autophagy targeting motif for incorporation into lysosomes (Kaushik and Cuervo, 2006). In this case a chaperone-cochaperone complex (hsc70) transports the specific substrate to the lysosomal membrane for translocation into the lysosome following binding to lysosomal-associated membrane protein type 2A.

The microautophagy, whereby cytoplasmic constituents are engulfed by invagination of the lysosomal membrane and subsequently degraded (Klionsky, 2007). The function of this process in higher eukaryotes is not known, whereas microautophagy-like processes in fungi are involved in selective organelle degradation.

The engulfment of peroxisomes and mitochondria by autophagosomes for transport to lysosomal degradation is referred to as pexophagy and mitophagy, respectively.

In the case of macroautophagy the cargoes are sequestered within a unique double-membrane cytosolic vesicle, an autophagosome.

Sequestration can be either non-specific, involving the engulfment of bulk cytoplasm, or selective, targeting specific cargoes such as organelles or invasive microbes.

### **Macroautophagy, the molecular machinery**

We focus our attention on macroautophagy (herein referred to as autophagy), the major regulated catabolic mechanism that eukaryotic cells use to degrade long-lived proteins and organelles.

This form of autophagy involves the delivery of cytoplasmic cargo sequestered inside double-membrane vesicles to the lysosome.

Autophagy begins with a *signal* (or stimuli), which can originate from extracellular stress, for example a shortage of essential nutrients, growth factors, ATP or oxygen, or an intracellular stress, such as an unfolded protein response (UPR) in the endoplasmic reticulum (ER), or an increase in unfolded cytoplasmic proteins. The signal to maintain basal autophagy might be a quality control sensor monitoring protein and organelle function. How these diverse signals are generated, integrated and propagated to downstream targets, including the Atg (Autophagy-related) proteins is not completely known, although some key regulators have been identified, including TOR (target of rapamycin), a negative regulator of autophagy. Downstream of mammalian TOR (mTOR) are Ulk (Unc51-like kinase) 1 and 2, which are the orthologues of yeast Atg1. Ulk1/2 are serine/threonine kinases required for autophagy that propagate the initiating signal via phosphorylation of so far unknown substrates, and possibly non-catalytic effectors.

The cellular events during autophagy follow distinct stages:

-vesicle nucleation (formation of the isolation membrane/phagophore) could be considered as the defining event that creates an active phagophore (or isolation membrane), the foundation of the

autophagosome. At the phagophore, Ulk1/2 function together with Atg9, a six-spanning membrane protein, and with the Class III phosphatidylinositol 3-kinase (PI3K) Vps34 complex, which comprises the Atg6 orthologue Beclin1 and its interactors, including Ambra1.

-vesicle elongation of an isolation membrane, which is called a phagophore. The edges of the phagophore then fuse (vesicle completion) to form autophagosome, a double-membraned vesicle that sequesters the cytoplasmic material and lysis of the autophagosome inner membrane and breakdown of its contents inside it.

-vesicle completion (growth and closure)

The expansion of the phagophore into an autophagosome requires two highly conserved ubiquitin (Ub)-like systems, which are essential for the posttranslational modification of Atg8. In mammalian cells, the most well studied homologue of Atg8 is MAP-LC3 (the microtubule associated protein light chain 3) or LC3. There are three forms of LC3, the newly synthesized full length protein, the Cterminally cleaved protein called LC3-I, and the PE-conjugated form of LC3-I, referred to as LC3-II which is generated by the ubiquitin-like system. The formation and recruitment of LC3-II during the expansion and sequestration step is dependent upon the Atg5-12-16 complex as well as the nucleation proteins. Sequestration of ubiquitinated cargo is facilitated by p62/SQSTM-1.

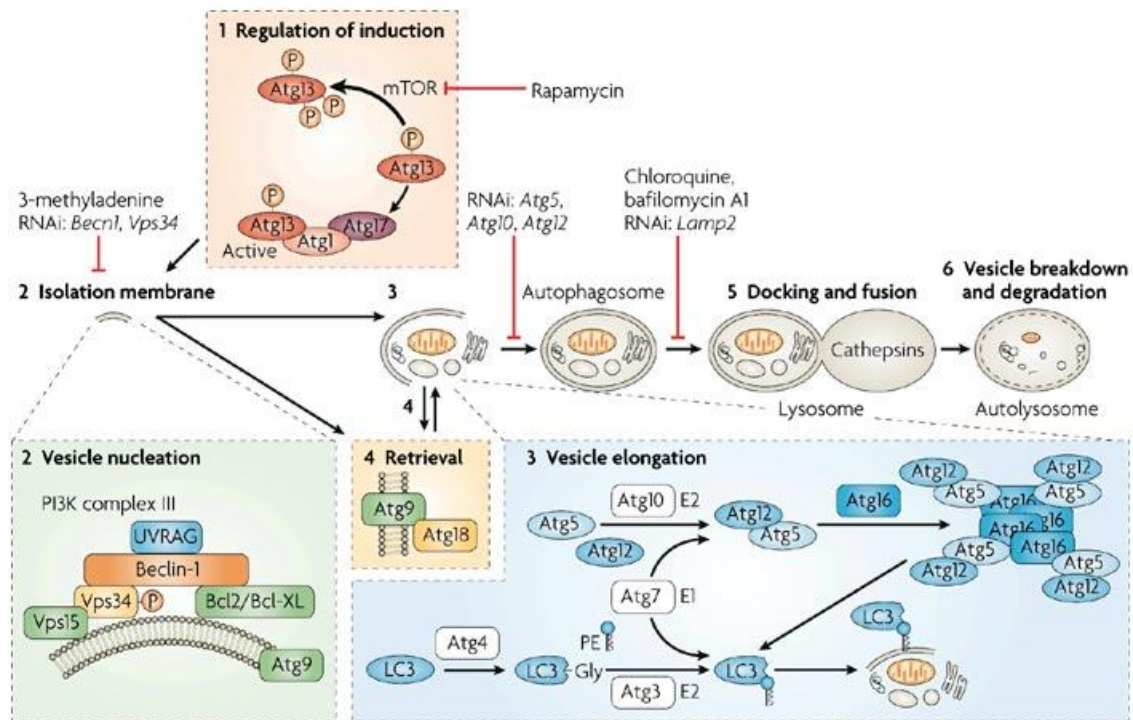


Fig 4: The three fuses for the autophagy induction

This is followed by fusion of the autophagosome with a lysosome to form an autophagolysosome where the captured material, together with the inner membrane, is degraded. This process occurs at low basal levels in virtually all cells to perform homeostatic functions such as protein and organelle turnover. It is rapidly upregulated when cells need to generate intracellular nutrients and energy, for example during starvation, growth factor withdrawal or high bioenergetic demands.

Autophagy is also upregulated when cells are preparing to undergo structural remodelling such as during developmental transitions or to rid themselves of damaging cytoplasmic components, for example during oxidative stress, infection or protein aggregate accumulation.

The identification of signals that regulate autophagy and genes that execute autophagy has facilitated detection and manipulation of the autophagy pathway.

Phosphatidylethanolamine (PE) conjugation of yeast Atg8 or mammalian LC3 during autophagy results in a nonsoluble form of Atg8 (Atg8-PE) or LC3 (LC3-II) that stably associates with the autophagosomal membrane. Consequently, autophagy can be detected biochemically (by assessing the generation of Atg8-PE or LC3-II) or microscopically (by observing the localization pattern of fluorescently tagged Atg8 or LC3) (Mizushima and Klionsky, 2007).

Autophagy can be pharmacologically induced by inhibiting negative regulators such as TOR with rapamycin (Rubinsztein et al., 2007); the antiapoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> that bind to the mammalian ortholog of yeast Atg6, Beclin1, with ABT-737 (Maiuri et al., 2007b) ; IP<sub>3</sub>R with xestospongin B, an IP<sub>3</sub>R antagonist; or lithium, a molecule that lowers IP<sub>3</sub>R levels (Criollo et al., 2007).

Autophagy can be pharmacologically inhibited by targeting the class III PI3K involved in autophagosome formation with 3-methyladenine or by targeting the fusion of autophagosomes with lysosomes, using inhibitors of the lysosomal proton pump such as bafilomycin A1 or lysosomotropic alkalines such as chloroquine and 3-hydroxychloroquine (Rubinsztein et al., 2007). It should be noted that all of these pharmacological agents lack specificity for the autophagy pathway. Therefore, although some of these agents such as rapamycin, lithium and chloroquine are clinically available and may be helpful for treating diseases associated with autophagy deregulation, genetic approaches to inhibiting autophagy, for example knockout of ATG genes by homologous recombination or knockdown by small-interfering RNA have yielded more conclusive information about the biologic roles of autophagy in health and disease.

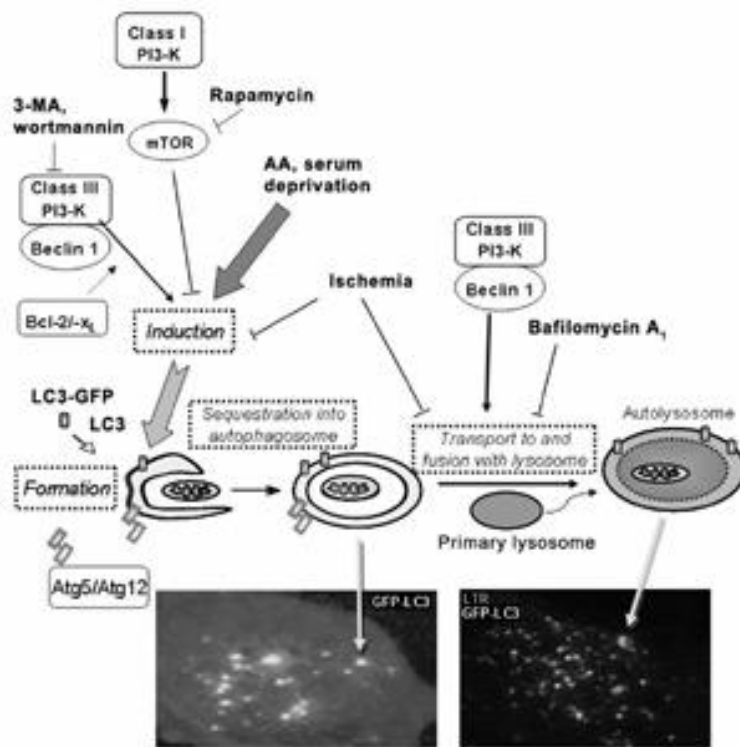


Fig 5: The LC3-lipidation



## **PHYSIOLOGICAL FUNCTION OF AUTOPHAGY**

### **Autophagy defends against metabolic stress**

Autophagy is activated as an adaptive catabolic process in response to different forms of metabolic stress, including nutrient deprivation, growth factor depletion and hypoxia. This bulk form of degradation generates free amino and fatty acids that can be recycled in a cell-autonomous fashion or delivered systematically to distant sites within the organism. Presumably, the amino acids generated are used for the novo synthesis of protein that are essential for stress adaptation. The molecular basis for the recycling function of autophagy has only recently begun to be defined with the identification of yeast Atg22 as a vacuolar permease required for the efflux of amino acids resulting from autophagic degradation (Mizushima and Klionsky, 2007). The amino acids liberated from autophagic degradation can be further processed and together with the fatty acids, used by the tricarboxylic acid cycle (TCA) to maintain cellular ATP production. The importance of autophagy in fueling the TCA cycle is supported by studies showing that certain phenotypes of autophagy-deficient cells can be reversed by supplying them with a TCA substrate such as pyruvate (or its membrane-permeable derivative methylpyruvate).

This role of autophagy in maintaining macromolecular synthesis and ATP production is likely a critical mechanism underlying its evolutionarily conserved prosurvival function. Gene knockout and knockdown studies provide strong evidence that autophagy plays an essential function in organismal during nutrient stress (Maiuri et al., 2007a) .

### **Autophagy works as a cellular housekeeper**

Autophagy can eliminate defective proteins and organelles, prevent the abnormal protein aggregate accumulation and remove the intracellular

pathogens. Such functions are likely critical for autophagy-mediated protection against aging, cancer, neurodegenerative disease and infection. Although some of these functions overlap with those of the ubiquitin-proteasome system, the other major cellular proteolytic system, the autophagy pathway is iniquely capable of degrading entire organelles such as mitochondria, peroxisomes and ER as well as intact intracellular microorganisms. Tissue-specific disruption of ATG genes has revealed a critical role for basal autophagy in protein quality control in murine post-mitotic cells. Atg7 deletion in hepatocytes, atg5 and atg7 deletion in neurons, and atg5 deletion in cardiomyocytes result in the accumulation of ubiquitin-positive protein aggregates in inclusion bodies that are associated with cellular degeneration.

Unlike proteosomal degradation, the autophagic breakdown of substrates is not limited by steric considerations and therefore autophagy can sequester and degrade entire organelles.

### **Autophagy may be a guardian of the genome**

Recent studies in ATG gene-deficient immortalized epithelial cells indicate that the autophagy machinery can limit DNA damage and chromosomal instability (Mathew et al., 2007).

Because these studies used cells with simultaneous defects in DNA checkpoints and apoptosis pathways, it is not yet known whether autophagy plays a primary function in preventing genomic instability in normal cells. However, in view of known functions of autophagy in energy homeostasis and in protein and organelle quality control, this seems likely. Failure to control the damage of checkpoint or repair proteins, deregulated turnover of centrosomes, insufficient energy for proper DNA replication, repair and excessive generation of reactive oxygen species due to inefficient removal of damaged mitochondria are

possible alterations that may contribute to genomic instability in autophagy-defective cells (Jin and White, 2007; Mathew et al., 2007).

### **Autophagy in life and death decisions of the cell**

Under most circumstances, autophagy constitutes a stress adaptation pathway that promotes cell survival. An apparent paradox is that autophagy is also considered a form of nonapoptotic programmed cell death called “type II” or autophagic cell death. The knockdown of ATG genes has recently defined whether autophagy functions in the execution of cell death in different settings (Maiuri et al., 2007a). It is not yet understood what factors determine if autophagy is cytoprotective or cytotoxic and if cytotoxicity occurs as the result of self-cannibalism, the specific degradation of cytoprotective factors or other as of yet undefined mechanisms.

However, cells subjected to prolonged growth factor deprivation or shortage of glucose and oxygen can lose the majority of their mass via autophagy and fully recover when placed in optimal culture conditions (Degenhardt et al., 2006) (Lum et al., 2005), suggesting that cell death via autophagy may not be simply a matter of crossing a quantitative threshold of selfdigestion.

## AUTOPHAGY IN DISEASES

### **Autophagy and Neurodegenerative diseases**

Early reports demonstrating that autophagosomes accumulate in the brains of patients with diverse neurodegenerative diseases, including Alzheimer's disease, transmissible spongiform encephalopathies, Parkinson's disease and Huntington's disease (Rubinsztein et al., 2007) (Williams et al., 2006), led to the initial hypothesis that autophagy contributed to the pathogenesis of these disorders. In mice with cerebellar degeneration due to mutations in glutamate receptor, autophagy was also postulated to be a mechanism of nonapoptotic cell death (Yue et al., 2002).

In contrast, more recent studies provide compelling evidence that at least in model organism autophagy protects against diverse neurodegenerative diseases and that the accumulation of autophagosomes represents the activation of autophagy as a beneficial physiological response or, in the case of Alzheimer's disease, the consequence of a defect in autophagosomal maturation (Martinez-Vicente and Cuervo, 2007) (Rubinsztein et al., 2007) (Williams et al., 2006).

Beyond its role in the clearance of misfolded proteins spontaneously generated during routine protein turnover, autophagy plays an important role in the clearance of aggregate-prone mutant proteins associated with several different neurodegenerative diseases. These include proteins with polyglutamine (polyQ) expansion tracts such as those seen in Huntington's disease and spinocerebellar ataxia, mutant  $\alpha$ -synucleins that cause familial Parkinson's disease and different forms of tau including mutations causing frontotemporal dementia (Williams et al., 2006).

Pharmacological activation of autophagy reduces the levels of soluble and aggregated forms of mutant huntingtin protein, protein mutated in spinocerebellar ataxia, mutant forms of  $\alpha$ -synucleins and mutant tau.

The role of autophagy in protection against neurodegenerative diseases is established in animal models but not yet in patients. Preclinical animal data provide a strong rationale for proceeding with clinical trials with autophagy-stimulatory agents; this is especially true as agents shown to be beneficial in reducing neurotoxicity of mutant aggregate-prone proteins are already in clinical use to treat other diseases. Rapamycin analogs, which are approved for the use of preventing organ transplant rejection and postangioplasty coronary artery restenosis and are in phase II oncology trials, protect against neurodegeneration seen in *Drosophila* and mouse polyQ disease models (Rubinsztein et al., 2007). Another group of new agents, small molecule enhancers or rapamycin (SMERs), enhance the clearance of mutant huntingtin and  $\alpha$ -synuclein and protect against neurodegeneration in a *Drosophila* Huntington's disease model (Sarkar et al., 2007). Given the lithium and SMERs both act independently of TOR, it is possible that they may be used therapeutically in combination with rapamycin analogs.

### **Autophagy and Liver disease**

Tissue-specific knockout studies in mice indicate an important role for basal hepatocyte autophagy in intracellular protein and organelle quality control. The protein quality-control function may be important in the pathogenesis of the most common genetic cause of human liver disease,  $\alpha$ 1-antitrypsin deficiency, which is associated with chronic inflammation and carcinogenesis (Perlmutter, 2006).

Perhaps similar to neurodegenerative disorders caused by aggregate-prone proteins, pharmacological activation of autophagy may be helpful in this setting.

### **Autophagy and Muscle disease**

Similar to neurodegenerative diseases, the pathogenesis of myodegenerative may involve either the failure of autophagosomes to fuse with lysosomes or the aggregation of misfolded proteins that exceed the autophagic clearance capacity of the myocyte. Danon disease, a genetic disease characterized by cardiomyopathy, myopathy and variable mental retardation, results from a mutation in the lysosomal protein LAMP-2 and is associated with extensive accumulation of autophagosomes in the muscles of LAMP-2-deficient mice and patients. There are several other histologically related diseases, such as X-linked myopathy with excessive autophagy, infantile autophagic vacuolar myopathy, adult-onset vacuolar myopathy with multiorgan involvement and X-linked congenital autophagic vacuolar myopathy that have unclear molecular defects (Nishino, 2006); however, the prediction is that these disorders may be due, at least in part, to an impairment in autophagosome-lysosome fusion.

Muscle disease in which autophagy may promote the clearance of disease-causing proteins inclusion body myositis, limb girdle muscular dystrophy type 2B and Miyoshi myopathy. In sporadic inclusion body myositis, the most common acquired muscle disease in patients above 50 years of age, overexpression of amyloid precursor protein (APP) and accumulation of its proteolytic fragment  $\beta$ -amyloid in vacuolated cells is thought to be a central pathogenetic mechanism (Askanas and Engel, 2006).

Both APP and  $\beta$ -amyloid colocalize with LC3 in cultured human muscle cells and in degenerating muscle fibers in human biopsy tissue, suggesting that these proteins are cleared by autophagy (Lunemann et al., 2007).

### **Autophagy and Cardiac disease**

Defective autophagy may play a role in relatively rare forms of inherited diseases of the heart (Dannon disease, Pompe disease). Of greater medical significance is the possibility that autophagy may constitute an important physiological or pathophysiological response to cardiac stresses such as ischemia or pressure overload, which are frequently encountered in patients with coronary artery disease, hypertension, aortic valvular disease and congestive heart failure. The accumulation of autophagosomes has been noted in cardiac biopsy tissues of patients with these disorders, rodent models of these cardiac diseases and isolated stressed cardiomyocytes (Terman and Brunk, 2005). After genetic studies, it was largely assumed that autophagy invariably contributed to myocyte degeneration in such settings. The cytoprotective effects of autophagy (either via ATP production, protein and organelle quality control or other mechanisms) may predominate in certain settings.

## CANCER

Cancer cells are defined by two heritable properties: they and their progeny reproduce in defiance of the normal restraints on cell division and invade and colonize territories normally reserved for other cells. It is the combination of these actions that makes cancers dangerous. Infact, if the proliferation is out of control, it will give rise to a tumour, or neoplasm.

As long as the neoplastic cells remain clustered together in a single mass, the tumour is said to be *benign*. At this stage, a complete cure can usually be achieved by removing the mass surgically. A tumour is considered a cancer only if it is *malignant* when its cells have acquired the ability to invade surrounding tissue. Invasiveness usually implies an ability to break loose, enter the bloodstream or lymphatic vessels with *metastases* and form secondary tumours at other sites of body.

Cancers are classified according to the tissue and cell type from which they arise. Cancers arising from epithelial cells are termed *carcinomas*; those arising from connective tissue or muscle cells are termed *sarcomas*. Cancers that do not fit in either of these two broad categories include the various *leukemias*, derived from hamopietic cells and cancers derived from cells of the nervous system.

There are good reasons to think that the vast majority of cancers are initiated by genietic changes. First, cells of a variety of cancers can be shown to a have a shared abnormality in their DNA sequence that distinguished them from the normal cells surrounding the tumour. Second, many of the agents known to give rise to cancer also cause genetic changes. Thus carcinogenesis (the generation of cancer) appears



to be linked with mutagenesis (the production of a change in the DNA sequence). This correlation is clear for three classes of agents:

**-chemical carcinogenesis**, which typically cause simple local changes in the nucleotide sequence.

**-ionizing radiations**, such as X-rays, which cause chromosome break and translocations.

**-viruses**, which introduce foreign DNA into the cell.

It is important to know that not all substances that favor the development of cancer are mutagens. Some of the clearest evidence comes from studies done long ago on the effects of cancer-causing chemicals on mouse skin, where it is easy to observe the stages of tumour progression.

In fact a single application of the carcinogen, usually does not by itself give rise to a tumour or any other obvious lasting abnormality. It can cause only latent genetic damage. This type of carcinogen is said to act as a *tumour initiator*. Only repeated exposure over a long period to certain substances known as *tumour promoters*, which are not themselves mutagenic, can cause cancer selectively in skin previously exposed to a tumour initiator.

### **The cancer-critical genes**

They are grouped into two broad classes, according to whether the cancer risk arises from too much activity of the gene product. The two classes are:

**-proto-oncogenes** and their mutant overactive forms are called oncogenes

**-tumor suppressor genes.**

Mutation of a single copy of a proto-oncogene can have a dominant, growth-promoting effect on a cell. Thus the oncogene can be detected by

its effect when it is added- by DNA transfection, for example, or through infection with a viral vector- to the genome of a suitable type of tester cells. In the case of the tumor suppressor gene, the cancer-causing mutations are generally recessive: both copies of the normal gene must be removed or inactivated in the diploid somatic cell before an effect is seen.

### **Colorectal cancer**

Colon carcinoma is the second largest cause of cancer-related mortality in the United States and both prevention and treatment of this disease are the focus of intensive research efforts. Diet has been recognized as an important modifiable risk factor for colon cancer for decades (Ellington and Bull, 2005). Specifically, a consistent inverse association between diets rich in plant foods and colon cancer incidence has been identified through epidemiologic studies.

Colorectal cancer arises from the epithelium lining the colon and rectum . It is common, currently causing over 60000 deaths a year in the United States, or about 11% of total deaths from cancer. Routine examination of normal adults with a colonoscope often reveals a small benign tumor, or adenoma, of the gut epithelium in the form of a protruding mass of tissue called a polyp. The progression of the disease is usually very slow, there is typically a period of 10-35 years in which the slowly growing tumor is detectable but has not yet turned malignant.

Gut epithelium is constantly regenerated from progenitor cells located near the bottom of intestinal and colonic crypts that produce transit amplifying cells that in turn give rise to the cell lineages required for gut function (Cheng and Leblond, 1974).

The most abundant differentiated cells are absorptive enterocytes, which have a highly organized cytoskeletal arrangement with an apical actin-rich brush border and cortical, lateral actin-rich junctions and highly ordered parallel arrays of microtubules (Cheng H and Leblond CP 1974). Maintenance of the gut epithelium depends on a combination of cell proliferation balanced by cell death, coupled with differentiation and active cell migration, while adhering to neighbouring cells and the basement membrane. It is therefore likely that changes that cause an imbalance between these processes can contribute to the initiation of tumours in this tissue.

Many genes are discovered to be involved in colorectal cancer: K-Ras, a member of the Ras gene family, p53 and APC. Others are involved in smaller numbers of colon cancer and still other critical genes remain to be identified.

Some mutations inactivating the APC (adenomatous polyposis coli) gene appears to be the first or at least a very early step in most cases. They can be detected already in small benign polyps at the same high frequency as in large malignant tumors. Loss of APC seems to increase the rate of cell proliferation in the colonic epithelium relative to the rate of cell loss, without affecting the way the cells differentiate or the details of the histological pattern they form. Mutations activating the K-Ras oncogene appear to take place a little later than those APC; they are rare in small polyps but common in larger ones that show disturbances of cell differentiations and histological pattern. When malignant colorectal carcinoma cells containing such Ras mutations are grown in culture, they show typical features of transformed cells, such as the ability to proliferate without anchorage to a substratum. Loss of cancer-critical genes on chromosome 18 and mutations in p53 may come still later. They are rare in polyps but common in carcinomas, suggesting that they may

often occur late in the sequence. Loss of p53 function is thought to allow the abnormal cells not only to avoid apoptosis and to divide, but also to accumulate additional mutations at a rapid rate by progressing through the cell cycle when they are not fit to do so, creating many abnormal chromosomes.

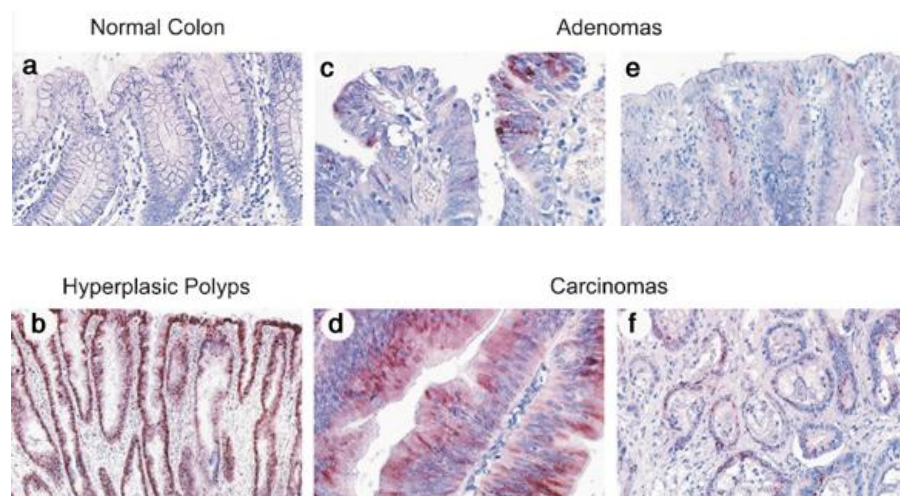


Fig 6: Histochemistry of tissues from normal colon to Carcinomas

## **APOPTOTIC CELL DEATH AND CARCINOGENESIS**

Apoptosis can be induced by various stimuli, and radiation or chemicals in particular have been used in cancer therapy (Johnstone et al., 2002) (Fesik, 2005). It is known two major signalling pathways that lead to apoptosis in mammalian cells: the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is controlled by antiapoptotic Bcl2 family proteins at the mitochondria and has a substantial role in chemotherapy and radiation-induced cell death. By contrast, the extrinsic death pathway is initiated through apoptotic signal transduction cascades mediated by members of the tumour necrosis factor (TNF) receptor superfamily.

In cancer cells, apoptosis induced by the extrinsic pathway complements that induced by the intrinsic pathway, so targeting death receptors is considered a useful new therapeutic approach.

### **TNF-related proteins**

The pathway involving TNF-related apoptosis inducing ligand (TRAIL, also known as APO2L and TNFSF10) and TRAIL receptors (TRAILRs) is most promising, as preclinical models suggest that apoptosis of tumour cells is achievable *in vivo* without lethal toxicities (Ashkenazi, 2002). In addition to triggering a pro-apoptotic signal through activation of caspases, TRAIL can activate diverse intracellular signalling pathways involving NF $\kappa$ B (Russo et al., 2004), phosphoinositide 3-kinase (Aggarwal and Shishodia, 2006) and mitogen activated protein kinase (Li et al., 2007) family proteins that can stimulate cell survival and proliferation.

TRAIL is an important immune effector molecule in the surveillance and elimination of developing tumours. Moreover, genetic lesions in various

components of the TRAIL pathway have been found in human tumour samples, suggesting that inactivation of the TRAIL pathway and/or escape from TRAIL-mediated immunosurveillance might have an important role in tumour onset and progression.

In preclinical trials, recombinant forms of TRAIL and agonistic anti-TRAIL receptor antibodies can have single-agent activity against TRAIL-sensitive tumour cells *in vitro* and *in vivo*. These agents can synergize with chemotherapeutic drugs and novel molecular therapeutic agents to more effectively kill TRAIL-sensitive tumour cells and TRAIL-resistant tumours.

Early-phase clinical trials using recombinant TRAIL and agonistic anti-TRAIL receptor antibodies indicate that these agents can be delivered safely and are generally well-tolerated. Although some objective anti-tumour responses have been reported with these agents as monotherapies, they probably hold greater promise for further clinical development when used in combination with other cancer treatments (Buchsbaum et al., 2007).

### **FLIP as an anti-cancer therapeutic target**

Antiapoptotic proteins overexpressed in tumor cells have been recognized as targeting points for anti-cancer therapeutic interventions, and their inhibitors at the levels of mRNA and protein have been developed, which are mostly antisense oligonucleotides and small molecule inhibitors (Fesik, 2005) (Debatin and Kramer, 2004).

Those drug candidate compounds are now mostly in the preclinical and early clinical stages.

FLIP is an another important antiapoptotic protein overexpressed in various types of tumor cells (Kataoka, 2005) but the agents directly targeting it have not yet been reportedly developed.

Elevated expression of c-FLIP has been found in various types of tumor cells which are often resistant (Kataoka, 2005) to death-receptor-mediated apoptosis.

Those tumors include colorectal carcinoma (Ullenhag et al., 2002) pancreatic carcinoma (Elnemr et al., 2001), Hodgkin's lymphoma (Valente et al., 2006), B cell chronic lymphocytic leukemia (Olsson et al., 2001), ovarian carcinoma (Abedini et al., 2004).

The expression of c-FLIP has been proven to be one of the major determinants of the resistance to death ligands such as FasL and TRAIL, and numerous reports have shown that down-regulation of c-FLIP results in sensitizing a various types of resistant tumor cells. Conversely, forced expression of c-FLIP renders cells resistant to Fas and/or TRAIL. These observations collectively imply that c-FLIP may be an attractive therapeutic target against at least the above mentioned kinds of tumors of which malignancy and resistance.

### **p53-based cancer therapies**

The tumor suppressor protein p53 is activated in response to stressful stimuli such as DNA damage, hypoxia and oncogene activation. p53 is often referred to as the 'guardian of the genome' because it plays a key role in determining a cell's fate following DNA damage. When DNA is damaged, p53 can trigger cell cycle arrest, senescence (permanent arrest) or apoptosis to eliminate the damaged cell. Cell cycle arrest provides time for repair of the damage thereby allowing the cell to recover and survive. The mechanisms governing whether a cell will arrest or die are not well understood, but these functions are essential to protecting an organism from the effects of aberrant cell divisions, and defects can lead to cancer development and progression.

The fact that p53 is defective in >50% of tumors supports the assertion that p53 is an important player in the prevention of tumor development. The pivotal role of p53 in regulating the decision of a cell to live or to die makes it an attractive target for cancer therapeutics. However, there are many conflicting ideas and approaches as to which would be the best therapeutic strategy to pursue. Initial attempts were based on the premise that reintroduction or activation of p53 would induce apoptosis in the tumor. Other approaches are based on the observation that cells with defective p53 are more sensitive to certain drugs and combinations.

Understanding the factors influencing the decision of a cell to undergo cell cycle arrest or die in response to DNA damage is essential to developing cancer therapies that specifically target tumor cells without damaging normal cells. There is a huge literature addressing both cell cycle arrest and cell death, but in the context of therapeutic strategies, the widely accepted model is that p53 is required for DNA damage-induced apoptosis.

There are numerous cases where p53-defective cells undergo apoptosis, and many cases in which the loss of p53 even sensitizes cells to DNA damage.

So pervasive has p53 become that it sometimes appears there are only two pathways of apoptosis, either p53-dependent or p53-independent, and this extends far beyond any role in response to DNA damage. It has frequently been implied that p53 is needed if cells are to succumb to anticancer drugs, yet many years of research have resulted in the discovery of drugs while using p53-defective tumor models, and the drugs have been used successfully to treat many patients with p53-defective tumors. Furthermore, radiation therapy is routinely administered to patients independent of their p53 status as it does not predict outcome.



Numerous studies have focused on activation of p53 as a strategy for cancer therapy. These studies have generally not considered the particular oncogenic context of each tumor and whether arrest or apoptosis would be the likely outcome.

However, re-introduction of p53 into p53-defective tumor cells has frequently been shown to increase apoptosis (Willis and Chen, 2002).

The consequence of over-expression of exogenous p53 may have little bearing on the pathways activated by DNA damage in the presence of a relatively low level of endogenous protein.

For example, overexpression of p53 may overwhelm any selective activation of growth arrest genes versus proapoptotic genes. Difficulties in this therapeutic strategy lie in controlling the level of p53 expression and in delivering the p53 gene effectively to tumor cells.

While much of the drug-induced phosphorylation of p53 is required to dissociate p53 from HDM2, other modifications of p53 that may not occur in the absence of DNA damage may be required for its full activity.

An alternate explanation provided for this tumor selectivity is that tumor cells are sensitized to apoptosis because of oncogene addiction (Issaeva et al., 2004). This is an important concept for which there is considerable support. For example, tumors almost invariably have elevated levels of Myc which is a known repressor of p21<sup>waf1</sup> (Seoane et al., 2002). Accordingly, p53 wild-type tumors (or those in which p53 has been reintroduced or reactivated) may still be defective in their ability to arrest compared to normal cells, and therefore more sensitive to DNA damage. Mice engineered to express reduced levels of MDM2, and as a consequence elevated p53, showed an increase in apoptosis of the thymus and small intestine when irradiated, although there was little toxicity in most tissues (Mendrysa et al., 2003).

There is another important variable to mention which will impact a potential therapeutic strategy . Specifically, it has been shown that p53 can also induce apoptosis independent of any transcriptional activity by directly interacting with Bcl-2 family members at the mitochondria (Mihara et al., 2003) (Chipuk et al., 2005) (Chipuk and Green, 2005) (Leu et al., 2004).

An alternate therapeutic strategy is based on the belief that activation of p53 in normal cells will indeed be toxic to the patient. p53 inhibitors were developed with the intent to protect normal tissues from the adverse effects of chemotherapeutic agents. The p53 inhibitor pifithrin- $\alpha$  (PFT- $\alpha$ ) rescued p53 wild-type cells from apoptosis induced by the DNA damaging agent etoposide and reduced lethality in mice following  $\gamma$ -radiation (Komarov et al., 1999).

PFT- $\alpha$  displays a number of chemo- and radiation-protective qualities, including neuroprotection, cardioprotection and renal protection (Gudkov and Komarova, 2005). These observations have led to the proposal that p53 inhibitors could be useful protective agents when used in combination with chemotherapy and radiation.

### **Targeting the IAPs for cancer therapy**

The IAPs are the only known endogenous proteins that regulate the activity of both initiator and effector caspases. Controlled expression of the IAPs has been shown to influence cell death in a variety of contexts and is believed to have important consequences with respect to human cancer. The mechanism by which the IAPs inhibit apoptosis was first interrogated in the laboratory of John Reed (Deveraux et al., 1998).

In these early studies, XIAP was found to prevent caspase-3 processing in response to caspase-8 activation. Therefore, XIAP was suggested to

inhibit the extrinsic apoptotic signaling by blocking the activity of the downstream effector caspases, as opposed to interfering directly with caspase-8 activation (Deveraux et al., 1998). Supporting this concept, XIAP was shown to specifically bind to caspase-3 and -7, but not to caspase-1, -6, -8 or -10. *In vitro* assays confirmed that XIAP, as well as cIAP1 and cIAP2, could prevent downstream proteolytic processing of pro-caspase-3, -6 and -7 by blocking cytochrome *c*-induced activation of pro-caspase-9 in the intrinsic pathway.

The role of the IAPs in apoptosis suppression is continuously being evaluated. The Salvesen's laboratory (Eckelman et al., 2006) suggests that only XIAP is a direct inhibitor of caspases, and that other IAPs simply bind caspases but do not inhibit them. This suggestion is based on the fact that XIAP is relatively stable and exhibits the greatest potency for caspase inhibition compared with the other IAPs.

A variety of cancer cell lines and primary tumor biopsy samples show elevated IAP expression levels (Ambrosini et al., 1997) (Fong et al., 2000).

The most dramatic example of IAP over-expression in tumors is seen with survivin. Survivin expression is limited to embryonic tissues and many different tumor types, but is absent in most adult differentiated tissues (Ambrosini et al., 1997). Furthermore, the presence of survivin in patient tumor biopsy samples correlates with poor prognosis, increased rates of treatment failure and relapse. The prognostic significance of IAP overexpression is less clear for some of the other IAPs. For example, XIAP protein levels correlate with disease severity and prognosis in acute myelogenous leukemia (AML) (Tamm et al., 2000) and renal cell carcinoma (Ramp et al., 2004), but not in non-small cell lung carcinoma (Ferreira et al., 2001) or cervical carcinoma (Liu et al., 2001).

XIAP levels have also been shown to increase in leukocytes with the transformation of myelodysplastic syndromes to overt AML (Yamamoto et al., 2004). High expression of XIAP or cIAP2 is associated with shorter overall survival, and lower complete response rates for AML (Hess et al., 2007). Other cross-validation and forward selection studies reveal a three-gene signature, consisting of cIAP2, Bax and BMF, to optimally predict overall survival in AML. Furthermore, an immunocytochemical survey of tumor tissue shows that XIAP immunostaining patterns allow for the ready distinction of malignant from benign populations in effusion washes (Wu et al., 2005). XIAP is also identified as part of a progression signature in prostate cancer using an immunoblot approach to characterize proteomic alterations in prostate tumors. XIAP protein is seen to increase in expression between benign prostatic tissue, and clinically localized prostate cancer, or metastatic prostate cancer (Varambally et al., 2005). In addition, XIAP protein expression is a strong predictor of prostate cancer recurrence (Seligson et al., 2007). Collectively, these results suggest that the expression levels of the IAPs could be expected to have a significant impact in the development and maintenance of cancer due to their central role in the regulation of apoptosis.

Furthermore, cIAP1 cooperates with the Myc oncogene in transformation, by acting as a ubiquitin ligase for Mad1, an antagonist of Myc. A recent report also suggests that cIAP1 and cIAP2 promote cancer cell survival by ubiquitinating RIP1, leading to constitutive RIP1 and NF- $\kappa$ B activity.

Applications of single-stranded antisense oligodeoxynucleotides (AS ODNs) as selective inhibitors of gene expression are being studied for efficacy in treating particular genetic disorders. Hybridization of the AS ODNs to the mRNA by Watson–Crick base pairing prevents the target

gene from being translated into protein, thereby blocking the action of the gene, and resulting in the degradation of the target mRNA (Galderisi et al., 1999) (Gleave et al., 2002) (Jansen and Zangemeister-Wittke, 2002).

A multitude of candidate genes, involved in apoptosis regulation, represents potential targets for antisense-based therapies.

Additional proof-of-principle studies performed with newer double-stranded RNAi-based technologies show that downregulating XIAP gene expression through siRNA increases apoptosis in cultured MCF-7 breast cancer cells and subsequently enhances the killing effects of etoposide and doxorubicin (Lima et al., 2004). Another study using short-hairpin RNAs as an RNAi approach directed against XIAP shows that XIAP mRNA can be reduced by as much as 85% in some breast carcinoma cell lines. This reduction in XIAP dramatically sensitizes these cell lines to TRAIL- and to taxane-induced killing (McManus et al., 2004).

Survivin is expressed in fetal tissues, becomes restricted during development, and is absent in most healthy, differentiated adult tissues (Adida et al., 1998), with some notable exceptions, such as stem cells, thymus, testes, regenerating hepatocytes and endothelial cells (Kobayashi et al., 1999) (Fukuda and Pelus, 2006). Significantly, survivin is re-expressed during malignant transformation and is found in nearly all tumor types, including neuroblastomas, pancreatic, prostate, gastric, colorectal, hepatocellular and breast carcinomas, as well as lung and bladder cancers, melanomas, B-cell lymphomas and esophageal cancer (Altieri, 2008). The expression of survivin in cancer is a predictor of both poor prognosis and decreased survival time, and is implicated in conferring chemo- and radio-resistance phenotypes to tumor cells. Numerous studies have addressed the diagnostic and prognostic potential of survivin expression, as well as that of its nuclear versus cytoplasmic localization, phosphorylation status and the presence of splice isoforms

(Baur and Sinclair, 2006) (Dohi et al., 2007) (Pannone et al., 2007) (Brennan et al., 2008).

MDM2 is a protein that binds to the tumour suppressor p53 and negatively regulates its activity and stability (Momand et al., 2000) .

The MDM2-mediated loss of p53 impairs a cell's ability to block cell-cycle progression and induce apoptosis in response to DNA damage, which can contribute to the cancerous phenotype.

The observation that MDM2 is amplified or overexpressed in several human tumour types (Momand et al., 2000) provides further support for the involvement of MDM2 in cancer.

Therefore, strategies aimed at blocking the interaction between MDM2 and p53 to inhibit the degradation of p53 could be important for cancer drug discovery (Lane and Lain, 2002). The X-ray crystal structure of the p53–MDM2 complex showed how p53 interacts with MDM2 and aided in the design of p53 mimetics that bind to MDM2 (Kussie et al., 1996).

In the first attempt to identify MDM2 inhibitors, peptide-based compounds were discovered that bind tightly to the MDM2 (Bottger et al., 1997).

However, these compounds lacked the ability to penetrate cells and did not possess drug-like properties. Although several small molecules such as polycyclic compounds, chlorofusin (Duncan et al., 2001), sulfonamides (Galatin and Abraham, 2004) and benzodiazepines (Parks et al., 2005) were also identified that bind to MDM2 and displace p53 peptides, these compounds only bind weakly to MDM2.

## **PI3K and apoptotic cell death in carcinogenesis**

There are many other compounds that induce apoptosis.

However, this might not be their primary function, or the mechanism by which they induce programmed cell death has not yet been defined. For example, in addition to its other functions, the phosphatidylinositol 3-kinase PI3K–AKT pathway can regulate both the intrinsic and extrinsic apoptotic pathways (Workman, 2004). Activated AKT decreases apoptosis by phosphorylating and inactivating BAD (Datta et al., 1997) (del Peso et al., 1997) and apoptosis signal-regulating kinase ASK. AKT also regulates FAS and IAP expression through phosphorylating the forkhead (FOXO) transcription factor family (Brunet et al., 1999) (Kops and Burgering, 1999) and nuclear factor of NF- $\kappa$ B (Gelfanov et al., 2001).

Furthermore, the PI3K–AKT pathway is deregulated in many cancers, indicating that this signalling pathway might contain possible anticancer targets. Although inhibitors of the PI3K family (such as LY294002) have been shown to inhibit the growth of both cancer cells *in vitro* and tumours in animal models, these compounds lack selectivity and have mostly served as pharmacological tools to study the functions of this pathway (Mitsiades et al., 2004). AKT inhibitors have also been reported that inhibit tumour growth when used as a monotherapy or in combination with paclitaxel. Unfortunately, compounds within these series were found to have a narrow therapeutic window and show significant metabolic toxicities that are probably mechanism-based. Another target in the PI3K–AKT pathway is mammalian target of rapamycin mTOR. Rapamycin analogues that inhibit mTOR slow the growth of tumours in animal models without displaying significant toxicity. These compounds are currently in clinical trials for the treatment of breast, colon and lung cancers (Rowinsky, 2004).

The proteasome could also be considered as a target for inducing apoptosis, and proteasome inhibitors are being used to treat cancer. They have been shown to promote apoptosis in part by inducing endoplasmic reticulum stress and reactive oxygen species in head and neck squamous cell carcinoma cells (Fribley et al., 2004). The proteasome degrades ubiquitylated proteins, including inhibitor of  $\kappa$ B $\alpha$  (NF- $\kappa$ BI $\alpha$ ), which inhibits NF- $\kappa$ B and modulates several pathways. However, the proteasome is not part of the core apoptotic pathway and degrades many other proteins besides NF- $\kappa$ BI $\alpha$ . Other compounds that indirectly induce apoptosis include inhibitor of  $\kappa$ B kinase (IKK) inhibitors (Burke, 2003), arsenic trioxide (Chen et al., 1996) and many chemotherapeutic agents that are widely used for cancer therapy.

## **ROLE OF AUTOPHAGY IN CANCER**

Cancer results from the accumulation of mutations that deregulate cell growth, checkpoints, cell death and conditions in the tumor microenvironment in a manner that favors tumor growth and progression. Cell death by apoptosis is an important means to purge abnormal, emerging cancer cells and thus is a prominent mechanism for tumor suppression (Cory et al., 2003) (Danial and Korsmeyer, 2004).

Apoptosis is also a common cellular response to stress and many of the molecular events that promote tumorigenesis create or amplify cellular stress. For these and other reasons, defects in apoptosis commonly evolve during tumorigenesis, which further promotes tumor growth and treatment resistance. Once apoptosis is inactivated, tumor cells clearly possess a survival advantage; however, it may be the survival of the damaged “undead” cells that promotes tumor progression through the



manifestation and preservation of genome damage and chromosome instability (Bianchi et al., 2004; Degenhardt et al., 2002).

An example of the induction of apoptosis in tumors is metabolic stress. It is a common feature of solid tumors caused by inadequate vascularization that results in nutrient, growth factor and oxygen deprivation. Once tumors exceed a diameter of 1-2mm, angiogenesis must occur to ameliorate metabolic stress associated with the central and most metabolically deprived hypoxic tumor domain. Metabolic stress also occurs in mature tumors, where interruption in the blood supply is common due to vascular collapse because vascularization is abnormal.

Solid tumors with defects in apoptosis survive this metabolic stress and autophagy localizes to these hypoxic tumor regions (Degenhardt et al., 2006).

Compromising autophagy in apoptosis-defective tumor cells substantially impairs survival in metabolic stress conditions in vitro and in tumors in vivo, establishing that autophagy is a survival pathway utilized to sustain viability during periods of nutrient limitation. Indeed, autophagy functions to sustain metabolism during periods of growth factor deprivation of hematopoietic cells (Lum et al., 2004) and upon nutrient deprivation in normal mouse development (Kuma et al., 2004).

### **Autophagy, cell growth and proliferation**

Several lines of evidence suggest that the downregulation of autophagy could play a role in the active proliferation of malignant cells, which is one of the major hallmarks of cancer. In proliferating cells, including cancer cells, cell size control relies on the coordinated regulation of cell growth and cell division (Jorgensen and Tyers, 2004) so that on average each cell division is accompanied by a doubling in cell mass.

In many cell types, cell cycle progression depends on growth-factor deprivation results in cell cycle arrest. It was also shown recently that mitotic cells shut down their autophagic protein pathway while their chromosomes and organelles are dividing and that the autophagic response reemerges during the late telophase/G<sub>1</sub> phase. These observations have been supported by mechanistic studies showing that several regulatory proteins exerting pivotal functions in autophagy are also involved in regulating cell proliferation.

The first specific link between the autophagy machinery and human cancer was the implication of Beclin1 in tumor progression. It was suggested by the observation that this gene is monoallelically deleted in a high percentage of human cancers and tumor cell lines (Liang et al., 1999).

Stable transfection of Beclin1 into MCF-7 human breast carcinoma cells their tumorigenicity in nude mice and slowed their proliferation rate. Furthermore Beclin1-haploinsufficient mice displayed an increased incidence of spontaneous tumors and the cell proliferative capacity was markedly increased in some of their tissues (Qu et al., 2003).

From the database frequent chromosomal aberrations of several other components of the autophagic machinery in human cancers are observed. Atg5, a component of the ubiquitin-like protein conjugation system, has tumor suppressor effects in a mouse xenograft model (Yousefi et al., 2006), and knock-out of atg4, a cysteine protease involved in processing LC3, increases chemically induced fibrosarcomas in mice. Other proteins that form part of the Beclin1/PI3K III complex are required for autophagy activity may also play a role in cell growth control and tumor suppression. UVRAG, originally identified through its ability to complement UV-radiation sensitivity in tumor cells, is monoallelically deleted at a high frequency in human colon cancers and suppresses the

proliferation and tumorigenicity of human colon cancer cell (Liang et al., 2006). Bif-1 interacts with Beclin 1 through UVRAG and activates the Beclin1/PI3K III complex, and its deletion in mice results in the development of spontaneous tumors. Another component of this complex that positively regulates autophagy is Ambra1 and its deficiency in mouse embryos leads to severe neural tube defects associated with autophagy impairment and uncontrolled cell proliferation, further supporting the concept that the beclin1/PI3K III complex regulates cell growth.

In mammalian cells mTOR integrates signals from nutrients and growth factors to produce coordinated regulation of cell growth and cell-cycle progression (Fingar and Blenis, 2004). mTOR regulates translation via its downstream targets: p70S6 kinase (which phosphorylates the S6 ribosomal protein) and 4E-BP1 (a binding protein for the translational initiation factor eIF4E) (Gingras et al., 1998).

In addition to these functions, mTOR also acts as a rheostat, adjusting the rate of autophagy in response to the levels of amino acids and ATP (Meijer and Codogno, 2004).

The class PI3KI/Akt pathway is an upstream nutrient- and growth factor-responsive regulator of mTOR that plays an evolutionarily conserved role in the regulation of autophagy (Rusten et al., 2004; Scott et al., 2004).

Akt activation phosphorylates mTOR and this in turn inhibits autophagy. PTEN, a phosphatase that counteracts the lipid kinase activity of PI3KI has recently been shown to promote autophagy in HT-29 colon cancer cells (Arico et al., 2001). Since many cancers display aberrantly high class PI3KI-dependent signalling, either via the constitutive activation of class PI3KI or Akt or via inactivation of PTEN, the decreased autophagic activity of tumor cells observed in these cases may result from activation of the mTOR pathway.

Several other tumor suppressors and oncogenes that were initially identified as regulators of cell growth and proliferation were recently shown to be capable of modulating autophagy. One of the most intriguing examples is p53, a tumor suppressor that plays an important role in preserving the integrity of the genome. Genotoxic stresses activate p53 which in turn initiates tumor suppressor processes such as growth arrest (Feng et al., 2005). Although p53 can affect cell growth and proliferation by activating cyclin-dependent kinase p21, recent observations indicate that once activated, p53 also inhibits mTOR activity and upregulates autophagy. These findings indicate that p53 and Mtor pathways can crosstalk, regulate cell growth, proliferation and autophagy and suggest that in cancers in which p53 is mutated, mTOR activation resulting from abnormal p53 activity may contribute to the lower autophagic capacities of the malignant cells.

A recent study has shown that c-myc, a proto-oncogene that controls cell division and cell growth, increases autophagic activity when overexpressed in rat 3Y1 fibroblast (Tsuneoka et al., 2003).

Ras is another oncogene involved in regulating cell proliferation and oncogenesis that could modulate autophagy. Ras activates pathways that produce conflicting effects on autophagy.

The possibility of interplay between autophagy and cell proliferation has been further supported by a recent report showing that p27, which belongs to the family of cyclin-dependent kinase inhibitors, is a potential regulator of autophagy. Overexpression of p27, by recombinant adenoviral vector, induced autophagic cell death in human glioma cell lines, whereas the same treatment did not affect the viability of non-malignant cultured astrocytes or induce autophagy (Komata et al., 2003).

## **Autophagy and growth factors**

It has recently been reported that growth factors are involved in the suppression of autophagy. These results are consistent with previous observations that:

- growth factors enhance cell growth and proliferation; this is closely linked to the concomitant stimulation of anabolism and the suppression of catabolism

- growth factors regulate the uptake of nutrients, such as glucose and aminoacids, which are downregulators of autophagy (Edinger and Thompson, 2002)

- growth factors regulate the activity of PI3KI/Akt/mTOR pathway that coordinates the regulation of both autophagy and growth factor signalling (Broker et al., 2005).

Inhibition of the autophagy genes *atg5* and *atg7* and the pharmacological blockade of autophagy both accelerate cell death, even in the presence of an abundant supply of extracellular nutrients. Cells can be rescued from death by the bioenergetic substrate, methylpyruvate, suggesting that in the absence of growth factors cells use autophagy-induced cell catabolism to maintain a sufficient level of ATP production and ensure cell survival.

It is known that autophagy is necessary to remove damaged macromolecules and organelles and its inhibiting in cancer cells by activating the growth factor signalling pathway would lead to an accumulation of such damage, and thereby contribute to the development of cancer.

Another possibility is that autophagy plays a more direct role in negative growth control, perhaps by degrading specific organelles or proteins essential for cell growth regulation.

It's important to point that the genetic links between deficiencies in the autophagy machinery and tumor susceptibility highlight the likely importance of autophagy in tumor suppression. However, the molecular mechanisms by which autophagy functions in tumor suppression are poorly defined. Increasing evidence suggests that the tumor suppressor functions of autophagy may be independent of both potential and prosurvival effects (Mathew et al., 2007).

The association between increased cell death and increased tumorigenic potential in the setting of ATG gene deficiency suggests that autophagy-dependent survival, at least in certain experimental models, does not promote tumorigenesis. When tumor cells cannot die by apoptosis upon exposure to metabolic stress, autophagy may prevent death from necrosis, a process that might exacerbate local inflammation and increase tumor growth rate (Degenhardt et al., 2006). ATG gene deletion may promote genomic instability in metabolically stressed cells, leading to oncogene activation and tumor progression. Indeed, immortalized mouse epithelial cells with ATG gene deficiency display increased DNA damage, centrosome abnormalities and gene amplification, especially during ischemic stress that is associated with increased tumorigenicity.

Another possibility is that autophagy plays a more direct role in negative growth control, perhaps by degrading specific organelles or proteins essential for cell growth regulation. In support of this theory, enforced Beclin1 expression slows the proliferation of tumor cell lines and causes a decrease in expression of cyclin E and phosphorylated Rb (Koneri et al., 2007; Liang et al., 1999).

Also, mice with a monoallelic deletion of beclin1 display hyperproliferation of both mammary epithelial cells and splenic lymphocytes (Qu et al., 2003).

Although it is presently unclear if cell survival/cell death effects are relevant to the tumor suppressor role of autophagy, such effects are likely important in cancer therapeutics. A large series of clinically approved and experimental anticancer therapies induce the accumulation of autophagosomes in tumor cell lines in vitro (Maiuri et al., 2007a).

For many years, it was thought that these therapies kill cells through autophagy. However, specific inhibition of autophagy with siRNAs targeted against ATG genes usually accelerates, rather than prevents, indicating that autophagy activation represents a cellular attempt to cope with stress induced by cytotoxic agents. The inhibition of autophagy might be beneficial in cancer treatment. Indeed, in mice harbouring c-Myc-induced lymphomas, the drug chloroquine, an alkalinizing lysosomotropic drug that impairs autophagic degradation, enhanced the ability of p53 or a DNA alkylating agent to induce tumor cell death and tumor regression (Amaravadi and Thompson, 2007), indicating a potential pro-survival and protumorigenic role for autophagy during cancer chemotherapy.

In vivo studies are needed with more specific inhibitors of autophagy to determine if the beneficial effects of blocking a tumor cell survival outweigh the potential detrimental effects of blocking a tumor suppressor pathway.

## LYSOSOMES IN CELL DEATH CONTROL

Cancer cells characteristically provide their own growth signals, ignore growth-inhibitory signals, replicate without limit, sustain angiogenesis, invade through basal membranes and capillary walls, proliferate in unnatural locations, and avoid cell death. Although resistance to cell death is attributed to the inhibition of classic apoptosis and its hallmarks, particularly caspase activation and mitochondrial outer-membrane permeabilization (MOMP) (Green and Kroemer, 2004), it is possible that alternative, non-apoptotic cell death pathways are also relevant to carcinogenesis (Jaattela, 2004). Therefore, changes in lysosomal trafficking and content that support invasion and angiogenesis could account for disorders in the regulation of apoptotic and non-apoptotic cell death, in particular through the aberrant cellular release of a class of proteases, cathepsins which are usually sequestered within the lysosomal lumen.

The importance of lysosomes and lysosomal hydrolases in the clean-up phase of apoptosis (that is, the engulfment and digestion of dying and dead cells by neighbouring cells or phagocytes), and in cellular and tissue autolysis during uncontrolled necrosis is well established (de Duve, 1983; Savill and Fadok, 2000). However, it has taken a long time to recognize the role of lysosomes in the more immediate events of programmed cell death (PCD). One of the reasons for this delay is that methylketone peptide inhibitors (for example, zVAD-fluoromethylketone (fmk)) that are commonly used to assess the role of caspases in PCD also inhibit other cysteine proteases, including several lysosomal cathepsins (Schotte et al., 1999) (Foghsgaard et al., 2001). Additionally, lysosomal involvement in PCD has been overlooked because the lysosomal



ultrastructure seems intact even when lysosomal hydrolases have leaked into the cytosol (Brunk et al., 2001).

Depending on the cell type and the extent of LMP, the resulting release of lysosomal hydrolases (coloured circles in the figure — different colours represent different hydrolases) and reactive oxygen species (ROS), as well as cytosolic acidification, then leads to the release of cytochrome c. This results in classical apoptosis, caspase-independent apoptosis-like or necrosis-like programmed cell death (PCD).

### **Lysosomes in invasion and angiogenesis**

Tumour invasion and metastasis are associated with altered lysosomal trafficking and increased expression of cathepsins, especially the cysteine cathepsins — cathepsin B and cathepsin L — as well as the aspartate cathepsin, cathepsin D (Joyce and Hanahan, 2004) (Roshy et al., 2003).

In cancer cells, particularly those at the invasive edges of tumours, the localization of lysosomes often shifts from a perinuclear to a peripheral pattern and the lysosomal contents can be secreted into the extracellular space.

### **Autophagy and cell death**

Autophagy begins when a flat membrane cistern wraps around cytoplasmic organelles and/or a portion of the cytosol, forming a closed double-membrane bound vacuole — the autophagosome — containing cytoplasmic material destined for degradation. Autophagosomes mature in a stepwise process that involves fusion events with endosomal and/or lysosomal vesicles that generate amphisomes or autolysosomes.

The final degradation step only takes place within autolysosomes when lysosomal hydrolases digest the luminal content of the autophagic vacuole.

It is important to note that the mere presence of autophagosomes in cells is not proof of increased autophagy because a reduced turnover of autophagic vacuoles — for instance, owing to a decreased fusion of autophagic vacuoles with lysosomes — suffices to increase the number of autophagic vacuoles.

When the late stages of autophagic vacuole maturation, namely the fusion with lysosomes, is inhibited — for instance, by addition of lysosomotropic agents (hydroxychloroquine or monensin), by inhibition of the vacuolar proton pump (V-H<sup>+</sup>-ATPase) with bafilomycin A1 (Boya et al., 2005) or by knock-down of the lysosome-associated membrane protein gene LAMP2 (Gonzalez-Polo et al., 2005). Under such conditions, nutrient-starved cells manifest high levels of cytoplasmic vacuolization with accumulation of autophagic vacuoles, as determined by electron microscopy or by following the autophagic vacuole marker LC3 fused to green fluorescent protein (GFP) (Kabeya et al., 2000). Protein turnover is also inhibited. So, autophagic vacuole accumulation will occur when biochemical signs of autophagy are inhibited. In spite of their textbook appearance of autophagic cell death, these cells can still be rescued by re-addition of nutrients and removal of the inhibitor, provided their nucleus appears normal and their mitochondria are energized. However, following prolonged nutrient starvation and autophagolysosome inhibition, vacuolated cells manifest hallmarks of apoptosis, including MOMP and caspase activation, indicating that the point-of-no-return has been reached (Boya et al., 2005).

### **Therapeutic opportunities**

The study of lysosomal changes in tumour progression and treatment is still very young, but the recent advances and increasing interest in the field promise rapid progress in the near future. A more defined role for

each of the cathepsins by dissecting their non-redundant functions in tumour progression and cell death is awaited. Important lessons might also be learned from wound-healing models where changes in lysosomal trafficking, similar to those seen in invasive tumours, give rise to cell migration and subsequent wound closure. The therapeutic exploitation of the differences between normal cell lysosomes and cancer cell lysosomes requires a better knowledge of the molecular changes that occur during tumori-genesis. A thorough mass-spectrometric analysis of the cancer-associated changes in lysosomal composition combined with systematic functional studies (employing, for example, RNAi technology) will probably reveal new and exciting targets for cancer therapy. A key question that needs to be answered is whether there are cancer-specific subpopulations of lysosomes and whether such populations account for lysosomal secretion and/or leakage in cancer cells.

### **Targeting endocytosis and/or lysosomal secretion**

Lysosomes in advanced tumours are abnormal in their content, subcellular localization and function. This offers an exciting possibility to target them for the specific eradication of tumours. The emerging data imply that cysteine cathepsins have a dual role in tumour progression as they increase malignancy by promoting invasion when outside the cell, but counteract it through their pro-apoptotic features when released into the cytosol. Therefore, inhibiting the secretion of cathepsins could give rise to populations of lysosomes that are more prone to membrane rupture, and thereby inhibit invasion and sensitize cells to the lysosomal cell death pathways. Indeed, genetic disorders that interfere with lysosomal trafficking and secretion — for example, Chediak Higashi Syndrome in humans and beige mice, both of which are caused by mutations in *LYST* (lysosomal trafficking regulator) — produce cells with

enlarged lysosomes that are more susceptible to LMP91, (Ono et al., 2003). Interestingly, mutations in *RAB27A* (which encodes a Rab GTPase involved in lysosomal secretion that causes Griscelli Syndrome Type when mutated in humans, and is also the gene that is disrupted in ashen mice) and in *RABGGT* are associated with disorders and cellular changes that resemble Chediak–Higashi syndrome (Dell'Angelica et al., 2000). *RABGGT* encodes RAB geranylgeranyl transferase, also known as GGT II — an enzyme that is essential and specific for the prenylation and activity of Rab GTPases — and is also the gene that is disrupted in gunmetal mice. Therefore, pharmaceutical inhibitors of RABGGT or inhibition of the other components of the secretory pathway might provide the means to give the tumour cells the double hit proposed above. The therapeutic potential of RABGGT inhibitors has been indicated by a recent study demonstrating an increased expression of RABGGT in tumours of various origins, and the potent p53-independent pro-apoptotic effects of RABGGT inhibition in various cancer cell lines.

### **Targeting HSP70**

As described above, immortalization and oncogene-driven transformation initially sensitize cells to lysosomal cell death pathways, and many commonly used chemotherapeutic agents trigger LMP in cancer cells. However, it remains to be shown whether lysosomes in advanced tumours are actually more fragile than those in normal cells or whether additional changes during tumorigenesis overcome the initial destabilization of lysosomes. The membrane localization of HSP70, found in 80% of human cancer biopsies, provides tumour lysosomes with increased resistance to LMP23 (Hantschel et al., 2000). As this subcellular localization of HSP70 seems to be cancer-specific, targeting HSP70 might have selective therapeutic effects. Specific inhibition of

HSP70 can be facilitated by decoy interactors such as apoptosis inducing-factor (AIF)-derived decoy of HSP70 (ADD70) (Schmitt et al., 2003), or by reducing the cellular concentrations of HSP70 with specific antisense constructs (Nylandsted et al., 2000), RNAi (Rohde et al., 2005) or U73122 (a pharmaceutical inhibitor of phospholipase C that increases the secretion of HSP70 from the cell). The therapeutic potential of such approaches has been demonstrated by the ability of an adenovirus expressing antisense *HSPA1A* (the gene that encodes HSP70-1) to induce LMP and to attenuate the growth of xenografted human tumours in immunodeficient mice (Nylandsted et al., 2004; Nylandsted et al., 2002). Similar to many novel experimental cancer therapies, the lack of effective methods for delivering macromolecules to the cancerous tissue poses the biggest limitation for the clinical success of DNA-, RNA- and polypeptide-based anti-HSP70 therapies. However, the cancer-specific localization of HSP70 to the inner leaflet of the lysosomal membrane offers a possible opportunity to interfere with its pro-survival function by introducing HSP70 inhibitors or molecules that interfere with the membrane-binding of HSP70 by the endocytic route.

### **Targeting lysosomal pH**

The low pH of the lysosomal compartment of tumour cells could be exploited by generating acid-labile pro-drugs that would be activated in the lysosomal compartment. This effect can be achieved by the incorporation of acid-sensitive spacers between the drug and carrier that are specifically cleaved by lysosomal proteases (Ulbrich and Subr, 2004). This approach could also be used to deliver compounds with direct membrane toxicity safely into the lysosomal lumen. On the other hand, raising the lysosomal pH by pharmacological inhibition of the vacuolar proton pump increases the cytoplasmic retention of cisplatin, 5-

fluorouracil and vinblastine, and ameliorates the nuclear targeting of doxorubicin, thereby sensitizing tumour cells to these treatments (Luciani et al., 2004). The neutralization of lysosomes can also sensitize cancer cells to other treatments, possibly by inhibiting autophagy-mediated cytoprotection (Kanzawa et al., 2003) (Kanzawa et al., 2004) (Paglin et al., 2001) or by exerting direct cytotoxic effects through the induction of lysosomal dysfunction, LMP and cathepsin-mediated apoptosis-like cell death.

## **RESVERATROL**

The polyphenolic compound Resveratrol (3,5,4'-trihydroxystilbene) is a phytoalexin found in a wide variety of dietary sources including grapes, plums and peanuts.

Numerous studies have reported interesting properties of this compound as a preventive agent against several important pathologies.

Resveratrol has been shown to have anti-inflammatory, antioxidant, antitumor, neuroprotective and immunomodulatory activities (Cos et al., 2003; Inoue et al., 2003; Jang and Pezzuto, 1999). The compound has also been examined in several model system for its potential effect against cancer (Agarwal et al., 2000; Hsieh and Wu, 2000; Mitchell and El-Deiry, 1999). Its anticancer effects include its role as a chemopreventive agent, its ability to inhibit cell proliferation, its direct effect in cytotoxicity by induction of apoptosis and on its potential therapeutics effect in preclinical studies (Bhat and Pezzuto, 2002).

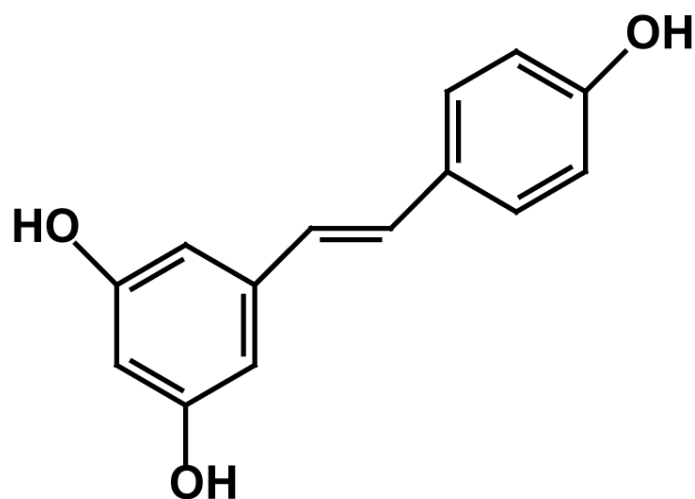


Fig 7: Resveratrol formula

Resveratrol is produced by plants in response to infection by the pathogen *Botrytis cinerea* (Delmas et al., 2006). It is also induced in response to a variety of stress conditions, such as vicissitudes in climate, exposure to ozone, sunlight and heavy metals (Bavaresco, 2003). It exists in both cis and trans isomeric forms. In plants, it mostly exists in glycosylated piceid forms (3-O-B-D-glucosides). Glycosylation is known to protect resveratrol from oxidative degradation, and glycosylated resveratrol is more stable and more soluble and readily absorbed in the human gastrointestinal tract (Regev-Shoshani et al., 2003). Although the biological positive effects of resveratrol are largely admitted, little is known about the transport and the distribution of the resveratrol through the body. Due to its low water solubility (Belguendouz et al., 1997), it must be bound to proteins and/or conjugated to remain at a high concentration in serum. Moreover, the efficiency of a therapeutic substance is related to its capacity (selectivity and affinity) to bind protein transporters (Khan et al., 2008). It is known that resveratrol is conjugated in intestine as glucoronide and sulfate derivates, but aglycone is also bioavailable.

Recently it has been shown that resveratrol uptake involves both passive diffusion and a carrier-mediated process. Moreover, an increasing lowering of resveratrol uptake was observed by addition of increasing concentration of BSA to a serum-free medium (Jannin et al., 2004).

## **MECHANISM OF ACTION**

### **Regulation of Bcl-2 family members.**

Overexpression of Bcl-2 and Bcl-X<sub>L</sub> protein protects a wide variety of cells from many death-inducing stimuli (Hickman et al., 1994; Srivastava et al., 1999).

Resveratrol inhibits the expression of antiapoptotic proteins such as Bcl-2 and Bcl-X<sub>L</sub>, and induces the expression of Bax, Bak, Bad, PUMA, Noxa and Bim (Aggarwal and Shishodia, 2006; Fulda and Debatin, 2004; Shankar and Srivastava, 2007) (Bhardwaj et al., 2007). These data suggest that the regulation of Bcl-2 family members plays a major role in resveratrol-induced apoptosis.

Further insights into the signaling network and interaction points modulated by resveratrol may provide the basis for novel drug discovery programs to exploit resveratrol for the prevention and treatment of human diseases.

### **Regulation of Cell cycle**

Resveratrol has been reported to modulate cell cycle and to induce apoptosis. Several authors have studied the effect of it on cell cycle-control. In colon cancer cells, a down-regulation of the cyclin D1/Cdk4 complex has been reported (Kotha et al., 2006), while in transplantable liver cancer H22 resveratrol decreased cyclin B1 and Cdc2 protein,



although no alteration of cyclin D1 was observed, G<sub>2</sub> arrest was itself been reported to be linked with the inhibition of Cdk7 and Cdc2. Moreover it has been shown that it is also involved in the modulation of p21<sup>/Cip/WAF1</sup> and p27<sup>/Kip1</sup> levels. However, the observed accumulation of cells in G1 could also imply the Rb or the p53 pathway.

In melanoma (Bruder et al., 2007; Ferrer et al., 2005), endothelial and fibroblastic cell line, the treatment with this compound led to an activation of p53 activity, which correlated with suppression of cell progression through the S and G2 phases of the cell cycle and apoptosis. The resveratrol effect on the G2-phase of cell cycle could be due to the action of resveratrol on the cytoskeleton (Bruder et al., 2001).

### **Regulation of Mitogen Activated Protein Kinase (MAPK)**

MAPK pathways are well understood and it is presented in mammalian signal pathways and includes p38, c-Jun N-terminal protein kinase (JNK) and extra cellular signal-regulated kinase (ERK). These MAP kinase pathways consist of several other kinases, which activate each other via phosphorylation cascades and thus activate several transcription factors (Bruder et al., 2001; Shih et al., 2002). The interaction between these MAPK pathways and resveratrol provides future beneficial therapies regarding its anticancerous property. It has been shown that resveratrol has its effects on upregulating p53 protein thereby downregulating the expression of NF-κB and AP-1 simultaneously (Huang et al., 1999).

In the last years it has found to induce apoptosis in mouse fibroblast cells with wild-type p53, but could not work out in absence of p53-protein in mouse fibroblast cells (Huang et al., 1999). Studies have shown that resveratrol-induced apoptosis by activating p53 expression (via phosphorylation) mediated through ERK and p38 pathways (She et al.,

2001; Shih et al., 2002). Similarly it downregulates the expression of AP-1 which is thought to be the key in inducing melanoma in humans.

### **Generation of Reactive Oxygen Species (ROS)**

Reactive oxygen species (ROS) include free oxygen ions, free radicals and both inorganic as well as organic peroxides. Ros are formed during natural metabolism as a byproduct and have important roles in cell signalling (Kannan and Jain, 2000).

During times of environmental stress Ros levels can dramatically increased, which can result in significant damage to cell structures due to presence of nascent molecule. These aggregates into a situation known as oxidative stress. Cells are normally able to defend themselves against ROS damage through the use of enzymes such as superoxide dismutases (SOD) and catalases. ROS have been implicated as a key factor in the activation of p53 by many chemotherapeutic drugs. Apoptosis triggered by p53 has been reported to be dependent on an increase in ROS and the release of apoptotic factors from mitochondrial damage. These studies suggest that ROS are downstream mediators in p53-dependent apoptosis in transcription-dependent or transcription-independent pathways. When cells are exposed to oxidative stress, p53 is expressed at high levels by posttranslational modifications, including phosphorylation, acetylation and glycosylation (Buccellato et al., 2004). These findings suggest the novel functions of ROS as p53 activators or p53 downstream effectors.

Moreover resveratrol has been reported to increase as well as decrease NO production (Chander and Chopra, 2006; Holian et al., 2002). The involvement of NOS has already been reported in cultured pulmonary (Holian et al., 2002), leading to inhibition of NOS activity reverted resveratrol action, indicating a direct relationship of increased NO production and inhibition of cell growth. In addition, resveratrol has been shown to modify iNOS expression (Das et al., 2005; Madar et al., 2005).

## **Regulation of PI3K/AKT pathway**

Loss of chromosome 10q is a frequently observed genetic defect in prostate cancer.

Recent work has indicated that loss of PTEN function occurs 10-20% of organ-confined and over 50% of advanced prostate cancer. Knockout mice lacking PTEN develop multiple cancers, including prostatic hyperplasia and prostatic intraepithelial neoplasia. This is supported by recent observation showing a correlation between the status of PTEN and activation of the serine/threonine kinase Akt. PTEN functions as a negative regulator of the PI3-kinase/Akt-signalling pathway.

In tumors, inactivating mutations in PTEN led to increase activity of Akt/PKB, one of the most well characterized downstream effectors of PI3K (Dahia et al., 1999). Akt/PKB exerts antiapoptotic effects through phosphorylation of substrates such as Bad (Datta et al., 1997) or caspase-9 (Cardone et al., 1997) that directly regulate the apoptotic machinery or substrates such as the human telomerase reverse transcriptase subunit. Moreover Akt plays an important role in the stimulation of cell proliferation, cell survival and it contributes to tumor growth and progression by promoting cell invasiveness and angiogenesis (Dudek et al., 1997; Kennedy et al., 1997). These data suggest that targeting a specific kinase that promotes survival such as Akt could change the apoptosis-inducing potential of TRAIL or resveratrol.

In the last years it has been shown that resveratrol induced the phosphorylation of Akt/PKB activating AMP-activated protein kinase (AMPK) and preventing cardiac myocyte hypertrophy via these two kinase system (Chan et al., 2008). Moreover the involvement of AMPK signalling cascade conditions of AMPK activated states and inactivated states. It has been shown ROS as an upstream regulator of AMPK in colon-carcinoma cell line HT29 (Hwang et al., 2007). Finally it has been

demonstrated for the first time that resveratrol inhibits Ang II-induced hypertrophy of vascular smooth muscle cells (VSMC) by interfering mainly with the PI3K/Akt and p70<sup>S6K</sup> (ribosomal protein) phosphorylation (Haider et al., 2002).

### **Regulation of Transcription Factors**

NFκB family is a group of structurally related and evolutionary conserved proteins subunits that have been identified and cloned in mammalian cells (Le Beau et al., 1992). The NFκB subunits form homo- or hetero-dimers through their rel homology domain (RHD), which is also responsible for the DNA binding of NFκB and interaction of NFκB with IκB, the family of inhibitory proteins of NFκB (Baldwin, 1996). NFκB usually exists in a latent state in the cytoplasm and its activation requires extracellular stimuli leading to the phosphorylation and subsequent proteasome-mediated degradation of inhibitory IκB proteins (Karin and Ben-Neriah, 2000). In mammalian cells, NFκB also regulates expression of more than 150 genes and some of them were linked to cancer initiation, proliferation, angiogenesis, survival and metastasis (Pahl, 1999). Mitotic cellular division is a crucial feature in the growth of normal as well as cancer cells. The cellular division can be characterized as a cycling process where cells are proceeding from the resting stage (G<sub>0</sub>) to DNA synthesis (S) and mitosis (M) stages of cell cycle. The check-points are controlled by a group of D cyclins and cyclins E and A. These check-points of the cell cycle are usually deregulated in oncogenesis and amplification or overexpression of cyclin D1 have been identified in the development of a subset of human cancers including a lot of kinds of cancer. It has been shown that the constitutive activation of NFκB is probably the major effector responsible for the overexpression of cyclin D1 in tumorigenesis .

For this reason many studies suggest that resveratrol may have a potential in the treatment and prevention of cancer, being involved in the regulation of cell cycle and in the inhibition of the nuclear transcription factor NF $\kappa$ B (Cheeke et al., 2006).

### **Regulation of TRAIL-Death Receptor Pathway**

TRAIL, belonging to the TNF cytokine family, is a type II membrane protein that induces apoptosis in a wide variety of transformed cells (Bodmer et al., 2000; Walczak et al., 1999).

Histological analysis of TRAIL-treated tumors revealed an increase in apoptotic cells and confirmed the ability of TRAIL to induce apoptosis in animal models without toxicity toward normal tissues. TRAIL is considered to be a tumor-selective, apoptosis-inducing cytokine and a promising new candidate for cancer prevention and treatment.

TRAIL binds several receptors with DR4 and DR5, the intracellular death domain, essential for the induction of apoptosis following receptor ligation (Golstein, 1997). The binding of TRAIL to DR4 and DR5 leads to the activation of caspase-8 or caspase-10 (Marsters et al., 1998), that in turn activates downstream effector caspases such as caspase-3 and caspase-7 (Muzio et al., 1998). After the activation of the caspase cascades, BID is cleaved and it triggers mitochondrial depolarization and subsequent release of mitochondrial proteins (cytochrome c, AIF, Smac/DIABLO) to the cytosol, and activation of the effector caspases after formation of apoptosome (Green and Amarante-Mendes, 1998).

Recently some TRAIL resistant cancer cell lines have been discovered but the molecular mechanisms responsible for the TRAIL resistance is still not very clear. It has been recently demonstrated that downregulation of Akt/PKB or NF $\kappa$ B sensitizes breast, prostate and lung cancer cells to TRAIL in vitro (Chen et al., 2003). These studies suggest that combined

chemotherapy or radiotherapy with TRAIL could be an effective treatment of epithelial cell-derived cancers. Study of the intracellular mechanisms that control TRAIL sensitivity may enhance the knowledge of death receptor-mediated signalling and help to develop resveratrol and/or TRAIL-based approaches to cancer prevention/treatment.

## **CLINICAL SIGNIFICANCE OF RESVERATROL**

Several studies indicate the importance of resveratrol in many diseases. It is involved in angiogenesis, in cardiovascular disease, in diabetes and it has antiviral effects and finally its ability to stop the metastatic processing in cancer.

Angiogenesis is the process or formation of new blood capillaries required to support the growth of most solid tumors. In various studies it has been observed that resveratrol inhibits tumour-induced formation of new blood capillaries. Its effects on inhibition of vascularization in the corneal micropocket assay in mice has also been found (Brakenhielm et al., 2001) (Brakenhielm E et al. 2001).

Moreover several studies suggest that resveratrol is an effective antioxidant (Chanvitayapongs et al., 1997). It inhibits lipid peroxidation of low-density lipoprotein (LDL), prevents the cytotoxicity of oxidized LDL and protects cells against lipid peroxidation. It is thought that because it contains highly hydrophilic and lipophilic properties, it can provide more effective protection than other well known antioxidants such as Vitamin C and E (Chanvitayapongs et al., 1997).

The cardioprotective effects of resveratrol may also be due, in part, to its vasorelaxation properties. This activity has been seen due to its ability to

stimulate  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels and to enhance NO signalling in the endothelium.

This compound was found to increase the potency of some antiretroviral drugs synergistically against HIV and herpes simplex viruses or can downregulate the expression of NF $\kappa$ B and suppress the activation of this transcription- and apoptosis-related protein (Aggarwal and Shishodia, 2006; Docherty et al., 2006).

In another study, the replication of severe acute respiratory syndrome (SARS) was totally inhibited by resveratrol derivatives in vitro (Li et al., 2006).

In inflammation process is mediated by prostaglandins (PGs) and their inhibition may be partially responsible for the chemopreventive and cardioprotective effects of resveratrol. It decreased the expression of cyclooxygenase-2 (COX-2), an enzyme that catalyzes PGsynthesis by inhibiting its expression via signal transduction pathways (Subbaramaiah et al., 1998). Moreover NF $\kappa$ B is involved in inflammation and tumorigenesis. Inhibition of NF $\kappa$ B activity is a possible mechanism by which resveratrol exerts its anti-inflammatory activity. Inhibition of TNF-induced NF $\kappa$ B activation by resveratrol has been observed in several cell lines (Holmes-McNary and Baldwin, 2000).

In other studies, this compound has a significant effect on plasma glucose level in normal and diabetic rats including the animal model of streptozotocin (STZ)-induced and nicotinamide-STZ-induced (NA-STZ) and insulin-resistant diabetic rats. It was observed that resveratrol produced a hypoglycaemic effect in a dose-dependent manner in normal as well as in diabetic rats, and it was also found that in both cases the insulin level was increased following resveratrol treatment. The results indicated that the mechanisms contributing to the hypoglycaemic effect of resveratrol include insulin-dependent and insulin-independent

pathway, along with PI3K-Akt-signaling pathways to enhance glucose uptake in skeletal muscle. Resveratrol has been suggested in treatment of diabetic neuropathy and its protective effect may be mediate through reduction in oxidative stress and DNA fragmentation (Sharma et al., 2006).

In addition to other properties, resveratrol has been observed to have its analgesics, protection against hearing loss and enhance lipopolysaccharide-induced anorexia in rats although it has no anorexic effect when given alone (Granados-Soto et al., 2002).



## **FIRST WORK**

In human colorectal cancer cells the polyphenol resveratrol activated the caspase-dependent intrinsic pathway of apoptosis. This effect was not mediated via estrogen receptors. Pepstatin A, an inhibitor of lysosomal cathepsin D, not E-64d, an inhibitor of cathepsins B and L, prevented resveratrol cytotoxicity. Similar protection was attained by small interference RNA-mediated knock-down of cathepsin D protein expression. Resveratrol promoted the accumulation of mature cathepsin D, induced lysosome leakage and increased cytosolic immunoreactivity of cathepsin D. Inhibition of cathepsin D or its post-transcriptional down-regulation precluded Bax oligomerization, permeabilization of mitochondrial membrane, cytosolic translocation of cytochrome c, caspase 3 activation and TUNEL positivity occurring in resveratrol-treated cells. The present study identifies the lysosome as a novel target of resveratrol activity and demonstrates a hierarchy of the proteolytic pathways involved in its cytotoxic mechanism in which the lysosomal cathepsin D acts upstream the cytosolic caspase activation. Our data indicate that metabolic or pharmacologic or genetic conditions affecting cathepsin D expression and/or activity could reflect on the sensitivity of cancer cells to resveratrol.

# **RESVERATROL INDUCES CELL DEATH IN COLORECTAL CANCER CELLS BY A NOVEL PATHWAY INVOLVING LYSOSOMAL CATHEPSIN D**

Nicol F. Trincheri, Giuseppina Nicotra, Carlo Follo, Roberta Castino and  
Ciro Isidoro<sup>1</sup>

Laboratorio di Patologia Molecolare, Università del Piemonte Orientale  
“A. Avogadro”, Dipartimento di Scienze Mediche, Novara (Italy)

## **INTRODUCTION**

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene, RV), a polyphenol enriched in red wine, grapes, peanuts and other food products, has recently attracted the interest of researchers owing to its protective effects in carcinogenesis (Bhat and Pezzuto, 2002). RV was shown to suppress cancer initiation and promotion (Jang et al., 1997), to reduce ascites tumor growth (Carbo et al., 1999) to prevent chemical carcinogen-induced epithelial cell transformation (Li et al., 2002) (Banerjee et al., 2002), and to inhibit neo-angiogenesis (Tseng et al., 2004) (Cao et al., 2004). In *in vitro* models, RV inhibited the growth of tumor cell lines derived from various human cancers (Hsieh et al., 1999) (Joe et al., 2002) (Pozo-Guisado et al., 2002) (Opipari et al., 2004). This effect has been associated with the ability of RV to arrest cell cycle progression (Hsieh and Wu, 1999) (Wolter et al., 2001), to promote cell differentiation

(Wolter and Stein, 2002) and to induce programmed cell death by caspase-independent or caspase-dependent apoptosis (Park et al., 2001) (Huang et al., 1999) (Tinhofer et al., 2001) or by autophagocytosis (Opipari et al., 2004). These properties make RV an attractive chemotherapy and chemopreventive drug for cancer treatment (Aggarwal et al., 2004). In this respect, it is interesting to note that RV exhibits estrogenic and anti-estrogenic activities (Bowers et al., 2000) (Bhat and Pezzuto, 2001), a property that might influence the dynamic of estrogen receptor (ER)-positive cancers, such as breast (Lu and Serrero, 1999) (Levenson et al., 2003) and colorectal (Schneider et al., 2000) (Qiu et al., 2002) (Chen and Donovan, 2004) cancers. To fully exploit its potential as anticarcinogenic drug it is mandatory to elucidate the cytotoxic pathways activated by and the molecules and organelles targeted by RV in cancer cells.

Two principal pathways of apoptosis have been described: in the ‘intrinsic’ pathway the cytotoxic stress affects primarily the mitochondrion inducing the release of molecules that promote the activation of caspases, while in the ‘extrinsic’ pathway the activation of caspases follows the stimulation of so-called ‘death receptors’ on the plasma membrane (Danial and Korsmeyer, 2004). It is now clear that programmed cell death pathways independent of caspases also exist (Broker et al., 2005). In various circumstances the initial trigger of the

death machinery was shown to be a protease normally resident within the endosomal-lysosomal compartment. Lysosomal cathepsins B and D (CB, CD) have been shown to mediate apoptotic cell death induced by TNF $\alpha$  (Deiss et al., 1996) (Guicciardi et al., 2000) (Foghsgaard et al., 2001) (Demoz et al., 2002) and cytotoxic drugs (Broker et al., 2004) (Bidere et al., 2003) (Emert-Sedlak et al., 2005). The type of lysosomal protease recruited in the death pathway seems to vary depending on the cell model and the trigger, and likely on the availability of specific substrates and other unknown metabolic factors. The aim of the present work was to examine the possible involvement of lysosomal cathepsins in the cytotoxic mechanism of RV in human colorectal cancer cells. Our data demonstrate that RV activates a lysosome-dependent cytotoxic pathway ending in caspase-dependent cell death. We provide evidences that CD, not CB or cathepsin L (CL), mediates RV cytotoxicity. The present data indicate the lysosome as a novel and primary target organelle of the cytotoxic activity of RV.

## **MATERIALS AND METHODS**

Unless otherwise specified all reagents were from Sigma-Aldrich Corp., St. Luis, MO, USA.

## **Cell cultures, treatments and evaluation of cytotoxicity**

Human colorectal cancer cell lines (DLD1 and HT29) were cultivated in standard culture conditions (37°C; 95% air: 5% CO<sub>2</sub>) in Dulbecco's modified Minimal Essential Medium supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA), 2 mM L-glutamine and 1% penicillin-streptomycin solution. Cells were seeded and let adhere on sterile plastic dishes for 24 h prior to start any treatment. Treatments included 1 to 100 µM RV, 100 µM Pepstatin A (Pst), 10 µM E-64d (Bachem AG, Bubendorf, Switzerland) and 30 µM ZVAD-fmk (Alexis Laboratories, San Diego USA). Inhibitors were added to the culture medium 12 h (Pst) or 1 h (E-64d, ZVAD-fmk) before the incubation with RV. In some experiments, the estrogen receptor antagonist fulvestrant (ICI 182,780 from Tocris, Bristol, UK) also was used at 1 µM concentration. Culture medium was changed and substances were re-added daily. At designated time-points, adherent and suspended cells were collected, diluted in a solution containing trypan blue and counted. Cell death was also assessed by cytofluorometer analysis. To this end, 10<sup>5</sup> cells were washed in PBS and incubated for 15 min at room temperature with 2 µl annexin V-FITC (fluorescein isothiocyanate, Alexis Laboratories, S. Diego, USA), 5 µl propidium iodide (PI, stock solution 50 µg/ml) and 98 µl of buffer (10mM HEPES/NaOH pH 7.4,

140mM NaCl, 2.5mM CaCl<sub>2</sub>). Alternatively, 10<sup>6</sup> cells were washed twice with cold PBS and fixed in ice-cold 70% ethanol for 1h at 4°C. Cells were then washed twice with PBS and incubated with RNase A (0.4mg/ml) for 30 min at 37°C and with PI (0.1 mg/ml) for 15 min in the dark, at room temperature. Cells with hypodiploid content of DNA (subG1 peak) were assumed as apoptotic. Cells (at least 10,000 per sample) were analyzed in a FacScan flow cytometer (Becton Dickinson, Mountain View, Ca, USA) equipped with a 488 nm argon laser. Data were interpreted with the winMDI software.

### **siRNA transfection**

Post-transcriptional silencing of CD expression was achieved by the small interference RNA (siRNA) technology. Duplexes of 21-nucleotide siRNA including two 3'-overhanging TT were synthesized by MWG Biotech AG (Washington, DC). The sense strand of siRNA was GAACAUCUUCUCCUUCUAC, corresponding to the positions 724-742 relative to the start codon of the CD mRNA (28). An inefficient CD9 oligonucleotide corresponding to the AGGUAGUGUAAUCGCCUUG sequence was used as a negative control of transfection (referred to as control-duplex). Transfection was performed with Lipofectamine 2000 (Invitrogen Corp.). Afterward, the transfection mixture was removed and cells were incubated for further 24 h in fresh medium prior to any treatment.

### **Protein expression analysis**

Cells were homogenized in buffer containing detergents and protease inhibitors. 30 µg of cell proteins were denatured with Laemmli sample buffer, separated by electrophoresis on a 12.5% polyacrylamide gel and then electroblotted onto nitrocellulose membrane (Biorad, Hercules, CA, USA). The filter was probed in two rounds with specific monoclonal antibodies against CD (EMD Biosciences, Calbiochem, San Diego, CA, USA) or Actin following standard Western blotting procedure (Faust et al., 1985). Immunocomplexes were revealed by incubation with peroxidase-conjugated goat-anti-mouse antibody and subsequent peroxidase-induced chemiluminescence reaction (Biorad). Intensity of the bands was estimated by densitometry analysis (Quantity one software).

### **Assessment of caspases activity**

Caspase-3 activity was assayed with the fluorogenic substrate DEVD-AFC following manufacturer's protocol (MBL, Naka-Ku, Nagoya, Japan). Total caspases activity was measured with the Caspases Detection kit (Merck Biosciences Ltd, Nottingham, UK) using FITC-VAD-fmk (Val-Ala-Asp-fluoromethylketone) as a substrate following the manufacturer's protocol. Stained cells (at least 10,000 per sample) were analyzed by flow cytometry and data were interpreted with the winMDI software.



### **Immunofluorescence studies**

Cells grown and treated on coverslips were fixed with methanol for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 15 min. The following primary antibodies were used: a monoclonal (EMD Biosciences) or a rabbit polyclonal anti-human CD (Demoz et al., 2006) (36); a rabbit polyclonal anti-Bax (Cell Signaling Technology, Danver, MA, USA); a mix of mouse monoclonal antibodies against alfa- and beta-tubulin; and a monoclonal anti-cytochrome c (Alexis). FITC- or TRITC (tetramethylrhodamine isothiocyanate)-conjugated secondary antibodies against mouse or rabbit IgG were used as appropriate. As negative control, the primary antibody was omitted or substituted with pre-immune antiserum.

### **TUNEL and DAPI staining**

Apoptotic cells were revealed by *in situ* Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay performed with the “In situ Cell Death Detection” fluorescent Kit (Roche Diagnostics Corporation Indianapolis, IN, USA) following manufacturer’s instructions. Apoptosis-associated chromatin alterations were detected by staining the cells with the DNA-labeling fluorescent dye 4-6-diamidino 2-phenylindol-dihydrochloride (DAPI, 1:100 in PBS/0,1% Triton X-100/4% fetal bovine serum).

### **Lysosomes and mitochondria integrity assessment**

Lysosomal membrane integrity was assessed by the Acridine Orange (AO) retention test. AO is an acidotropic fluorochrome that emits red fluorescence when accumulates in its protonated form within lysosomes, while it emits a green fluorescence when localizes in the cytosol and in the nucleus. Cells adherent on coverslips were loaded with AO (1:200 from 0,5 mg/ml in distilled water for 10 min at 37°C) and immediately observed and photographed under the confocal fluorescence microscope. Lysosome leakage was quantified by cytofluorometer analysis of cells labeled with AO in suspension. The integrity of mitochondrial membrane was tested by using either the fluorescent dye Mitotracker (Invitrogen Corp.) or Rhodamine-123. Briefly, cells on cover-slips were incubated with 0.2 µl of mitotracker solution for 15 min at 37°C, then fixed in 3.7% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 15 min for further fluorescence staining. Alternatively, cells on coverslips were incubated with Rhodamine 123 (50 nM in culture medium; 10 min) and immediately observed under the fluorescence microscope. Loss of mitochondrial membrane integrity was also assessed by cytofluorometer analysis of rhodamine-labeled cells.

### **Fluorescence microscope imaging**

Coverslips were mounted in mowiol (1% in PBS). Images were captured with a Zeiss fluorescence microscope equipped with a digital camera or

with Leica DMIRE2 confocal fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with Leica Confocal Software v. 2.61. Three coverslips were prepared for each experimental condition. Representative images, selected by two independent investigators, are shown.

### **Statistical analysis**

All experiments were independently replicated at least three times. Data are presented as means  $\pm$  SD. The InStat-3 Statistical software (Graphpad Software Inc, San Diego, CA, USA) was used. Significance was calculated by the test of Mann-Whitney.

## **RESULTS**

### **Resveratrol cytotoxicity in colorectal cancer cells is concentration- and time-dependent, is not mediated via estrogen receptors and is prevented by caspase inhibition**

The present study was conducted in DLD1 and HT29 cells, two cell lines derived from human coloncarcinomas that express ER $\beta$ , not ER $\alpha$ , isoforms (Qiu et al., 2002) (Fiorelli et al., 1999) (Campbell-Thompson et al., 2001). RV has been shown to bind to both  $\alpha$  and  $\beta$  isoforms of ER (Bowers et al., 2000) (Bhat and Pezzuto, 2001) and to exert estrogenic activity when used at very low doses (i.e., 1 to 10  $\mu$ M) (Levenson et al., 2003). We tested the concentration- and time-dependency of inhibitory or

stimulating effects of RV on the growth of human colorectal cancer cells. To assess whether such effects were mediated through ER, cells were treated with concentrations of RV ranging from 1 to 100  $\mu\text{M}$  in the absence or the presence of 1  $\mu\text{M}$  fulvestrant (ICI 182,780), which has been shown to inhibit the nuclear translocation of ERs and to promote their cytoplasmic degradation at a concentration of 100 nM (Dauvois et al., 1992). A treatment for 24 to 48 h with 1  $\mu\text{M}$  or 10  $\mu\text{M}$  RV did not affect cell vitality or final cell density in DLD1 and HT29 cultures (Fig. 1A and not shown). At 24 h treatment with 100  $\mu\text{M}$  RV some 35% reduction of cell culture growth was observed (not shown and Fig. 3C), while cell death, as assessed by cell counting and cytofluorometer evaluation of annexin V positivity, was apparent only at 48 h (Fig. 1A and not shown). Based on the results obtained with low doses of RV and on the lack of effects of ICI 182,780, we conclude that RV cytotoxic effects were not mediated via ER (Fig. 1A). In the following experiments RV was used at 100  $\mu\text{M}$ . With this concentration, on average, annexin V-positive apoptotic cells in DLD1 cultures amounted to about 50% and 75% after 48 h and 96 h of treatment, respectively (Fig. 1B). In parallel cultures in which the potent and broad-range caspase inhibitor ZVAD-fmk was added daily along with RV cell death was practically absent (Fig. 1B). HT29 cells showed more sensitivity to RV than DLD1, the apoptotic cells amounting to 50-70% and 90% at 48 h and 72 h of

treatment, respectively. Also in the case of HT29 cells ZVAD-fmk effectively prevented RV-induced cell death starting at 48 h (Fig. 1B). It is to note that ZVAD-fmk, while inhibiting occurrence of cell death at 48 h, could not prevent cell growth inhibition induced by RV in the first 24 h of treatment both in DLD1 and in HT29 cultures (not shown).

### **Resveratrol cytotoxicity is associated with mitochondrial permeabilization and caspases activation**

To better assess the significance of ZVAD-fmk protective effects, we monitored the time-dependent activation of caspases during RV treatment in DLD1 cells. The cleavage of a fluorogenic peptide substrate of different caspases remained at basal levels in the first 24 h of RV treatment and increased by a factor of approximately 2.2 by 48 h (Fig. 2A). By this time the percentage of cells in which caspase activation occurred roughly corresponded to that of cells positive for annexin V, a biomarker of apoptotic-like programmed cell death (Fig. 2B). In apoptotic cells (identified on the basis of chromatin alteration) mitochondrial integrity was lost, indicating that RV activated the intrinsic pathway of cell death (Fig. 2C). The time-dependency of mitochondrial permeabilization was assessed by cytofluorometry in DLD1 and HT29 cells treated with RV for up to 48 h and labeled with rhodamine. No changes in the cytofluorometry profiles were observed during the first 24 h of treatment. Mitochondrial permeabilization, as shown by loss of

rhodamine retention, was observed starting at 36 h of treatment (Fig. 2D). The microscope images of these cells shown in Fig. 2E well reflect the phenomenon as quantified by cytofluorometry. It is to note that mitochondrial permeabilization occurred more rapidly and more extensively in HT29 than in DLD1 cells, accordingly with the highest sensitivity of these cells toward RV.

### **Cathepsin D is an essential mediator of RV cytotoxicity in colorectal cancer cells**

CB, CD and CL, the most abundant lysosomal proteases, have recently been involved in the activation of the intrinsic pathway of programmed cell death (Bidere et al., 2003) (Emert-Sedlak et al., 2005) (Stoka et al., 2005) (Guicciardi et al., 2004), and therefore were considered possible mediators of RV cytotoxicity. To determine their involvement we employed the inhibitors E64d, which is specific for the cysteine-type proteases CB and CL, and Pst, which is specific for the aspartic-type protease CD. In preliminary experiments in which cathepsins activity was assayed with specific fluorogenic substrates 10  $\mu$ M E64d and 100  $\mu$ M Pst were shown to effectively inhibit (by approximately 85%) the activity of CB and CL and of CD, respectively, in the cells. DLD1 cells exposed to RV for up to 48 h in the absence or the presence of Pst or E64d were tested for annexin V positivity and PI labeling. Cytofluorometer analysis of these samples indicated that Pst, not E64d, protected DLD1 cells from

RV cytotoxicity (Fig. 3A). We then checked whether the CD-dependent pathway of RV cytotoxicity was also operative in the other human colorectal cancer cell line. HT29 cells were treated for up to 48 h with RV in the absence or the presence of Pst or E64d and cytotoxicity was assessed by cytofluorometer estimation of the hypodiploid (not shown) and annexin V-positive cell population (Fig. 3B). In the first 24 h of RV treatment cell death was negligible (not shown), but by 48 h the treatment was toxic for >60 % of the culture. At this time, inhibition of CB and CL by E64d revealed itself toxic and did not protect from RV; by contrast, Pst protected a large fraction of the cell population from RV cytotoxicity. In parallel samples cell survival was assessed by counting viable (trypan blue excluding) adherent cells. Data shown in Fig. 3C indicate that: i) RV reduced the rate of cell proliferation in the first 24 h. This effect could not be prevented by Pst or E64d; ii) at 48 h RV induced a dramatic cell loss in the monolayer. This effect was largely prevented by Pst, not by E64d; iii) Pst and E64d were not toxic on itself in DLD1 cells, while E64d only revealed toxic to HT29 cells after 48 h incubation. It is to note that Pst, while unable to prevent the growth inhibitory effect of RV in the first 24 h, showed very effective in preventing RV-induced cell death in the following 24 h both in DLD1 and HT29 cells. In this respect, Pst behaved much alike ZVAD-fmk. Not only Pst prevented RV-induced cell death, but it also allowed to rescue cell proliferation. Pst inhibits not only

CD, but also cathepsin E, another aspartic protease resident in endosomes and lysosomes. To definitely prove the active role of CD in the death pathway activated by RV we specifically down-regulated the expression of this protease by transient transfection with a siRNA 21-mer duplex. As controls, parallel cultures were not transfected or transfected with an inefficient duplex oligonucleotide not targeting CD mRNA (sham transfected). Proper conditions were assessed to achieve optimal down-regulation of CD expression, which was monitored by assaying the proteolytic activity at acid pH on fluorogenic substrate (not shown) and by immunoblotting determination of CD protein level (Fig. 3D, upper panel). siRNA transfection successfully down-regulated CD expression in not treated as well as in RV-treated cells. By densitometry, in siRNA-transfected cells CD protein level, normalized against actin protein level, was down-regulated by >95% (average of three independent experiments). A parallel set of cultures was used to estimate cell vitality. Transfection in itself was not harmful, as cell viability in control duplex-transfected cultures was not dissimilar from that reported in untransfected cultures (Fig. 3D, lower panel). After exposure to RV, viable cells recovered from untransfected or sham-transfected cultures amounted to approximately 50% and those recovered from CD-siRNA-transfected culture amounted to almost 90% of those initially present in the control culture, indicating that no cell loss occurred when CD expression was



down-regulated (Fig. 3C, lower panel). Cytofluorometry analysis of annexin V-labeled cells confirmed the protection by CD-siRNA against RV cytotoxicity (not shown). The fact that Pst and siRNA elicited the same protective effect rules out the involvement of any aspartic protease other than CD and permits to exclude any role of CD polypeptides other than the enzymatically-active one in RV cytotoxicity.

### **Cathepsin D mediates Resveratrol-induced caspase 3 activation, Bax oligomerization on mitochondria and cytosolic release of cytochrome c**

Data so far obtained demonstrate that both CD-mediated and caspase-mediated pathways are involved in the cytotoxic mechanism of RV. Whether the two proteolytic death pathways are activated independently or are recruited in series remains to be determined. Caspase 3 is a key effector protease of the apoptotic machinery. To address the link between the cathepsin-dependent and caspase-dependent proteolytic pathways we determined the level of caspase 3 activity in cells that had been incubated or not with RV in the presence or the absence of Pst for 48 h. As shown in Fig. 4A inhibition of CD largely precluded the activation of caspase 3 in RV-treated cells. To definitely prove the involvement of CD in caspase 3 activation we further checked for the presence of TUNEL-positive cells in cultures exposed to RV along with Pst. The TUNEL technique evidences the presence of nicked DNA in apoptotic cells, which results

from the caspase 3-mediated cleavage of poly(ADP-ribose) polymerase, an enzyme involved in DNA repair. As shown in Fig. 4B, Pst prevented the occurrence of TUNEL-positive cells in cultures treated with RV for 48 h. These data confirm that in RV-treated cells activation of the CD-mediated proteolytic pathway precedes that of the caspase cascade. One of the main pathways leading to chemotherapy-induced activation of the caspase cascade relies on the permeabilization of mitochondria outer membrane and cytosolic translocation of cytochrome c. Bax, a protein belonging to the Bcl-2 family, has been involved in mitochondrial permeabilization and cytosolic relocation of cytochrome c and other pro-apoptotic proteins (Broker et al., 2004). In colon cancer cells RV was shown to up-regulate Bax expression, to promote the exposure of its occluded N-terminus and to trigger its translocation into mitochondria (Dauvois et al., 1992) (Stoka et al., 2005). CD has been shown capable to induce conformational changes and oligomerization on mitochondrial membrane of Bax in apoptotic lymphocytes (Bidere et al., 2003). Therefore, it seemed of obvious interest to look at Bax localization in RV-treated colon cancer cells. Both in DLD1 and HT29 cells, a 48 h exposure to RV resulted in increased immunoreactivity and changes in cellular distribution of Bax (Fig. 4C and not shown). To clearly assess the involvement of CD in Bax-mediated permeabilization of mitochondria induced by RV we performed a fluorescent double-staining for

mitotracker and Bax in cells pre-treated with Pst and in cells in which CD expression had been silenced by siRNA technology. In most cells treated with RV only (Fig. 4C, upper panels) mitotracker staining is weak and diffuse, while Bax immunoreactivity is increased and appears as spots that in some cases co-localizes with the mitotracker stain. This pattern is compatible with oligomerization of Bax on mitochondrial membrane. By contrast, in cells pre-treated with Pst (middle panels) and in CD-siRNA transfected cells (lower panels) mitochondria appear well preserved (as shown by mitotracker staining) and Bax immunostaining is diffuse in the cytoplasm, whether or not treated with RV for 48 h. We finally investigated whether RV-induced mitochondrial permeabilization was followed by cytosolic translocation of cytochrome c. The fluorescence staining of cytochrome c appears confined in mitochondria in control cells, while it is diffused throughout the entire cytoplasm after 48 h treatment with RV (Fig. 4D). The cytosolic relocation of cytochrome c is not observed in cultures treated with RV in the presence of Pst (Fig. 4D).

### **RV induces the cellular accumulation and the cytosolic release of mature CD in colorectal cancer cells**

How to explain the mitochondrial and cytosolic events depending on the activity of a lysosome resident protease ? We reasoned that RV, like other chemotherapy drugs, could alter the permeability of lysosomal membrane without inducing the indiscriminate release of necrogenic hydrolases. We

thus tested the lysosome integrity by looking at lysosomal retention of acridine orange (AO), an acidophilic fluorochrome that upon protonation is retained within lysosomes and becomes intensively red fluorescent. DLD1 and HT29 cells on coverslip were exposed to RV and observed under the fluorescence microscope at intervals of 6-8 h after AO staining. In control cells (time 0) AO fluorescence appeared as intense red spots, indicating that it was confined within acid organelles (Fig. 5A). No changes were observed in cells treated with RV for a period of time up to 36 h, beside an increase in size and number of red fluorescent organelles. At this time-point of RV treatment, however, some cells showed a diffuse cytosolic staining of AO fluorescence, which appeared yellow-colored and less intense (Fig. 5A). The proportion of cells showing such staining features was increased at 48 h of RV treatment (Fig. 5A). We quantified this phenomenon by cytofluorometry. Data shown in Fig. 5B confirm the occurrence of lysosome leakage in cells treated with RV for 36 h or longer. We then ascertained that CD indeed re-located into the cytosol under treatment with RV. Immunofluorescence studies showed that this was in fact the case in injured DLD1 and HT29 cells exposed for 48 h to RV (Fig. 5C). Finally, considering the involvement of CD in RV cytotoxicity, we wondered if RV could affect its expression and maturation. DLD1 and HT29 cells were thus exposed for up to 72 h to RV and the expression of CD polypeptides in cell homogenate was

analyzed at designated time-points by immunoblotting. As shown in Fig. 5D, RV induced a time-dependent accumulation of the mature double-chain (31 + 13 kDa) form of CD in both the colorectal cancer cell lines. Cell-associated mature CD greatly accumulated between 24 and 48 h of RV treatment: by this time CD increased of about 2.5-fold in DLD1 and of about 3.5-fold in HT29 cells. In separate experiments we also checked the expression of CD in DLD1 cells exposed for up to 48 h to 1  $\mu$ M and 10  $\mu$ M RV, doses that have been shown to induce CD synthesis in ER-positive breast cancer cells (Vyas et al., 2006). However, no changes in CD expression level were observed under these conditions (not shown). It is interesting to note that in our study cell death by (100  $\mu$ M) RV became evident in cultures after 48 h of treatment and that HT29 showed more sensitive to RV cytotoxicity than DLD1 cells. The data suggest a time-dependent correlation between the induction of CD accumulation and the toxic effect by RV.

## **DISCUSSION**

The naturally occurring polyphenol RV possesses potential chemopreventive and chemotherapy activities in colorectal cancer (Bodmer et al., 2000) (Tessitore et al., 2000) (Mahyar-Roemer et al., 2002). Its mechanism of cytotoxicity is not fully understood, as yet. RV has been categorized as a phytoestrogen, because of its ability to act as an

agonist or antagonist of ERs (Bowers et al., 2000) (Bhat and Pezzuto, 2001). Thus, its effects could be mediated via ER, as it has been shown to occur for genistein, another alimentary phytoestrogen (Chen and Donovan, 2004). Here we show that in human colorectal cancer DLD1 and HT29 cells, which are known to express ER $\beta$  isoforms, the death pathway activated by RV is not mediated through ERs, rather it involves primarily the lysosome. The findings of the present study lead to the proposed mechanism of RV toxicity reported in Fig. 6. Based on the effects of E64d, any involvement of lysosomal CB and CL in RV cytotoxic activity was excluded. By contrast inhibition of CD by Pst or post-transcriptional silencing of CD by specific siRNA completely suppressed RV-induced cell death. In the first 24 h of treatment RV slowed down the growth of cell cultures, and this effect could not be reverted by inhibitors of caspases or cathepsins. Consistently, during this period caspases activity and the cellular content of CD did not change. RV-induced cell death became apparent at 48 h of treatment, and it was paralleled by induction of caspases activity and cellular accumulation of CD. These observations indicated that, while not playing an active role in the growth inhibitory effect, both caspases and CD were involved in the death effect of RV. Since Pst prevented caspase activation in RV-treated cells, we argued that CD was the lethal trigger and therefore we investigated on its downstream targets. Cytotoxicity by chemotherapy

drugs is associated with the intrinsic apoptotic pathway (Wei et al., 2001) (Castino et al., 2003) (Hu and Kavanagh, 2003). This death pathway is initiated by loss of mitochondrial transmembrane potential followed by cytosolic release of pro-apoptotic molecules such as cytochrome c, SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI) and apoptosis-inducing factor, which eventually results in activation of the caspase proteolytic cascade. CD was shown to play an active role in such mitochondrial events during cell death induced by staurosporine and chemotherapy drugs (Bidere et al., 2003) (Emert-Sedlak et al., 2005). The question still unanswered concerns the molecular targets of CD proteolysis that link this lysosomal protease with the mitochondrial release of pro-apoptotic mediators and subsequent activation of cytosolic caspase. Two candidates have been proposed for such a role: Bax (Bidere et al., 2003) and Bid (Heinrich et al., 2004). Several experimental evidences favor Bax as the likely target of CD in RV cytotoxicity. In an animal model of colon carcinogenesis RV chemopreventive effect was associated with Bax hyper-expression (Tessitore et al., 2000) and in colorectal cancer cell lines RV-induced activation of the apoptotic intrinsic pathway relied on Bax conformational activation (Mahyar-Roemer et al., 2002). Moreover, Bax gene appears frequently mutated in human colorectal cancers, and it has been suggested that this may affect their responsiveness to

chemotherapy drugs (Zhang et al., 2000). In the present study RV caused the oligomerization and relocation onto mitochondrial membranes of Bax, the permeabilization of mitochondria and the consequent release of cytochrome c; all these events were associated with the permeabilization of lysosomes and the cytosolic relocation of CD and could be prevented by Pst or siRNA-mediated down-regulation of CD. In conclusion we have identified a novel pathway of RV cytotoxic mechanism in which the lysosomes act as death signal integrators. In colorectal cancer cells RV cytotoxicity was associated with up-regulation of CD expression and, conversely, siRNA-mediated down-regulation of CD expression abolished the cytotoxic effect of RV, indicating that this protease was the master trigger of RV lethal activity (Fig. 6). The present findings might therefore be of relevance when assessing the responsiveness or the resistance of tumors to RV-based therapy. In this respect, we notice that RV is fast and extremely metabolized in the body, so that predicted serum concentrations achieved when RV is injected for therapeutic purposes (at 100 mg per kg body weight) are much lower (probably 10 times) than those utilized in the 'in vitro' experiments here described (Baur and Sinclair, 2006). Yet, in intestinal mucosa a 30-fold enrichment of RV over serum concentration has been reported (Sale et al., 2004). These observations emphasize the need to develop RV analogues with improved bioavailability, as well as to engineer drug delivery systems that allow the



efficient accumulation of RV within the target diseased tissue.

**Acknowledgments:** The authors wish to thank Prof. BF Sloane (Detroit) and Prof. G Pisani (Novara) for helpful discussion and support, and D. Longhi for artwork. Researches supported by grants from Università del Piemonte Orientale, Regione Piemonte (ricerca Sanitaria Finalizzata), Fondazione Cassa di Risparmio di Torino and Lega per la Lotta contro i Tumori (sez. Novara).

## **FIGURE LEGENDS**

**Figure 1. RV-induced cell death in colorectal cancer cells is not mediated by estrogen receptors and is prevented by the pan-caspases inhibitor ZVAD-fmk.**

A) DLD1 cells exposed to increasing concentrations of RV for 48 h in the absence or the presence of the ER disruptor ICI 182,780. Substances were added at time zero and re-added in fresh medium at 24 h. Cytofluorograms of annexin V-labeled cells and histograms of adherent viable cell counts are shown (upper and lower panels, respectively). Cell counting data show that initial cell density (at day 0) is almost halved after a 48 h exposure to 100  $\mu$ M RV. This effect was not prevented by ICI 182,780. No effects on cell growth were elicited by 1 and 10  $\mu$ M RV.

B) DLD1 and HT29 cells were incubated with 100  $\mu$ M RV in the absence

or the presence of 30  $\mu$ M ZVAD-fmk for up to 96 h. Substances were added at time zero and re-added in fresh medium every 24 h. Typical cytofluorograms of cells double-labeled with annexin V-FITC and PI. In the cytogram apoptotic cells (positive for annexin V) are distributed in the lower and upper right panels (values in percent are given). ZVAD-fmk prevented cell death by RV in both DLD1 and HT29 cell cultures.

**Figure 2. Time-dependent permeabilization of mitochondria and activation of caspases by RV.**

A) Cleavage of fluorogenic peptide substrate of different caspases at 0 to 48 h of RV treatment in DLD1 cells. B) Caspases fluorescence assay (upper panels) and annexinV-FITC staining (lower panels) in DLD1 cells treated or not with RV for 48 h. Representative cytofluorograms are shown. C) Control (Co) and 48 h RV-treated DLD1 cells double-stained for chromatin with DAPI (blue fluorescence) and for mitochondria with mitotracker (red fluorescence). D) cytofluorograms of DLD1 and HT29 cells incubated for up to 48 h with 100  $\mu$ M RV and labeled with Rhodamine-123. Loss of mitochondrial retention of the fluorescent dye was apparent at 36 h of treatment. E) Images at the fluorescence confocal microscope of cells treated for 48 h with RV and labeled with Rhodamine.

**Figure 3. CD mediates RV cytotoxicity in colorectal cancer cells. A)**

Cells were incubated with RV in the absence or the presence of Pst or

E64d for 48 h. Medium was changed and substances re-added every 24 h. At the end of treatments cells were double-labeled with annexin V-FITC and PI and analyzed by cytofluorometry. Typical cytofluorograms are presented. Apoptotic cells (in lower and upper right panels of the cytofluorogram) amounted to approximately 40-48 % in RV- and RV plus E64d-treated cultures, and to approximately 10% in RV plus Pst-treated cultures. B) HT29 cells were incubated with 100  $\mu$ M RV in the absence or the presence of 10  $\mu$ M E64d or 100  $\mu$ M Pst for 48 h, then adherent and suspended cells were labeled with annexin V-FITC and analyzed by cytofluorometry. Medium was changed and substances re-added every 24 h. Representative cytofluorograms (of four experiments) are shown. At 48 h >60 % of the cell population in RV-treated culture shows apoptotic features. RV-induced apoptosis was largely prevented by Pst, not by E64d. The latter inhibitor revealed itself cytotoxic to HT29 cells. C) Histograms of cell counting in DLD1 and HT29 cultures treated or not for 24 h and 48 h with RV in the absence or the presence of Pst or E64d. Medium was changed and substances re-added every 24 h. Control un-treated cells roughly doubled every 24 h. RV slowed down the rate of cell proliferation in the first 24 h. Compared to the initial cell density (day 0), final cell density (48 h) in RV-treated cultures was nearly halved. Cell loss from the monolayer did not occur when RV treatment was performed in the presence of Pst. Not only, in Pst pre-treated cultures

cells also recovered from growth arrest despite the presence of RV. D) Silencing of CD by siRNA prevents RV cytotoxicity. Cells were plated, un-transfected (UT) or transfected with CD-siRNA or with control-duplex oligonucleotides as indicated and incubated or not with RV for 48 h. In the upper panel a representative (of three) western blotting of CD is shown (symbols: P, precursor; I, intermediate; LM, large chain of mature form). The filter was stripped and re-probed for actin to prove equal loading of protein homogenates; in the lower panel, data on adherent viable cells in cultures exposed to RV for 48 h are given as percentage of cell number in the control culture prior to the incubation (assumed as 100 %).

**Figure 4. Cathepsin D triggers caspase 3 activation, Bax oligomerization on mitochondria and cytosolic release of cytochrome c induced by Resveratrol.**

A) Histogram of caspase-3 activity in DLD1 cells exposed for 48 h to RV with or without Pst. B) TUNEL staining of DLD1 cells plated on coverslips and incubated for 48 h with RV in the absence or the presence of Pst (representative images are shown). C) Control, Pst-pretreated and CD-siRNA transfected DLD1 cells plated on coverslips were exposed to RV for 48 h. Cells were then double-stained for mitotracker and Bax and photographed under the fluorescence confocal microscope. Compared to un-treated cells, RV-treated cells show a weak and diffuse staining of

mitotracker. In these cells Bax immunoreactivity is increased and appears as spots that in some cases co-localizes with the mitotracker stain. This pattern, indicative of Bax oligomerization on mitochondrial membrane, is not observed in cells pre-treated with Pst (middle panels) and in CD-siRNA transfected cells (lower panels). In the latter cases mitochondria appear well preserved and Bax immunostaining is diffuse in the cytoplasm also in the presence of RV. Cells transfected with CD-unrelated siRNA duplexes behaved as control un-transfected cells. D) DLD1 cells on coverslips incubated for 48 h with RV in the absence or the presence of Pst and labeled for immunofluorescence detection of cytochrome c. The experiments demonstrate that Pst prevents mitochondrial permeabilization and cytosolic release of cytochrome c induced by RV. Images in C and D are representative of four independent experiments.

**Figure 5. Resveratrol cytotoxicity in colorectal cancer cells is preceded by lysosome leakage and up-regulation of the expression of and by cytosolic relocation of CD.**

A) DLD1 and HT29 cells plated on coverslips were incubated for increasing time with RV and then stained with the acidotropic AO fluorochrome and immediately observed under the fluorescence microscope (representative images are shown). Intense red fluorescent spots are indicative of intralysosomal retention of AO. At 36 and 48 h of

RV treatment a large proportion of cells show a weak yellow-green fluorescence indicative of cytosolic diffusion of the fluorochrome. B) Cells treated and labeled with AO as above and analyzed by cytofluorometry. C) DLD1 and HT29 cells plated on coverslips were incubated for 48 h with RV and double-labeled for immunofluorescence detection of CD (red fluorescence) and of tubulin (green fluorescence). D) Western blotting analysis of mature CD (LM, large chain of the double-chain) in a time-course treatment with RV of DLD1 and HT29 cells and relative densitometry (representative of three experiments). Filters were stripped and re-probed for actin to quantify protein loading in the lanes.

**Figure 6. Interpretative scheme of the results.** RV induces the up-regulation of CD expression and the cytosolic relocation of mature active CD from lysosomes. CD was shown essential to induce mitochondria permeabilization (associated with Bax oligomerization onto mitochondrial membrane), cytochrome c release, caspase 3 activation and appearance of annexin V and TUNEL positive cell death.

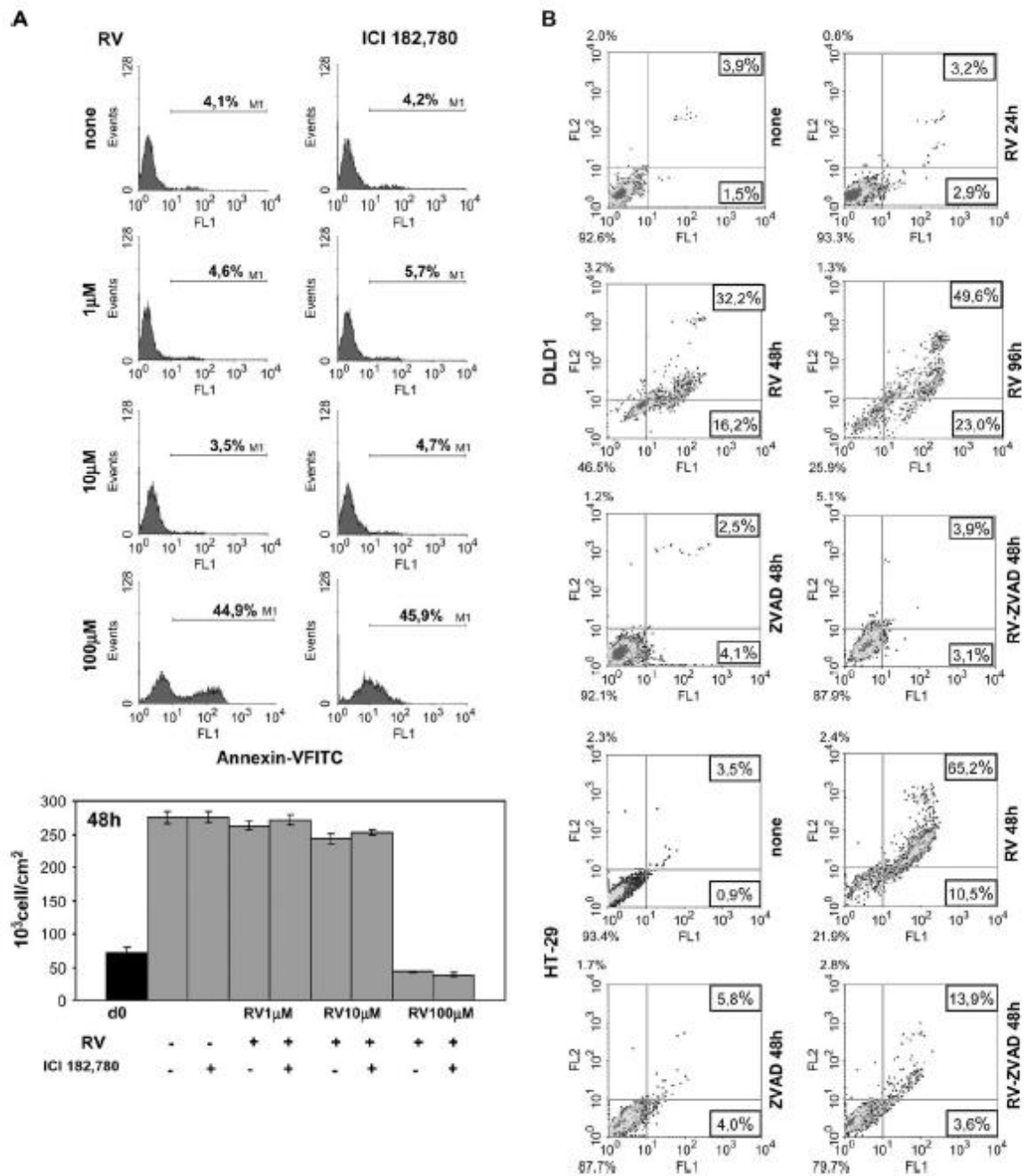


Figure 1

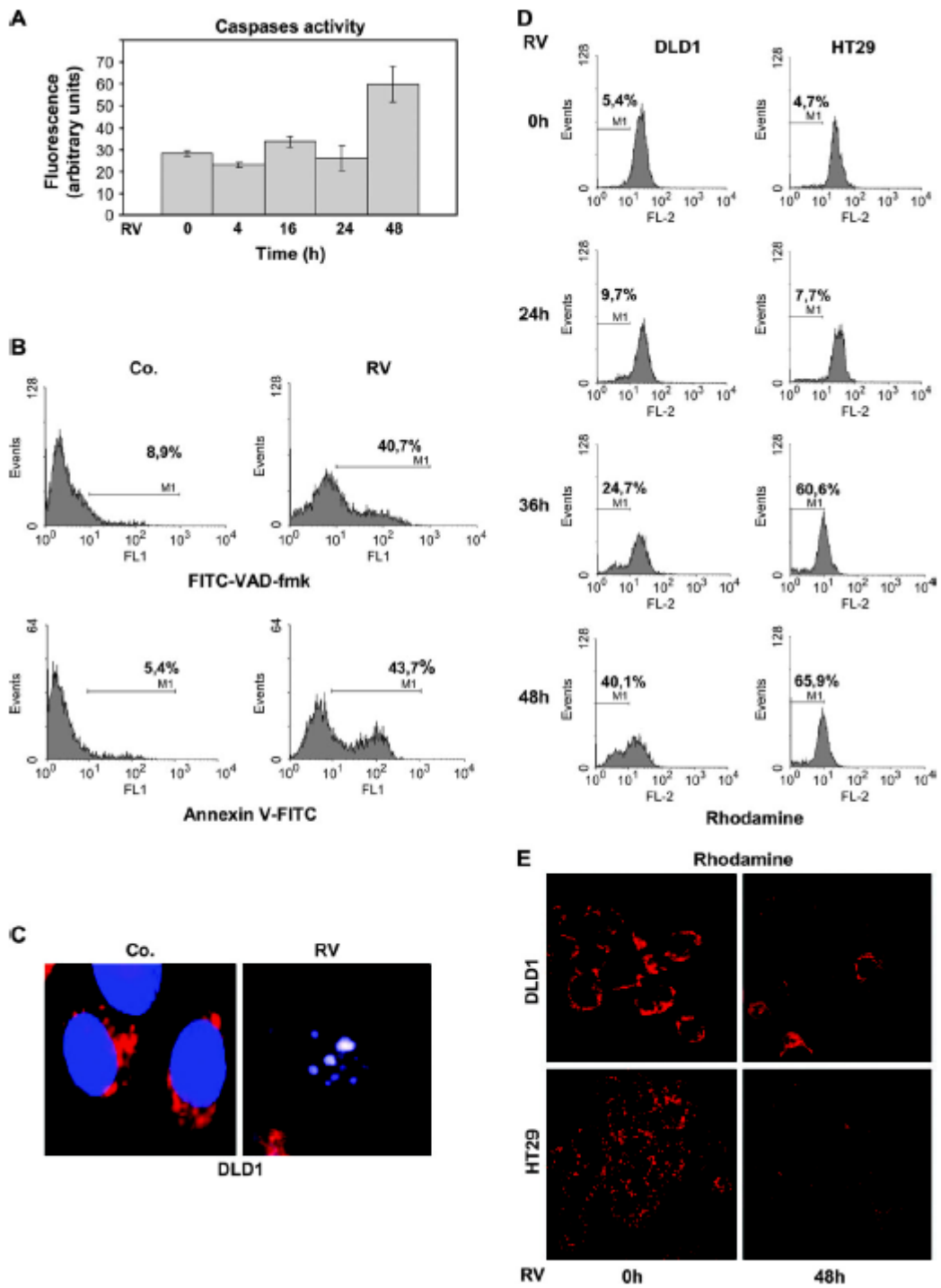


Figure 2



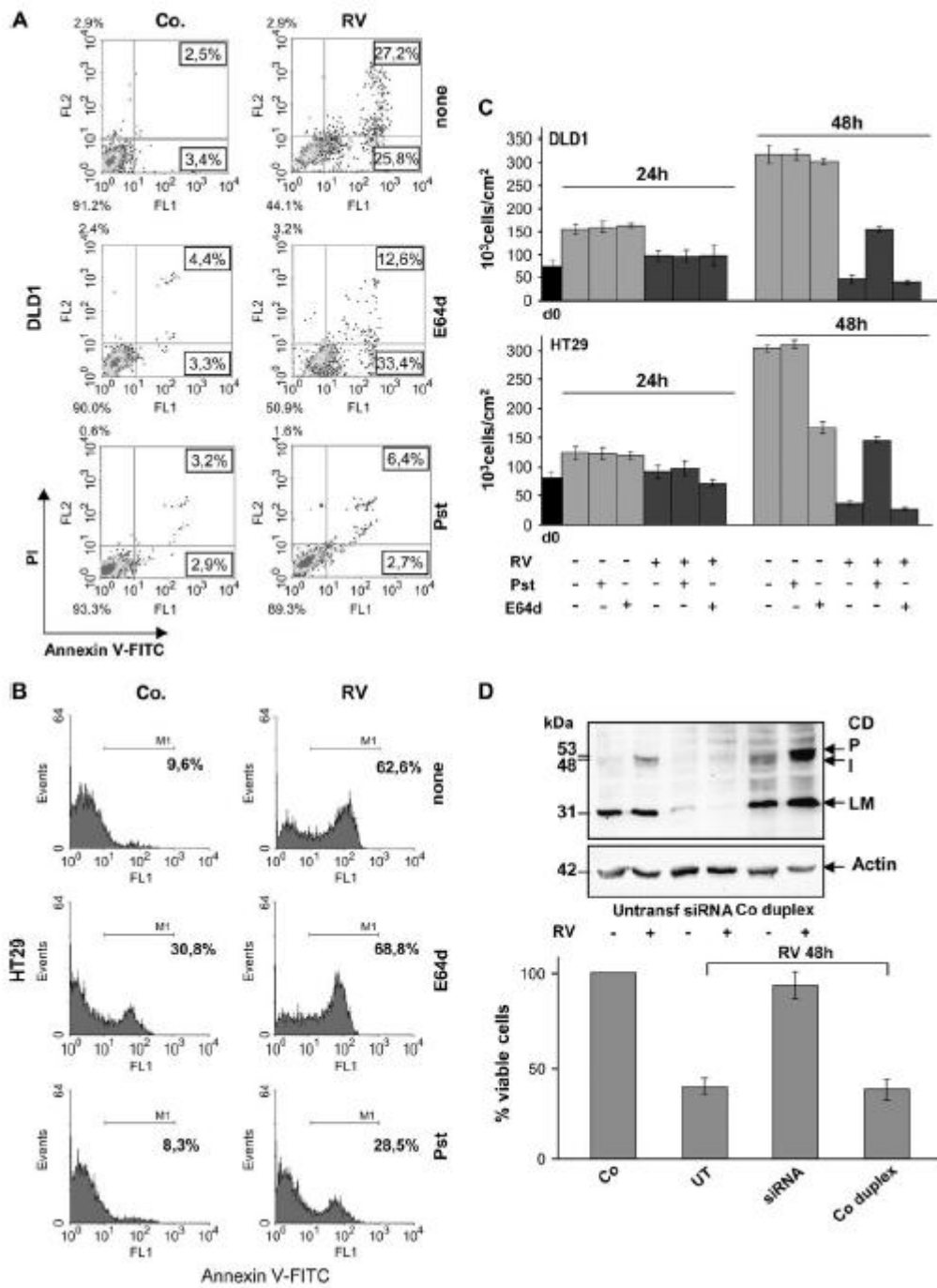
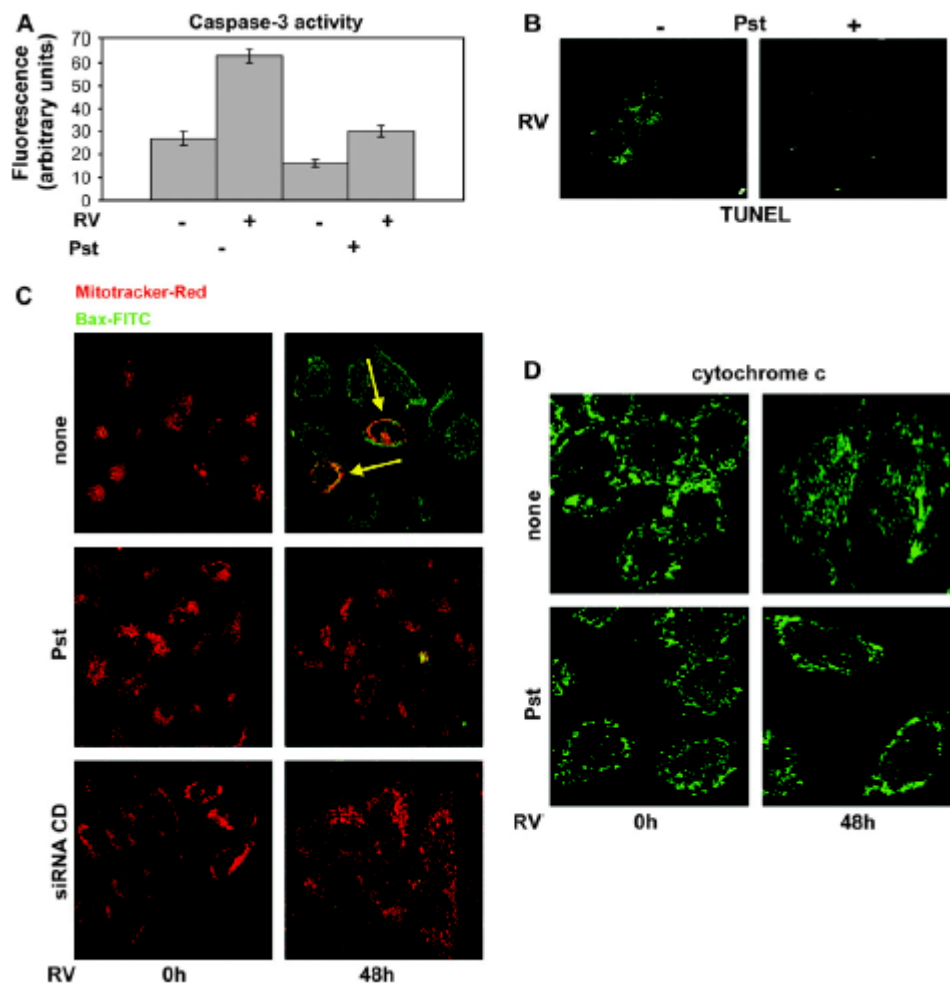


Figure 3



**Figure 4**

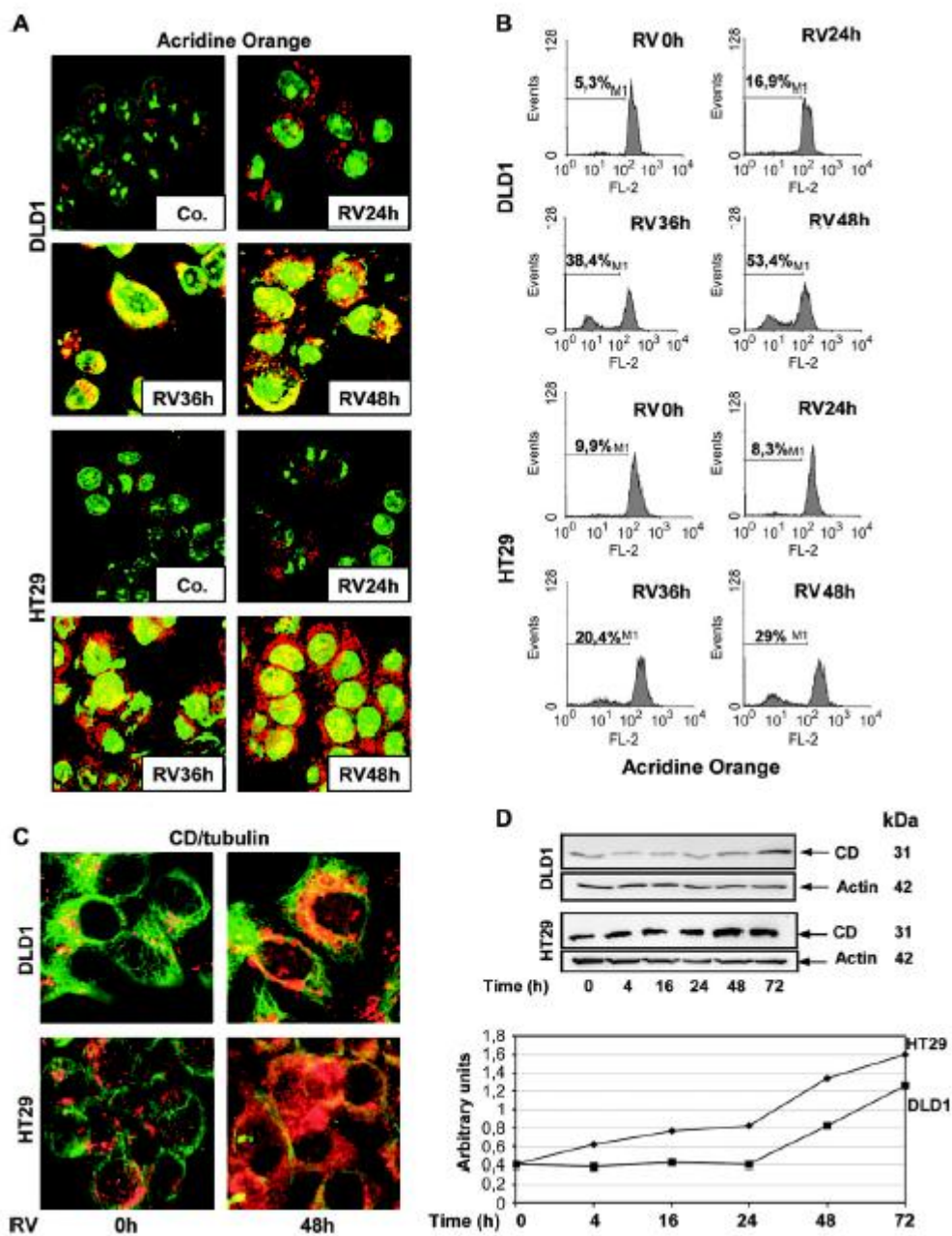
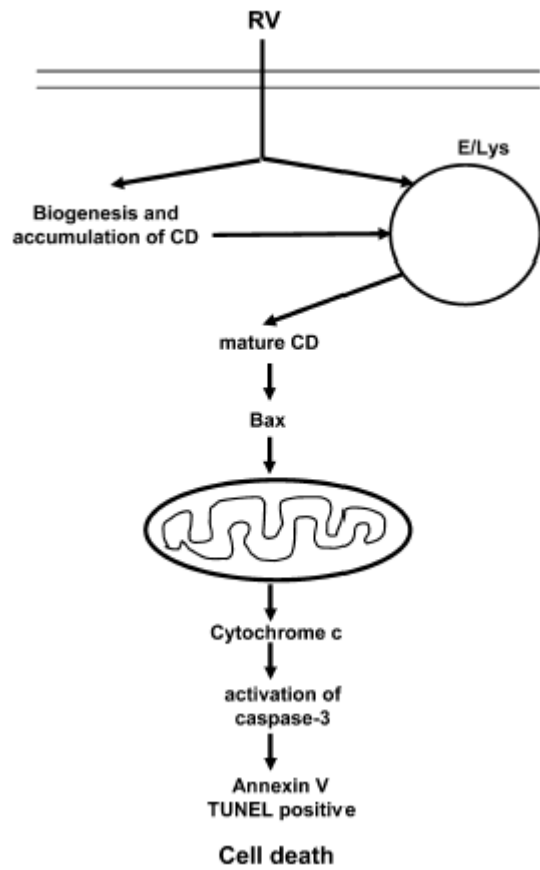


Figure 5



**Figure 6**

## **SECOND WORK**

In human colorectal DLD1 cancer cells, the dietary bioflavonoid Resveratrol (RV) rapidly induced autophagy. This effect was reversible (on removal of the drug), and was associated with increased expression and cytosolic redistribution of the proteins Beclin1 and LC3 II. Supplementing the cells with asparagine abrogated the Beclin-dependent autophagy. When applied acutely (2 h), RV was not toxic, however reiterate chronic (48 h) exposure to RV eventually led to annexin V- and TUNEL-positive cell death. This toxic effect was autophagy-dependent, as it was prevented either by asparagine, by expressing a dominant negative lipid kinase-deficient class III PI3k or by RNA-interference knock-down of Beclin1. Lamp2b silencing abolished the fusion of autophagosomes with lysosomes and preserved cell viability despite the ongoing formation of autophagosomes in cells chronically exposed to RV. The pan-caspase inhibitor ZVAD-fmk inhibited RV-induced cell death, but not autophagy. These results uncover a novel pathway of RV cytotoxicity in which autophagy plays a dual role: (i) at first, it acts as a pro-survival stress response and (ii) at a later time, it switches to a caspase-dependent apoptosis pathway. The present data also indicate that genetic or epigenetic inactivation of autophagy proteins in cancer cells may confer resistance to RV-mediated killing.

# **RESVERATROL-INDUCED APOPTOSIS DEPENDS ON THE LIPID KINASE ACTIVITY OF Vps34 AND ON THE FORMATION OF AUTOPHAGOLYSOSOMES**

Nicol F. Trincheri, Carlo Follo, Giuseppina Nicotra, Claudia Peracchio, Roberta Castino and Ciro Isidoro<sup>1</sup>

Laboratorio di Patologia Molecolare, Università del Piemonte Orientale “A. Avogadro”, Dipartimento di Scienze Mediche, Novara (Italy)

## **INTRODUCTION**

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene, RV) is a polyphenolic antioxidant compound present in grapes, red wine, berries, peanuts and other alimentary products (Baur and Sinclair, 2006; Fremont, 2000). In 1997, Pezzuto and co-workers provided the first compendious evidence that RV possesses chemopreventive activity against the three steps of carcinogenesis (i.e., initiation, promotion and progression) in an animal model of skin cancer (Jang et al., 1997). The fact that about 70 to 90% of colorectal cancers seems associated to dietary habits stimulates the interest on dietary factors that can exert cancer chemopreventive action on the intestinal mucosa (Schatzkin and Kelloff, 1995). In this respect, RV is particularly appealing as preliminary *in vitro* and *in vivo* studies have shown no overt toxicity toward normal cells when administered at doses high enough to achieve a pharmacological effect (Boocock et al.,

2007; Gusman et al., 2001). In addition, although it is fast and extensively metabolized in the body, in intestinal mucosa a 30-fold enrichment of RV over serum concentration can be reached (Sale et al., 2004). Several *in vivo* studies have shown that RV can actively contrast the development and/or progression of colorectal cancers (Schneider et al., 2001; Tessitore et al., 2000), and *in vitro* studies have confirmed the ability of RV (at concentration comparable to that found in some foods) to halt cell proliferation and to induce enterocyte-like differentiation and cell death of human coloncarcinoma cells (Liang et al., 2003; Mahyar-Roemer et al., 2001; Schneider et al., 2000; Wolter et al., 2001; Wolter and Stein, 2002) (Kotha et al., 2006; Mahyar-Roemer et al., 2002; Mohan et al., 2006; Trincheri et al., 2007).

In ovarian cancer cells, RV was shown to induce both autophagocytosis and caspase-independent cell death (Opipari et al., 2004). Yet, it remained unexplained whether the induction of autophagy by RV represented an epiphenomenal stress response or it was actively involved in the toxic mechanism of RV. Autophagy preserves cell survival under unfavourable environmental conditions by ensuring the lysosomal degradation of aged or damaged proteins, membranes and cytoplasmic structures (Yorimitsu and Klionsky, 2005). Whether this pathway contributes to or counteracts the toxic outcome of chemotherapy drug treatments is still a matter of investigation. Autophagy might confer



resistance to chemotherapy drugs in cancer cells by actively removing the proteins and the organelles that are damaged under antitumor treatment (Abedin et al., 2007; Amaravadi and Thompson, 2007). Still, the toxic effects of some anticancer drugs has been associated with induction of autophagy (Bursch et al., 1996; Kessel and Reiners, 2007). In the present study we report the following findings: i) autophagy is rapidly and reversibly induced by an acute exposure to RV; ii) the prolonged exposure to RV eventually activates a caspase-mediated cell death pathway; iii) genetic inactivation of the autophagy proteins class III PI3k, Beclin 1 and Lamp2b abrogates RV toxicity. These data emphasize the role of autophagy in the response to chemotherapy drugs and the strict relationship between autophagy and apoptosis in the execution of the death program.

## **MATERIALS AND METHODS**

Unless otherwise specified all reagents were from Sigma-Aldrich Corp., St. Luis, MO, USA.

### **Cell cultures, treatments and evaluation of cytotoxicity**

Human colorectal cancer DLD1 cells were cultivated in standard culture conditions (37°C; 95% air: 5% CO<sub>2</sub>) in Dulbecco's modified Minimal Essential Medium supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA), 2 mM L-glutamine and 1% penicillin-

streptomycin solution. Cells were seeded and let adhere on sterile plastic dishes for 24 h prior to start any treatment. Treatments included 100  $\mu$ M RV, 50 mM Asparagine (Asn) and 30  $\mu$ M ZVAD(OMe)-fmk (ZVAD) (Alexis Laboratories, San Diego USA). In experiments lasting 48 h, the culture medium was changed and the substances were re-added after the first 24 h of incubation. At designated time-points, adherent viable (trypan blue-excluding) cells were counted. Cell death was assessed by cytofluorometry analysis of cells labeled either with annexin V-FITC (Alexis Laboratories, S. Diego, USA) or propidium iodide (PI); for this purpose, adherent cells were trypsinized and mixed with the suspended cells recovered from the medium (Trincheri et al., 2007).

### **siRNA transfection**

Post-transcriptional silencing of Beclin1 and Lamp2b expression was achieved by the small interference RNA (siRNA) technology. Duplexes of 27-nucleotide siRNA including two 3'-overhanging TT were synthesized by MWG Biotech AG (Washington, DC). An inefficient CD9 oligonucleotide corresponding to the AGGUAGUGUAAUCGCCUUG sequence was used as a negative control of transfection (referred to as 'sham'). The sense strands of siRNA targeting Beclin1 and Lamp2b mRNAs were GGAACUCACAGCUCCAUAUACUUACCAC and AAGAGUGUUCGCUGGAUGAUGACACCA, respectively. Transfection was performed with Lipofectamine 2000 (Invitrogen Corp.).

Afterward, the transfection mixture was removed and cells were incubated for further 24 h in fresh medium prior to any treatment.

### **PI3K III dominant negative adenoviral vector**

The recombinant adenoviral vector directing the synthesis of the dominant negative form of Vps34 (the yeast homolog of class III PI3k), which is devoid of lipid kinase activity, was kindly provided by Dr D. Murphy (University of Bristol). The vector also bears the coding sequence for the enhanced Green Fluorescent Protein, eGFP), thus allowing to monitor cell transfection. As a control, sham infection was performed with an empty paired adenoviral vector.

### **Western blotting analysis**

Expression of proteins of interest was assessed by standard western blotting procedure (*Trincheri et al., 2007*). The filter was probed with the following antibodies: a rabbit polyclonal anti-Beclin1 (Santa Cruz Biotechnology, CA, USA); a polyclonal anti-MAPLC3 (Santa Cruz Biotechnology, CA, USA); a mouse monoclonal antibody specific for  $\beta$ -Actin. Immunocomplexes were revealed by incubation with peroxidase-conjugated goat-anti-rabbit or goat-anti-mouse antibody, as appropriate, and subsequent peroxidase-induced chemiluminescence reaction (Biorad, Hercules, CA, USA). Intensity of the bands was estimated by densitometry analysis (Quantity one software).

### **Immunofluorescence studies**

Cells grown and treated on coverslips were fixed with methanol for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 15 min. The following primary antibodies were used: a polyclonal anti-Lamp1 (BD Biosciences); a polyclonal anti-MAPLC3 (Santa Cruz Biotechnology, CA, USA). FITC- or TRITC-conjugated secondary antibodies against rabbit IgG were used. As negative control, the primary antibody was omitted.

### **TUNEL staining**

Apoptotic cells were revealed by *in situ* Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay performed with the “In situ Cell Death Detection” fluorescent Kit (Roche Diagnostics Corporation Indianapolis, IN, USA) following manufacturer’s instructions.

### **Fluorescence microscope imaging**

Coverslips were mounted in mowiol (1% in PBS). Images were captured with a Zeiss fluorescence microscope equipped with a digital camera or with Leica DMIRE2 confocal fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with Leica Confocal Software v. 2.61. Three coverslips were prepared for each experimental condition and were independently examined by two investigators. Representative images are shown.

## **Fluorescence assessment of autophagy**

Autophagolysosomes were detected with the fluorescent dye monodansylcadaverine (MDC) (Munafò and Colombo, 2001). Living cells were incubated with 0.05 mM MDC in PBS at 37° C for 15 min. After incubation, cells were washed twice with PBS and immediately analyzed by fluorescence microscopy (excitation: 380-420, barrier filter 450 nm). Induction of AV formation was directly monitored in living cells transiently transfected with a plasmid encoding the fluorescence chimeric protein GFP-LC3 or Beclin-GFP. Microtubule to vacuole translocation of LC3 is associated with limited proteolysis and lipidation of its C-terminus (Kabeya et al., 2000). Therefore, the fusion protein is made so that GFP (green fluorescent protein) is placed at the N-terminus of LC3. The DNA encoding human LC3 was cloned by PCR from the total cDNA of human OVCAR-3 cells (sense primer 5'-CAACAAGCTTCACCATGCCGTCGGAGAAGACC-3'; antisense primer 5'-AGATCTCGAGTTACTGACAATTCATCCCG-3'). The cDNA was subcloned into the expression vector pEGFPC2 (Clontech Laboratories, Mountain View, California, USA). The cDNA of Beclin1 was cloned by PCR from the total cDNA of human OVCAR-3 cells (sense primer 5'-GCCCGAATTCGGGATGGAAGGGTCTAAGA-3'; antisense primer 5'-GCCCGGGATCCTTTTCAGACTGCAGCAAATC-3') and it was subcloned into the expression vector pEGFPN1 (Clontech

Laboratories). LC3 and Beclin1 DNAs were checked by automated sequencing.

### **Statistical analysis**

All experiments were independently replicated at least three times. Data are presented as means  $\pm$  SD. The Microsoft Excel XLStats software was used.

## **RESULTS**

### **Autophagy is an early and reversible stress response to Resveratrol**

To monitor the induction of autophagy by RV we employed DLD1 transfected cells that transiently express the chimeric fluorescent protein GFP-LC3 or Beclin1-GFP. LC3 is the mammalian equivalent of yeast atg 8 and is normally associated with the cytoskeleton; on induction of autophagy this protein translocates onto the membrane of the nascent AV (Kabeya et al., 2000). Images in Figure 1A show that both endogenous LC3 and transfected GFP-LC3 assume a punctate vacuolar-like localization in RV-treated cells. Cytosol to vacuole translocation of LC3 is associated with proteolytic processing and subsequent lipidation of the C-terminus of the precursor MAP-LC3, a process leading to the 16 kDa LC3 II isoform (Kabeya et al., 2000) (Mizushima et al., 2001). This process in fact occurred in RV-treated cells (Figure 1B). Beclin1, the homolog of yeast Vps30 (also known as atg 6), interacts with class III

PI3-kinase (the homolog of yeast Vps34) and UVRAG to start the recruitment of autophagy proteins on the membrane of the pre-autophagosomal structure (Kihara et al., 2001) (Liang et al., 2006). We therefore also monitored the localization of Beclin1 in cells transiently expressing the Beclin-GFP fluorescent chimera. Beclin-positive aggregates were soon detectable after 15 min of exposure to RV (Figure 1C). To see whether the autophagy process triggered by a short exposure to RV lasted for long time, DLD1 cells were exposed to RV for 2 h, then the cells were washed and further incubated in fresh medium in the absence or the presence of RV and collected at 24 and 48 h. In parallel, cells were exposed to RV throughout the 24 or 48 h period of incubation. In the latter case, the medium was replaced and RV was re-added at 24 h, to avoid aspecific induction of autophagy due to nutrient consumption. The presence of autophagolysosomes was visualized by labeling the cells with the auto-fluorescent dye monodansylcadaverine (MDC) (Kabeya et al., 2000; Munafo and Colombo, 2001) (Bampton et al., 2005; Kihara et al., 2001; Liang et al., 2006; Mizushima et al., 2001). A 2 h incubation with RV led to the accumulation of autophagolysosomes, as indicated by the increased MDC staining (Figure 1D). On removal of RV, the cell recovered from the toxic stress and down-regulated autophagy to (basal) control level within 24 h. By contrast, the chronic presence of RV sustained the autophagy process, as demonstrated by the intense MDC

labeling of cells exposed to RV for 24 and 48 h (Figure 1D). From these data we conclude that: i) autophagy induced by RV has the characteristics of a rapid and reversible stress response; ii) RV retains its stimulatory activity on autophagy for at least 24 h.

### **Asparagine prevents the hyper-regulation of Beclin-dependent autophagy induced by chronic exposure to Resveratrol**

To confirm the induction of autophagy by RV we attempted to interfere with the process using the inhibitor 3-methyladenine (3MA) (Seglen and Gordon, 1982) This drug, at appropriate concentrations (5-10 mM), effectively inhibited the RV-induced formation of Beclin-positive macrocomplexes (not shown), yet it revealed itself toxic in prolonged incubation (Opipari et al., 2004), thus precluding its use in further experiments. Extra supplementation of certain aminoacids has been proved to down-regulate autophagy (Hoyvik et al., 1991; Seglen et al., 1980). 50 mM asparagine prevented the formation of Beclin-GFP macrocomplexes (Figure 2A) and the vacuolar localization of GFP-LC3 (not shown) in cells exposed for 2 h to RV. Asparagine also prevented the accumulation of AVs positive for Beclin1, Rab24, and LC3 in cells chronically exposed (for 24 and 48 h) to RV (not shown). Further, the content of Beclin1 greatly increased in cells chronically exposed to RV, yet this effect was not observed in cells co-treated with asparagine



(Figure 2B). Thus, raising the intracellular concentration of asparagine efficiently impaired the induction of autophagy by RV.

### **Asparagine prevents apoptosis induced by Resveratrol**

We have recently shown that chronic and reiterate administration of 100  $\mu$ M RV induces caspase-dependent apoptosis of DLD1 cells through a pathway driven by lysosomal cathepsin D and bax (Trincheri et al., 2007). While a 24 h incubation with RV provoked cell growth arrest and no apparent cell death, the incubation with RV for further 24 h produced ~50% cell death in the monolayer (Trincheri et al., 2007). We asked whether the induction of autophagy by RV was finalized to protect the cells (thus accounting for the ~ 50% of cells that survived at the end of the treatment) or was part of the death pathway (thus accounting for the ~ 50% of cells that succumbed at the end of the treatment). Hyper-activation of autophagy is in fact *per se* deleterious for the cell, as excessive self-digestion ends up in autophagic cell death (ACD) (Bursch, 2001; Feng et al., 2005). To better assess the real contribution of apoptosis and autophagy in the cytotoxic mechanism of RV, DLD1 cells were exposed to 100  $\mu$ M RV for up to 48 h in the absence or the presence of the pan-caspase inhibitor ZVAD-fmk and/or of the aminoacid asparagine. Cell death was assessed by counting the viable and necrotic cells and by cytofluorometry of cells labeled with annexin V-FITC, which is assumed as an early marker of apoptosis (Martin et al., 1995). In

agreement with published data (Trincheri et al., 2007), cell counting data (not shown) and cytofluorometry quantification of annexin V-positive cells confirmed the occurrence of ~50 % cell loss (compared to day0 cell density) in the RV-exposed culture; cell death was completely abrogated by co-treating with ZVAD-fmk or asparagine or both the inhibitors (Figure 3A). We then looked at the effect of these inhibitors on the regulation of autophagy. Fluorescence localization of LC3 in GFP-LC3 expressing cells (data not shown) and MDC staining of autophagolysosomes (Figure 3B) demonstrated the inability of ZVAD-fmk to halt RV-induced autophagy, whereas asparagine confirmed its ability to prevent the early formation of AVs.

### **Genetic down-regulation of Beclin1 abrogates the formation of autophagosomes and apoptosis induced by Resveratrol**

The findings that asparagine was able to prevent the hyper-regulation of Beclin1 expression, autophagy and cytotoxicity by RV are suggestive of a functional link between Beclin-dependent autophagy and caspase-dependent apoptosis. We tested this hypothesis. The expression of Beclin1 protein was knocked-down through the expression of a specific siRNA (Figure 4A). MDC staining proved that under this condition the formation of autophagolysosomes was impaired in the cells exposed to RV for 48 h (Figure 4B). Cell counting (not shown) and cytofluorometry data (Figure 4C) demonstrated that while control (sham-transfected) cells

were largely sensitive to a 48 h treatment with RV, Beclin1-siRNA-transfected DLD1 cells were not. To definitely implicate Beclin-dependent autophagy in RV-induced apoptosis we stained Beclin1-knocked-down cultures with the TUNEL technique, which evidences the presence of nicked DNA that accumulates in dying cells. On treatment with RV, TUNEL positive cells were detectable in sham-transfected, but not in Beclin-siRNA-transfected, cultures (Figure 4D).

### **Resveratrol toxicity depends on the lipid kinase activity of class III PI3 kinase**

We further investigated the molecular pathways through which RV induces both the up-regulation of autophagy and cell death. The interaction of Vps34-Class III PI3k with Atg6-Beclin1 is a key step for membrane nucleation and formation of the AV at the level of the pre-autophagosomal structure (Kihara et al., 2001; Suzuki et al., 2001). We asked about the need of PI3k III activity for the formation of beclin-macrocomplexes under RV treatment. To overcome the unspecific toxic effects of 3MA, we interfered with the activation of PI3k III by ectopic over-expression of its dominant negative. To this end, we employed a recombinant adenoviral vector coding for a mutant Vps34 protein devoid of lipid kinase activity (Ad-Vps34dn). Adherent DLD1 cells were sham- or Ad-Vps34dn-infected and then incubated for 48 h with or without RV. On average, > 90% of cells were effectively infected, as estimated by

GFP staining. Despite the chronic exposure to RV, the ectopic expression of Vps34dn completely prevented the induction of autophagy, as demonstrated by MDC staining (Figure 5A), and contemporary inhibited the occurrence of cell death, as demonstrated by the lack of annexin V-labeling (Figure 5B). Cell counting data confirmed the full protection attained by Vps34dn expression toward RV cytotoxicity (not shown).

### **Genetic down-regulation of Lamp2b abrogates the formation of autophagolysosomes and cell death induced by Resveratrol**

An important point is to determine whether autophagy-dependent cell death associated with RV exposure is due to the excessive accumulation of AVs and/or of autophagolysosomes in the cell. To address this issue we interfered with the last step of autophagy, i.e. the formation of the autophagolysosome. Both Lamp1 and Lamp2 proteins are involved in the process of reciprocal recognition and fusion of AVs and endosomal-lysosomal organelles (Eskelinen, 2006). We were able to inhibit the AV-lysosome fusion by siRNA-mediated knock-down of Lamp2b, as indicated by immunofluorescence co-staining of LC3 and Lamp1, which respectively identifies AVs and endosomal-lysosomal organelles (Figure 6A). MDC staining further proved that the formation of autophagolysosomes by RV was impaired in DLD1 cells transfected with an siRNA targeting Lamp2b (Figure 6B). We then assessed the toxic effect of RV under this condition. Cytofluorometry data (and cell

counting data, not shown) demonstrated that silencing Lamp2b prevents the induction of annexin V-positive cell death by chronic and reiterate exposure to RV (Figure 6C).

## **DISCUSSION**

Autophagy is a lysosomal-mediated degradative pathway that plays a major role in cell and tissue homeostasis. How and to what extent autophagy contributes to cancer development and progression/regression is still a matter of investigation (Ogier-Denis and Codogno, 2003). Defective autophagy favors tumorigenesis, and genes involved in the regulation of autophagy, such as BECLIN 1 and PTEN are frequently mutated in carcinomas and are therefore regarded as oncosuppressors (Di Cristofano and Pandolfi, 2000; Liang et al., 1999; Qu et al., 2003). On the other hand, functional autophagy helps the cancer cell to survive when the blood supply is reduced, thus preventing necrosis and inflammation-associated tumor progression (Degenhardt et al., 2006). The clarification of the real contribution of autophagy in the cellular response to anti-carcinogenic drugs is of obvious utility when designing chemotherapy and chemopreventive pharmacological strategies. Here we show that the pro-apoptotic activity of RV in DLD1 colorectal cancer cells is mediated by proteins involved in the regulation and execution of autophagy. In DLD1 cells RV promptly induced autophagy, which was reversible and

not harmful when the exposition to the drug was temporally limited. The reiterate and prolonged incubation with RV led to a cell death with the morphological and biochemical features of apoptosis (annexin V-positive, TUNEL-positive, caspase-mediated), yet it was dependent on the lipid kinase activity of class III PI3k. The signaling pathway linking RV and activation of class III PI3k definitely deserves further studies. Post-transcriptional down-regulation of either Beclin1 and Lamp2b, which respectively led to inhibition of AV and autophagolysosome formation, also protected the cells from RV toxicity. It is to note that the Lamp2b silencing condition inhibited the formation of autophagolysosomes and determined the accumulation of AVs in RV-treated cells, yet it did not precipitate an ACD as was shown to occur in starved cells (Gonzalez-Polo et al., 2005). Inhibition of autophagy prevented the occurrence of TUNEL-positive cell death, whereas inhibition of caspases, though saving the cells, did not impair autophagy in RV-treated cells. Again, in this latter condition ACD did not occur despite the chronic exposure to RV and ongoing autophagy. These observations suggest that the autophagy and apoptosis pathways were not independent, rather they were strictly linked and merged at the execution point, the caspases acting as death executioners downstream autophagy. We have previously shown that RV induces the cytosolic relocation of

cathepsin D, which in turn activates the bax-mitochondrial intrinsic pathway of caspase-dependent cell death (Trincheri et al., 2007).

Taken together with the present findings, we propose a model of RV cytotoxicity in which initially autophagy represents an adaptive response with pro-survival function, but on chronic intoxication autophagy is hyper-stimulated and the lysosomal membrane becomes permeable, thus allowing the cytosolic relocation of pro-apoptotic cathepsins. The present results indicate that genetic or epigenetic inactivation of proteins involved in the regulation and/or execution of autophagy in cancer cells confer resistance to RV-mediated killing.

**Acknowledgments:** Researches supported by grants from Università del Piemonte Orientale, Regione Piemonte (Ricerca Sanitaria Finalizzata), Fondazione Cassa di Risparmio di Torino and Lega per la Lotta contro i Tumori (sez. Novara). The authors wish to thank Prof. Dr D. Murphy (University of Bristol) for kindly providing the recombinant adenoviral vector encoding for Vps34 dominant negative.

## LEGENDS TO FIGURES

**Figure 1. Resveratrol rapidly induces an autophagy response.** A) Untransfected and GFP-LC3 transfected DLD1 cells plated on coverslips were exposed to RV for 1 and 2 h. RV provokes the rapid changes in LC3 distribution from a diffuse cytoplasmic staining to a vacuolar-like staining. B) Western blotting analysis of MAP-LC3 processing in DLD1 cells. On RV treatment, the vacuolar-associated LC3 II isoform is rapidly generated. C) DLD1 cells were transfected with a plasmid encoding Beclin-GFP and exposed to RV for the time indicated. Vacuolar-like aggregates of Beclin1 could be detected after a 15 min exposure to RV. D) DLD1 cells plated on coverslips were exposed to RV for 24 h or 48 h (refreshing the medium and re-adding RV at 24 h) or for 2 h and then incubated in fresh medium (without RV) and collected at 24 h or 48 h. Cells were then stained with MDC and immediately imaged under the fluorescence microscope. Uptake and accumulation of MDC reflect the presence of autophagolysosomes. The experiment reveals that induction of autophagy is an early and reversible response to RV.

**Figure 2. Asparagine prevents the induction of Beclin-dependent autophagy by Resveratrol.** Beclin-GFP-transfected DLD1 cells were exposed for 2 h to RV in the absence or the presence of asparagine (Asn). Images show that the vacuolar-like localization of Beclin-GFP induced by RV does not occur in the presence of Asn. B) Western blotting



analysis (one out of three is shown) of Beclin1 expression in cells exposed to RV for 24 or 48 h in the absence or the presence of Asn. RV induces the accumulation of Beclin1 protein (at 24 h this effect is higher than at 48 h). Inhibition of autophagy by Asn prevents the cellular accumulation of Beclin 1 induced by RV.

**Figure 3. Asparagine prevents the toxic effect induced by chronic administration of Resveratrol.** A) Annexin V-FITC flow cytometry profiles of cells exposed to RV for 48 h in the absence (none) or the presence of ZVAD-fmk or Asn or the two inhibitors together, as indicated. Inhibition of caspases or of autophagy elicited complete protection against RV toxicity, and no synergistic protection was attained by co-treating with both inhibitors. B) MDC staining of DLD1 cells incubated for 2 to 48 h with RV and in the absence or the presence of Asn or ZVAD-fmk. Images confirm the inability of the pan-caspases inhibitor to prevent the formation and accumulation of autophagolysosomes induced by RV, while Asn maintains its inhibitory effect on autophagy induction in cells exposed to RV for as long as 48 h.

**Figure 4. Ectopic expression of a PI3k III dominant negative protects from Resveratrol cytotoxicity.** DLD1 cells (adherent either on coverslips or Petri dishes) were infected with the recombinant vector Ad-Vps34dn (which directs the expression of a lipid kinase-deficient mutant of class III PI3k) or with an empty adenoviral vector (sham) and then

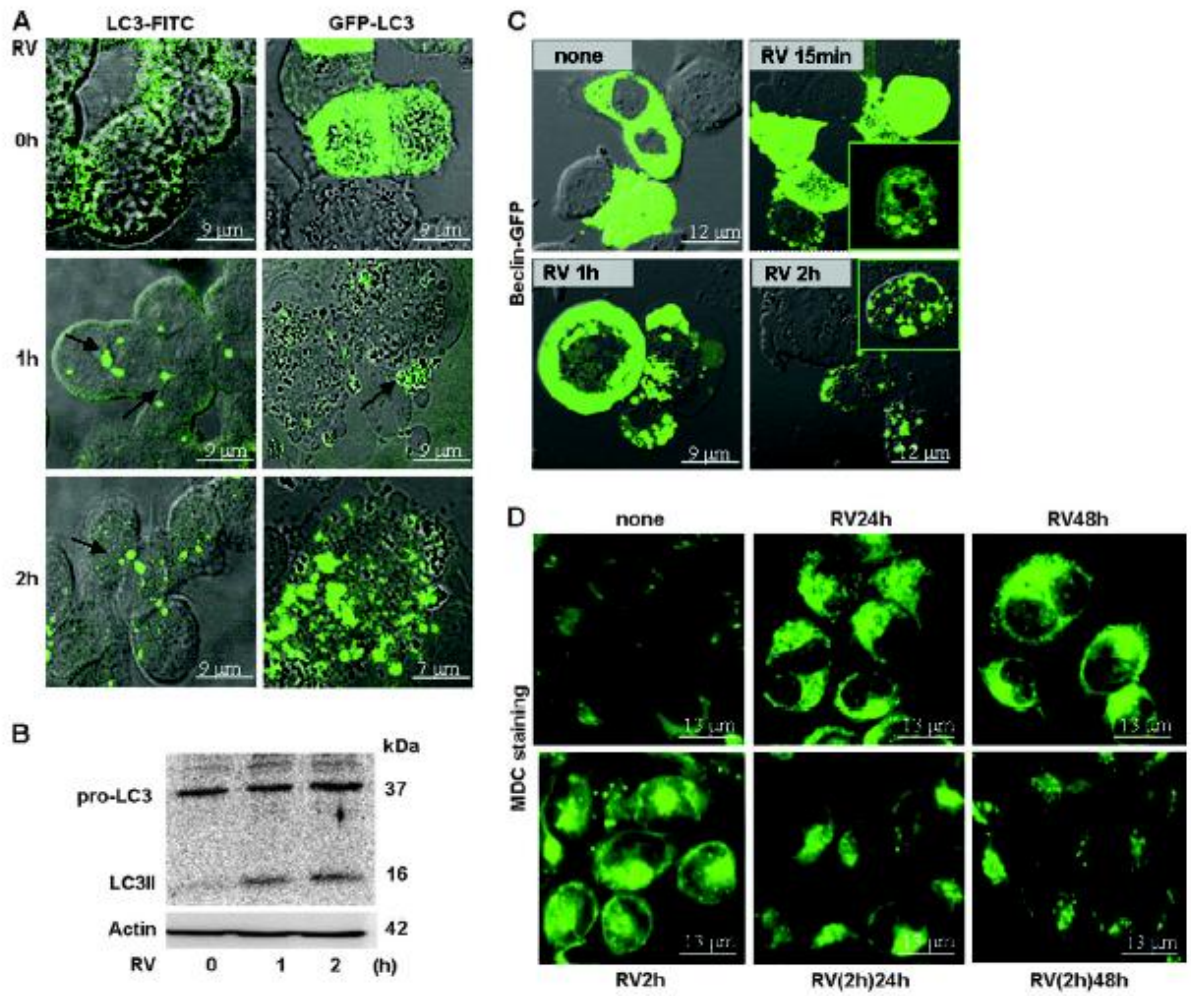
exposed to RV for 48 h. A) At the end, cells on coverslips were stained with MDC and immediately observed under the fluorescence microscope. The images show that expression of Vps34dn efficiently abolished the induction of autophagy by RV. B) At the end of the treatment, the cells were labeled with annexin V-FITC and analyzed by flow cytometry. The cytofluorometry profiles demonstrate that in Ad-Vps34dn-infected cultures RV did not induce annexin V-positive cell death. In a parallel set of cultures, adherent viable cells were counted. The data (not shown) confirmed the protection attained by Vps34dn against RV cytotoxicity.

**Figure 5. siRNA-mediated silencing of Beclin 1 prevents Resveratrol-induced apoptosis.** DLD1 cells were transfected with an inefficient duplex oligoRNA (sham) or with an siRNA specific for Beclin1. A) Western blotting analysis demonstrates the efficient knock-down of Beclin1 protein expression attained with the specific siRNA. B) The cells, plated on coverslips, were exposed to RV for 48 h and stained with MDC. The images confirm that silencing the expression of Beclin1 prevents the accumulation of autophagolysosomes in RV-treated cells. C) The cells plated in Petri dishes were exposed to RV for 48 h and then trypsinized, counted and labeled with annexin V-FITC for flow cytometry evaluation of cell death. Data demonstrate that a complete protection against RV cytotoxicity was obtained by knocking-down the expression of Beclin 1. D) Cells plated on coverslips were exposed to RV for 48 h

and then stained with the TUNEL technique. The images show that in sham-infected cultures, but not in Beclin1-silenced cultures, RV provokes the appearance of TUNEL-positive cells.

**Figure 6. siRNA-mediated silencing of Lamp2b prevents autophagolysosome formation and cell death induced by Resveratrol.**

Sham- and Lamp2b-siRNA-transfected cells were incubated for 48 h with RV and at the end cells were assayed by immunofluorescence for the formation of AVs (LC3-positive) and of autophagolysosomes (LC3-Lamp1 positive) and for occurrence of annexin V-positive cell death. A) Images show that siRNA-mediated knock-down of Lamp2b abolishes the formation of vacuoles double-positive for both LC3 and Lamp1, which are seen in sham-infected cells. B) The cells were stained with MDC. The images confirm that silencing the expression of Lamp2b is sufficient to prevent the accumulation of autophagolysosomes in RV-treated cells. C). The cells were labeled with annexin V-FITC and analyzed by flow cytometry. Data demonstrate that the complete protection against RV cytotoxicity obtained by knocking-down the expression Lamp2b.



**Figure 1**

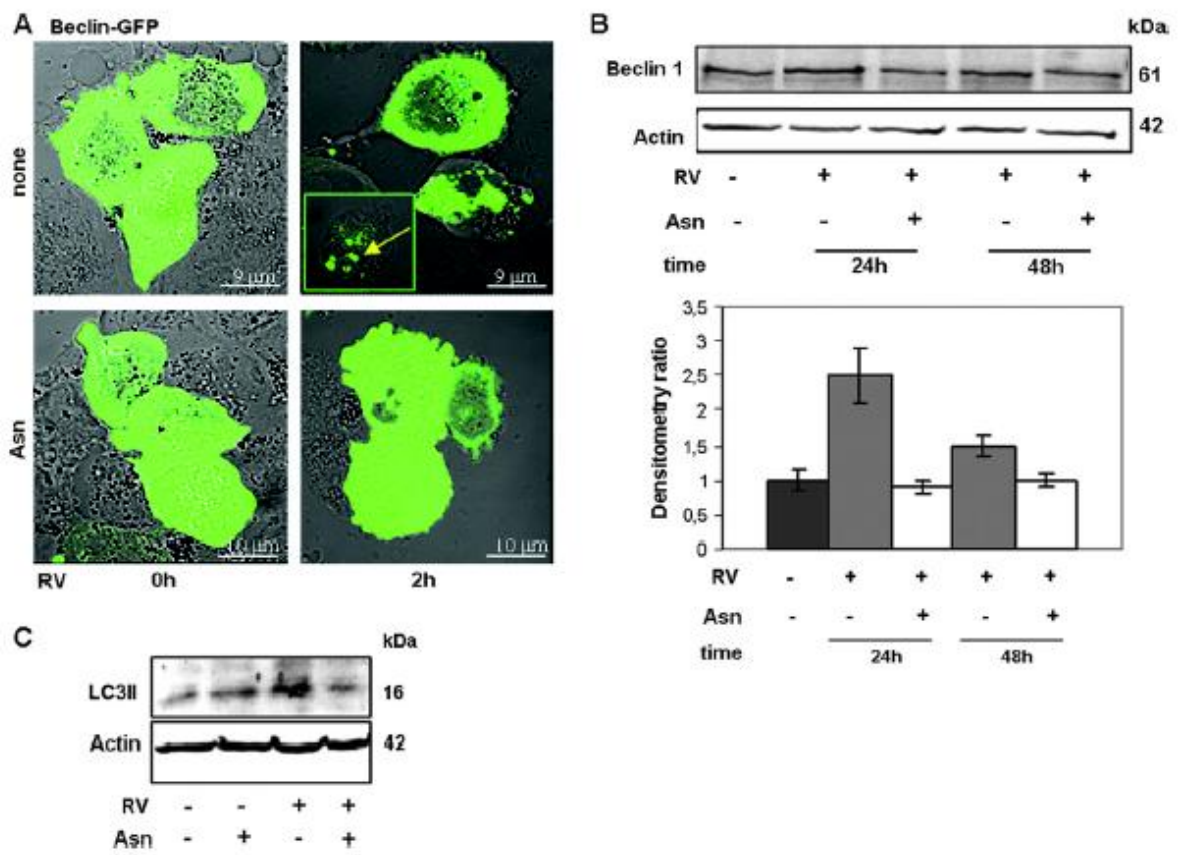
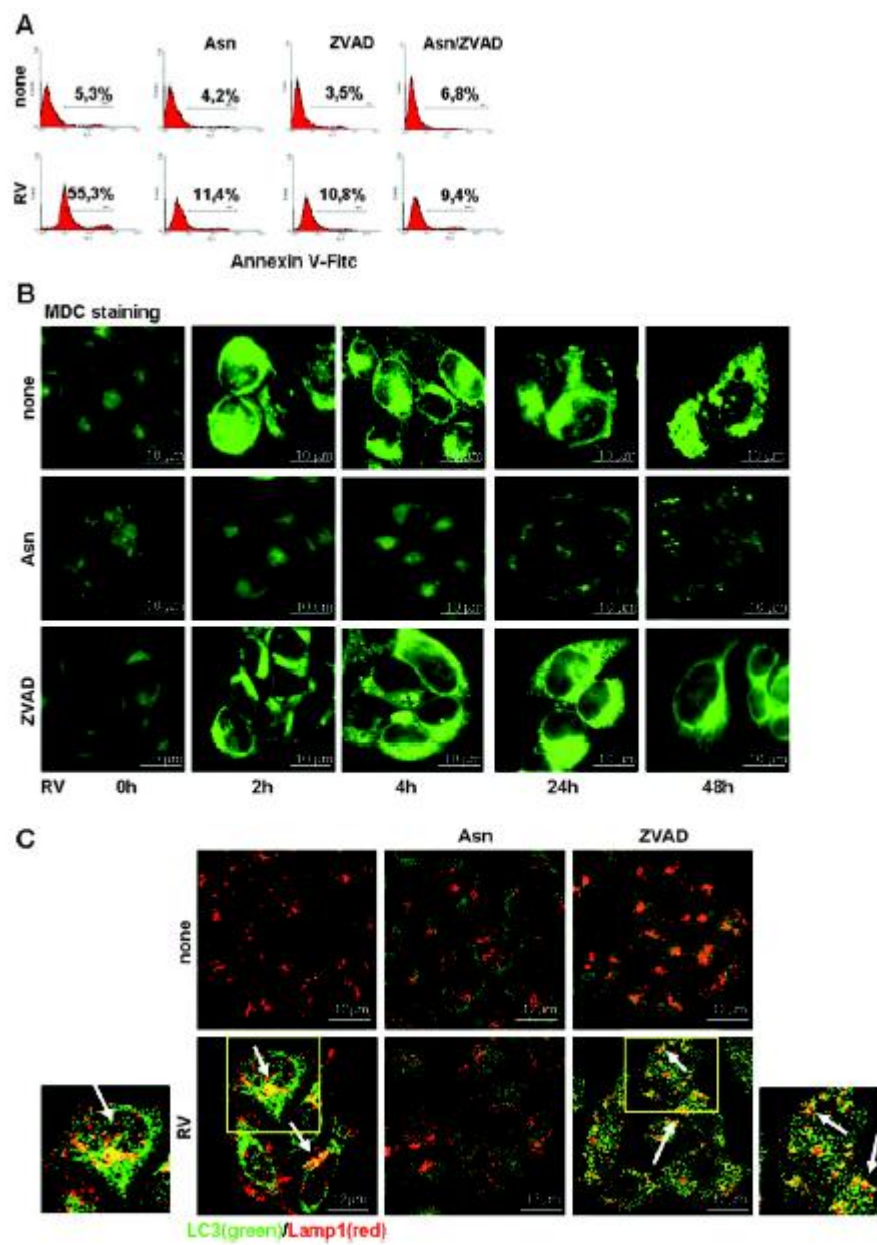


Figure 2



**Figure 3**

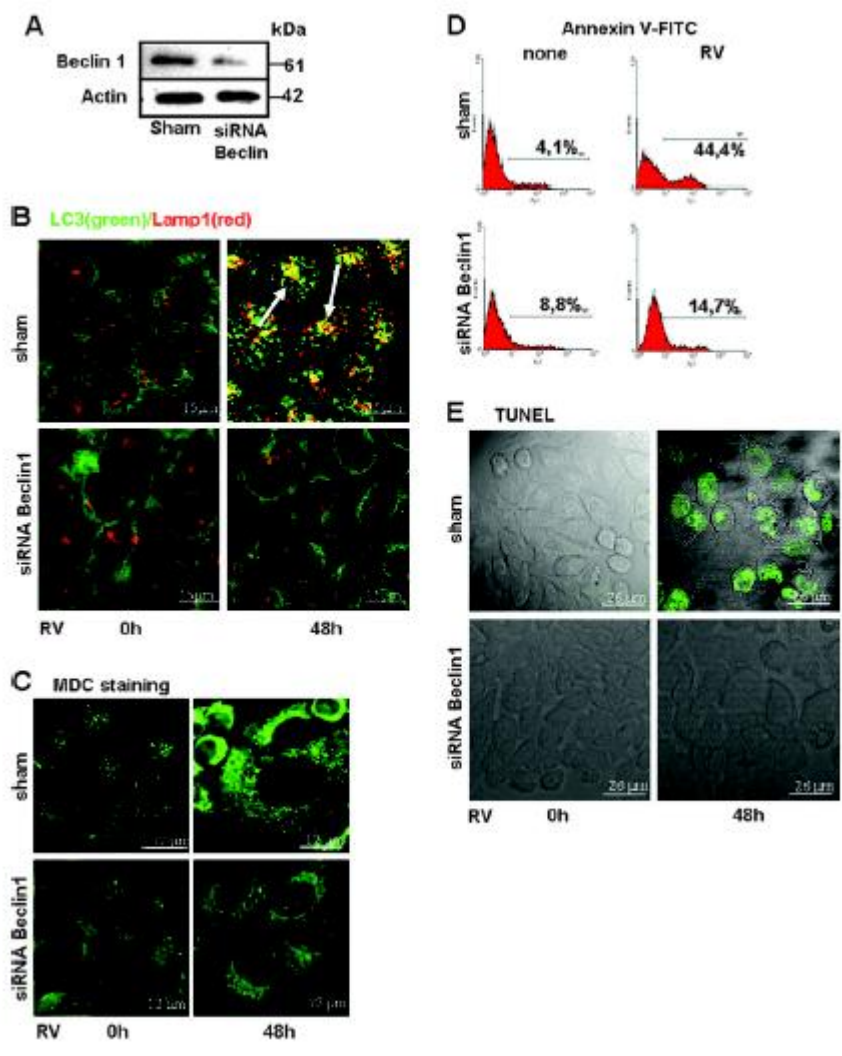
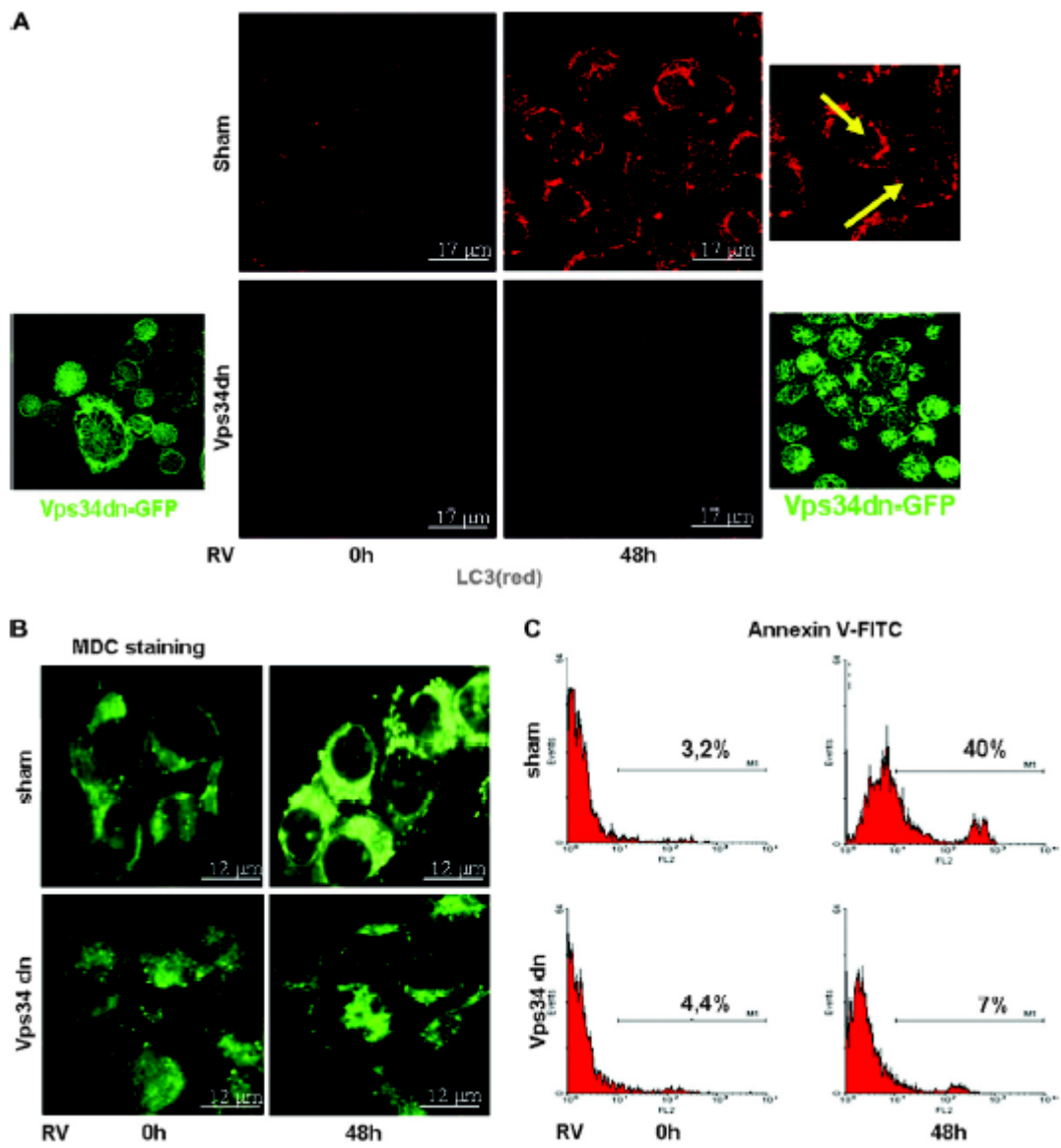


Figure 4



**Figure 5**



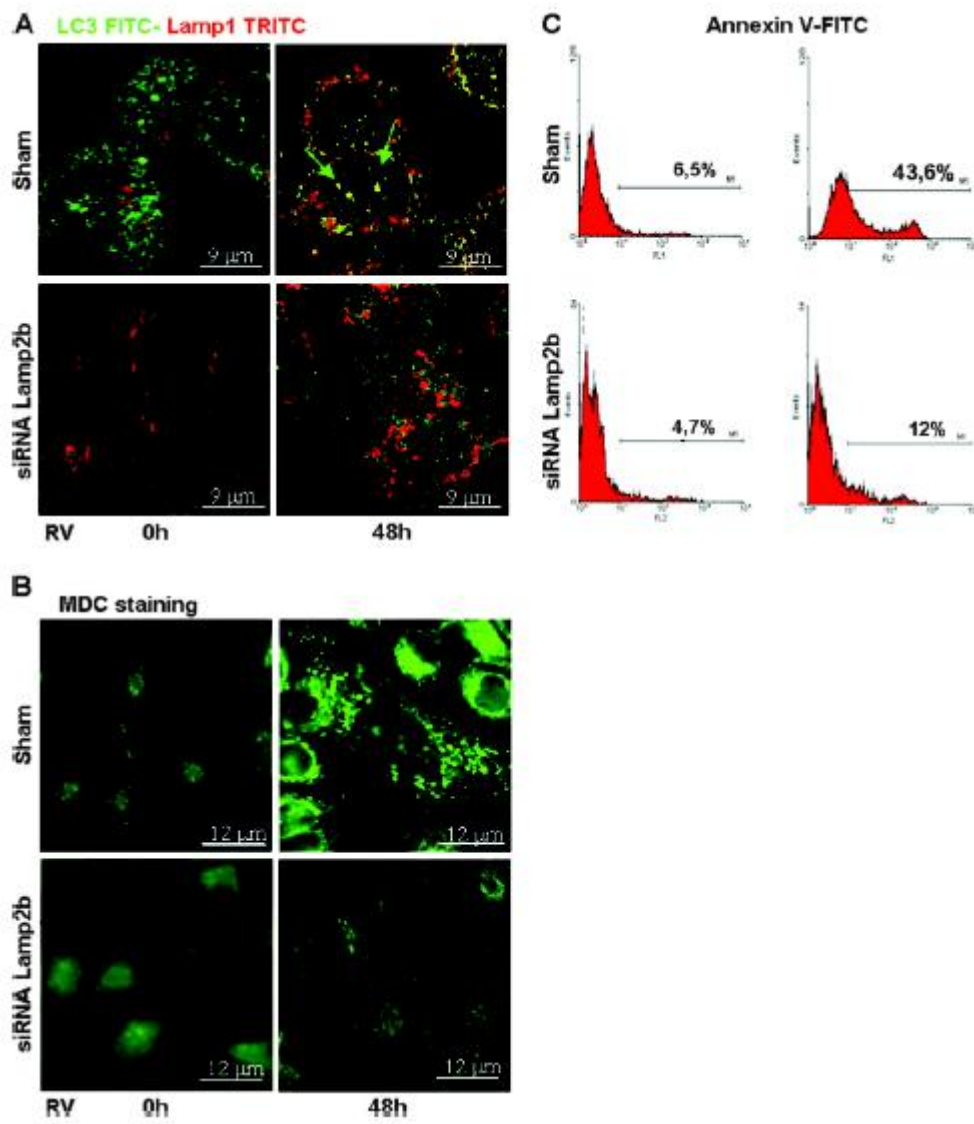


Figure 6

## OTHER WORKS

-Rita Carini, **Nicol Francesca Trincheri**, Elisa Alchera, Maria Grazia De Cesaris, Roberta Castino, Roberta Splendore, Emanuele Albano, Ciro Isidoro. PI3K-dependent lysosome exocytosis in nitric oxide-preconditioned hepatocytes. *Free Radic Biol Med.* 2006 May 15;40:1738-48.

-C. Follo, R. Castino, G. Nicotra, **N.F. Trincheri**, C. Isidoro. Folding, activity and targetting of mutated human cathepsin D that cannot be processed into the double - chain form.  
*Int J Biochem Cell Biol.* 2007;39:638-49. Epub 2006 Nov 25.

-R. Castino, N. Bellio, G. Nicotra, **N.F. Trincheri**, C. Isidoro. Cathepsin D-Bax death pathway in oxidative stressed neuroblastoma cells.  
*Free Radic Biol Med.* 2007 May 1;42:1305-16. Epub 2007 Jan 8.

-R. Castino, C. Peracchio, A. Salini, G. Nicotra, **N.F. Trincheri**, M. Demoz, G. Valente, C. Isidoro. Chemotherapy drug response in ovarian cancer cells strictly depends on a cathepsin D- Bax activation loop.  
2008 *Journal of cellular and Molecular Medicine*

-G. Nicotra, F. Manfroi, F. Mercalli, **N.F. Trincheri**, R. Castino, S. Kerim, G. Valente, C. Isidoro. High expression of cathepsin D predicts poor prognosis in patients bearing aggressive non Hodgkin's Lymphomas.  
2008 In submission.

## BIBLIOGRAPHY

- Abedin, M.J., Wang, D., McDonnell, M.A., Lehmann, U. and Kelekar, A. (2007) Autophagy delays apoptotic death in breast cancer cells following DNA damage. *Cell Death Differ*, **14**, 500-510.
- Abedini, M.R., Qiu, Q., Yan, X. and Tsang, B.K. (2004) Possible role of FLICE-like inhibitory protein (FLIP) in chemoresistant ovarian cancer cells in vitro. *Oncogene*, **23**, 6997-7004.
- Adida, C., Crotty, P.L., McGrath, J., Berrebi, D., Diebold, J. and Altieri, D.C. (1998) Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. *Am J Pathol*, **152**, 43-49
- Agarwal, C., Sharma, Y. and Agarwal, R. (2000) Anticarcinogenic effect of a polyphenolic fraction isolated from grape seeds in human prostate carcinoma DU145 cells: modulation of mitogenic signaling and cell-cycle regulators and induction of G1 arrest and apoptosis. *Mol Carcinog*, **28**, 129-138.
- Aggarwal, B.B., Bhardwaj, A., Aggarwal, R.S., Seeram, N.P., Shishodia, S. and Takada, Y. (2004) Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer Res*, **24**, 2783-2840.
- Aggarwal, B.B. and Shishodia, S. (2006) Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem Pharmacol*, **71**, 1397-1421.
- Allavena, P., Sica, A., Solinas, G., Porta, C. and Mantovani, A. (2008) The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Crit Rev Oncol Hematol*, **66**, 1-9.
- Altieri, D.C. (2008) Survivin, cancer networks and pathway-directed drug discovery. *Nat Rev Cancer*, **8**, 61-70.
- Amaravadi, R.K. and Thompson, C.B. (2007) The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clin Cancer Res*, **13**, 7271-7279.
- Ambrosini, G., Adida, C. and Altieri, D.C. (1997) A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med*, **3**, 917-921.
- Arico, S., Petiot, A., Bauvy, C., Dubbelhuis, P.F., Meijer, A.J., Codogno, P. and Ogier-Denis, E. (2001) The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem*, **276**, 35243-35246.
- Ashkenazi, A. (2002) Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer*, **2**, 420-430.
- Askanas, V. and Engel, W.K. (2006) Inclusion-body myositis: a myodegenerative conformational disorder associated with Abeta, protein misfolding, and proteasome inhibition. *Neurology*, **66**, S39-48.

- Baldwin, A.S., Jr. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol*, **14**, 649-683.
- Bampton, E.T., Goemans, C.G., Niranjana, D., Mizushima, N. and Tolkovsky, A.M. (2005) The dynamics of autophagy visualized in live cells: from autophagosome formation to fusion with endo/lysosomes. *Autophagy*, **1**, 23-36.
- Banerjee, S., Bueso-Ramos, C. and Aggarwal, B.B. (2002) Suppression of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-kappaB, cyclooxygenase 2, and matrix metalloproteinase 9. *Cancer Res*, **62**, 4945-4954.
- Baur, J.A. and Sinclair, D.A. (2006) Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov*, **5**, 493-506.
- Bavaresco, L. (2003) Role of viticultural factors on stilbene concentrations of grapes and wine. *Drugs Exp Clin Res*, **29**, 181-187.
- Belguendouz, L., Fremont, L. and Linard, A. (1997) Resveratrol inhibits metal ion-dependent and independent peroxidation of porcine low-density lipoproteins. *Biochem Pharmacol*, **53**, 1347-1355.
- Bertazza, L. and Mocellin, S. (2008) Tumor necrosis factor (TNF) biology and cell death. *Front Biosci*, **13**, 2736-2743.
- Bhardwaj, A., Sethi, G., Vadhan-Raj, S., Bueso-Ramos, C., Takada, Y., Gaur, U., Nair, A.S., Shishodia, S. and Aggarwal, B.B. (2007) Resveratrol inhibits proliferation, induces apoptosis, and overcomes chemoresistance through down-regulation of STAT3 and nuclear factor-kappaB-regulated antiapoptotic and cell survival gene products in human multiple myeloma cells. *Blood*, **109**, 2293-2302.
- Bhat, K.P. and Pezzuto, J.M. (2001) Resveratrol exhibits cytostatic and antiestrogenic properties with human endometrial adenocarcinoma (Ishikawa) cells. *Cancer Res*, **61**, 6137-6144.
- Bhat, K.P. and Pezzuto, J.M. (2002) Cancer chemopreventive activity of resveratrol. *Ann N Y Acad Sci*, **957**, 210-229.
- Bianchi, L., Gerstbrein, B., Frokjaer-Jensen, C., Royal, D.C., Mukherjee, G., Royal, M.A., Xue, J., Schafer, W.R. and Driscoll, M. (2004) The neurotoxic MEC-4(d) DEG/ENaC sodium channel conducts calcium: implications for necrosis initiation. *Nat Neurosci*, **7**, 1337-1344.
- Bidere, N., Lorenzo, H.K., Carmona, S., Laforge, M., Harper, F., Dumont, C. and Senik, A. (2003) Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation in T lymphocytes entering the early commitment phase to apoptosis. *J Biol Chem*, **278**, 31401-31411.

- Boatright, K.M. and Salvesen, G.S. (2003) Caspase activation. *Biochem Soc Symp*, 233-242.
- Bodmer, J.L., Holler, N., Reynard, S., Vinciguerra, P., Schneider, P., Juo, P., Blenis, J. and Tschopp, J. (2000) TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat Cell Biol*, **2**, 241-243.
- Boocock, D.J., Faust, G.E., Patel, K.R., Schinas, A.M., Brown, V.A., Ducharme, M.P., Booth, T.D., Crowell, J.A., Perloff, M., Gescher, A.J., Steward, W.P. and Brenner, D.E. (2007) Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. *Cancer Epidemiol Biomarkers Prev*, **16**, 1246-1252.
- Bottger, A., Bottger, V., Garcia-Echeverria, C., Chene, P., Hochkeppel, H.K., Sampson, W., Ang, K., Howard, S.F., Picksley, S.M. and Lane, D.P. (1997) Molecular characterization of the hdm2-p53 interaction. *J Mol Biol*, **269**, 744-756.
- Bowers, J.L., Tyulmenkov, V.V., Jernigan, S.C. and Klinge, C.M. (2000) Resveratrol acts as a mixed agonist/antagonist for estrogen receptors alpha and beta. *Endocrinology*, **141**, 3657-3667.
- Boya, P., Gonzalez-Polo, R.A., Casares, N., Perfettini, J.L., Dessen, P., Larochette, N., Metivier, D., Meley, D., Souquere, S., Yoshimori, T., Pierron, G., Codogno, P. and Kroemer, G. (2005) Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol*, **25**, 1025-1040.
- Brakenhielm, E., Cao, R. and Cao, Y. (2001) Suppression of angiogenesis, tumor growth, and wound healing by resveratrol, a natural compound in red wine and grapes. *Faseb J*, **15**, 1798-1800.
- Brennan, D.J., Rexhepaj, E., O'Brien, S.L., McSherry, E., O'Connor, D.P., Fagan, A., Culhane, A.C., Higgins, D.G., Jirstrom, K., Millikan, R.C., Landberg, G., Duffy, M.J., Hewitt, S.M. and Gallagher, W.M. (2008) Altered cytoplasmic-to-nuclear ratio of survivin is a prognostic indicator in breast cancer. *Clin Cancer Res*, **14**, 2681-2689.
- Broker, L.E., Huisman, C., Span, S.W., Rodriguez, J.A., Kruyt, F.A. and Giaccone, G. (2004) Cathepsin B mediates caspase-independent cell death induced by microtubule stabilizing agents in non-small cell lung cancer cells. *Cancer Res*, **64**, 27-30.
- Broker, L.E., Kruyt, F.A. and Giaccone, G. (2005) Cell death independent of caspases: a review. *Clin Cancer Res*, **11**, 3155-3162.
- Bruder, J.L., Hsieh, T., Lerea, K.M., Olson, S.C. and Wu, J.M. (2001) Induced cytoskeletal changes in bovine pulmonary artery endothelial cells by resveratrol and the accompanying modified responses to arterial shear stress. *BMC Cell Biol*, **2**, 1.

- Bruder, J.M., Lee, A.P. and Hoffman-Kim, D. (2007) Biomimetic materials replicating Schwann cell topography enhance neuronal adhesion and neurite alignment in vitro. *J Biomater Sci Polym Ed*, **18**, 967-982.
- Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J. and Greenberg, M.E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*, **96**, 857-868.
- Brunk, U.T., Neuzil, J. and Eaton, J.W. (2001) Lysosomal involvement in apoptosis. *Redox Rep*, **6**, 91-97.
- Buccellato, L.J., Tso, M., Akinci, O.I., Chandel, N.S. and Budinger, G.R. (2004) Reactive oxygen species are required for hyperoxia-induced Bax activation and cell death in alveolar epithelial cells. *J Biol Chem*, **279**, 6753-6760.
- Buchsbaum, D.J., Forero-Torres, A. and LoBuglio, A.F. (2007) TRAIL-receptor antibodies as a potential cancer treatment. *Future Oncol*, **3**, 405-409.
- Burke, J.R. (2003) Targeting I kappa B kinase for the treatment of inflammatory and other disorders. *Curr Opin Drug Discov Devel*, **6**, 720-728.
- Bursch, W. (2001) The autophagosomal-lysosomal compartment in programmed cell death. *Cell Death Differ*, **8**, 569-581.
- Bursch, W., Ellinger, A., Kienzl, H., Torok, L., Pandey, S., Sikorska, M., Walker, R. and Hermann, R.S. (1996) Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. *Carcinogenesis*, **17**, 1595-1607.
- Campbell-Thompson, M., Lynch, I.J. and Bhardwaj, B. (2001) Expression of estrogen receptor (ER) subtypes and ERbeta isoforms in colon cancer. *Cancer Res*, **61**, 632-640.
- Cao, Z., Fang, J., Xia, C., Shi, X. and Jiang, B.H. (2004) trans-3,4,5'-Trihydroxystibene inhibits hypoxia-inducible factor 1alpha and vascular endothelial growth factor expression in human ovarian cancer cells. *Clin Cancer Res*, **10**, 5253-5263.
- Carbo, N., Costelli, P., Baccino, F.M., Lopez-Soriano, F.J. and Argiles, J.M. (1999) Resveratrol, a natural product present in wine, decreases tumour growth in a rat tumour model. *Biochem Biophys Res Commun*, **254**, 739-743.
- Cardone, M.H., Salvesen, G.S., Widmann, C., Johnson, G. and Frisch, S.M. (1997) The regulation of anoikis: MEKK-1 activation requires cleavage by caspases. *Cell*, **90**, 315-323.
- Castino, R., Demoz, M. and Isidoro, C. (2003) Destination 'lysosome': a target organelle for tumour cell killing? *J Mol Recognit*, **16**, 337-348.

- Castino, R., Peracchio, C., Salini, A., Nicotra, G., Trincheri, N.F., Demoz, M., Valente, G. and Isidoro, C. (2008) Chemotherapy Drug Response in Ovarian Cancer Cells Strictly Depends on a Cathepsin D - Bax Activation Loop. *J Cell Mol Med*.
- Chan, A.Y., Dolinsky, V.W., Soltys, C.L., Viollet, B., Baksh, S., Light, P.E. and Dyck, J.R. (2008) Resveratrol inhibits cardiac hypertrophy via AMP-activated protein kinase and Akt. *J Biol Chem*, **283**, 24194-24201.
- Chander, V. and Chopra, K. (2006) Possible role of nitric oxide in the protective effect of resveratrol in 5/6th nephrectomized rats. *J Surg Res*, **133**, 129-135.
- Chanvitayapongs, S., Draczynska-Lusiak, B. and Sun, A.Y. (1997) Amelioration of oxidative stress by antioxidants and resveratrol in PC12 cells. *Neuroreport*, **8**, 1499-1502.
- Cheeke, P.R., Piacente, S. and Oleszek, W. (2006) Anti-inflammatory and anti-arthritic effects of *Yucca schidigera*: a review. *J Inflamm (Lond)*, **3**, 6.
- Chen, A.C. and Donovan, S.M. (2004) Genistein at a concentration present in soy infant formula inhibits Caco-2BBE cell proliferation by causing G2/M cell cycle arrest. *J Nutr*, **134**, 1303-1308.
- Chen, G.Q., Zhu, J., Shi, X.G., Ni, J.H., Zhong, H.J., Si, G.Y., Jin, X.L., Tang, W., Li, X.S., Xong, S.M., Shen, Z.X., Sun, G.L., Ma, J., Zhang, P., Zhang, T.D., Gazin, C., Naoe, T., Chen, S.J., Wang, Z.Y. and Chen, Z. (1996) In vitro studies on cellular and molecular mechanisms of arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) in the treatment of acute promyelocytic leukemia: As<sub>2</sub>O<sub>3</sub> induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. *Blood*, **88**, 1052-1061.
- Chen, Q., Ray, S., Hussein, M.A., Srkalovic, G. and Almasan, A. (2003) Role of Apo2L/TRAIL and Bcl-2-family proteins in apoptosis of multiple myeloma. *Leuk Lymphoma*, **44**, 1209-1214.
- Cheng, H. and Leblond, C.P. (1974) Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *Am J Anat*, **141**, 537-561.
- Chinnaiyan, A.M., O'Rourke, K., Tewari, M. and Dixit, V.M. (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell*, **81**, 505-512.
- Chipuk, J.E., Bouchier-Hayes, L., Kuwana, T., Newmeyer, D.D. and Green, D.R. (2005) PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. *Science*, **309**, 1732-1735.
- Chipuk, J.E. and Green, D.R. (2005) Do inducers of apoptosis trigger caspase-independent cell death? *Nat Rev Mol Cell Biol*, **6**, 268-275.

- Cory, S., Huang, D.C. and Adams, J.M. (2003) The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene*, **22**, 8590-8607.
- Cos, P., De Bruyne, T., Apers, S., Vanden Berghe, D., Pieters, L. and Vlietinck, A.J. (2003) Phytoestrogens: recent developments. *Planta Med*, **69**, 589-599.
- Criollo, A., Maiuri, M.C., Tasdemir, E., Vitale, I., Fiebig, A.A., Andrews, D., Molgo, J., Diaz, J., Lavandero, S., Harper, F., Pierron, G., di Stefano, D., Rizzuto, R., Szabadkai, G. and Kroemer, G. (2007) Regulation of autophagy by the inositol trisphosphate receptor. *Cell Death Differ*, **14**, 1029-1039.
- Dahia, P.L., Aguiar, R.C., Alberta, J., Kum, J.B., Caron, S., Sill, H., Marsh, D.J., Ritz, J., Freedman, A., Stiles, C. and Eng, C. (1999) PTEN is inversely correlated with the cell survival factor Akt/PKB and is inactivated via multiple mechanisms in haematological malignancies. *Hum Mol Genet*, **8**, 185-193.
- Danial, N.N. and Korsmeyer, S.J. (2004) Cell death: critical control points. *Cell*, **116**, 205-219.
- Das, S., Alagappan, V.K., Bagchi, D., Sharma, H.S., Maulik, N. and Das, D.K. (2005) Coordinated induction of iNOS-VEGF-KDR-eNOS after resveratrol consumption: a potential mechanism for resveratrol preconditioning of the heart. *Vascul Pharmacol*, **42**, 281-289.
- Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. and Greenberg, M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*, **91**, 231-241.
- Dauvois, S., Danielian, P.S., White, R. and Parker, M.G. (1992) Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover. *Proc Natl Acad Sci U S A*, **89**, 4037-4041.
- de Duve, C. (1983) Lysosomes revisited. *Eur J Biochem*, **137**, 391-397.
- Debatin, K.M. and Krammer, P.H. (2004) Death receptors in chemotherapy and cancer. *Oncogene*, **23**, 2950-2966.
- Degenhardt, K., Chen, G., Lindsten, T. and White, E. (2002) BAX and BAK mediate p53-independent suppression of tumorigenesis. *Cancer Cell*, **2**, 193-203.
- Degenhardt, K., Mathew, R., Beaudoin, B., Bray, K., Anderson, D., Chen, G., Mukherjee, C., Shi, Y., Gelinas, C., Fan, Y., Nelson, D.A., Jin, S. and White, E. (2006) Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell*, **10**, 51-64.
- Deiss, L.P., Galinka, H., Berissi, H., Cohen, O. and Kimchi, A. (1996) Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. *Embo J*, **15**, 3861-3870.



- del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R. and Nunez, G. (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science*, **278**, 687-689.
- Dell'Angelica, E.C., Mullins, C., Caplan, S. and Bonifacino, J.S. (2000) Lysosome-related organelles. *Faseb J*, **14**, 1265-1278.
- Delmas, D., Lancon, A., Colin, D., Jannin, B. and Latruffe, N. (2006) Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. *Curr Drug Targets*, **7**, 423-442.
- Demoz, M., Castino, R., Cesaro, P., Baccino, F.M., Bonelli, G. and Isidoro, C. (2002) Endosomal-lysosomal proteolysis mediates death signalling by TNFalpha, not by etoposide, in L929 fibrosarcoma cells: evidence for an active role of cathepsin D. *Biol Chem*, **383**, 1237-1248.
- Demoz, M., Castino, R., Follo, C., Hasilik, A., Sloane, B.F. and Isidoro, C. (2006) High yield synthesis and characterization of phosphorylated recombinant human procathepsin D expressed in mammalian cells. *Protein Expr Purif*, **45**, 157-167.
- Deveraux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, S.M., Alnemri, E.S., Salvesen, G.S. and Reed, J.C. (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *Embo J*, **17**, 2215-2223.
- Di Cristofano, A. and Pandolfi, P.P. (2000) The multiple roles of PTEN in tumor suppression. *Cell*, **100**, 387-390.
- Docherty, J.J., Sweet, T.J., Bailey, E., Faith, S.A. and Booth, T. (2006) Resveratrol inhibition of varicella-zoster virus replication in vitro. *Antiviral Res*, **72**, 171-177.
- Dohi, T., Xia, F. and Altieri, D.C. (2007) Compartmentalized phosphorylation of IAP by protein kinase A regulates cytoprotection. *Mol Cell*, **27**, 17-28.
- Donepudi, M., Mac Sweeney, A., Briand, C. and Grutter, M.G. (2003) Insights into the regulatory mechanism for caspase-8 activation. *Mol Cell*, **11**, 543-549.
- Du, C., Fang, M., Li, Y., Li, L. and Wang, X. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell*, **102**, 33-42.
- Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R. and Greenberg, M.E. (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science*, **275**, 661-665.
- Duncan, S.J., Gruschow, S., Williams, D.H., McNicholas, C., Purewal, R., Hajek, M., Gerlitz, M., Martin, S., Wrigley, S.K. and Moore, M. (2001) Isolation and

- structure elucidation of Chlorofusin, a novel p53-MDM2 antagonist from a *Fusarium* sp. *J Am Chem Soc*, **123**, 554-560.
- Earnshaw, W.C. (1999) Apoptosis. A cellular poison cupboard. *Nature*, **397**, 387, 389.
- Eckelman, B.P., Salvesen, G.S. and Scott, F.L. (2006) Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Rep*, **7**, 988-994.
- Edinger, A.L. and Thompson, C.B. (2002) Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. *Mol Biol Cell*, **13**, 2276-2288.
- El-Deiry, W.S. (2003) The role of p53 in chemosensitivity and radiosensitivity. *Oncogene*, **22**, 7486-7495.
- Ellington, A.D. and Bull, J.J. (2005) Evolution. Changing the cofactor diet of an enzyme. *Science*, **310**, 454-455.
- Elnemr, A., Ohta, T., Yachie, A., Kayahara, M., Kitagawa, H., Fujimura, T., Ninomiya, I., Fushida, S., Nishimura, G.I., Shimizu, K. and Miwa, K. (2001) Human pancreatic cancer cells disable function of Fas receptors at several levels in Fas signal transduction pathway. *Int J Oncol*, **18**, 311-316.
- Emert-Sedlak, L., Shangary, S., Rabinovitz, A., Miranda, M.B., Delach, S.M. and Johnson, D.E. (2005) Involvement of cathepsin D in chemotherapy-induced cytochrome c release, caspase activation, and cell death. *Mol Cancer Ther*, **4**, 733-742.
- Eskelinen, E.L. (2006) Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. *Mol Aspects Med*, **27**, 495-502.
- Faust, P.L., Kornfeld, S. and Chirgwin, J.M. (1985) Cloning and sequence analysis of cDNA for human cathepsin D. *Proc Natl Acad Sci U S A*, **82**, 4910-4914.
- Feng, Z., Zhang, H., Levine, A.J. and Jin, S. (2005) The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci U S A*, **102**, 8204-8209.
- Ferreira, C.G., van der Valk, P., Span, S.W., Jonker, J.M., Postmus, P.E., Kruijt, F.A. and Giaccone, G. (2001) Assessment of IAP (inhibitor of apoptosis) proteins as predictors of response to chemotherapy in advanced non-small-cell lung cancer patients. *Ann Oncol*, **12**, 799-805.
- Ferrer, P., Asensi, M., Segarra, R., Ortega, A., Benlloch, M., Obrador, E., Varea, M.T., Asensio, G., Jorda, L. and Estrela, J.M. (2005) Association between pterostilbene and quercetin inhibits metastatic activity of B16 melanoma. *Neoplasia*, **7**, 37-47.

- Fesik, S.W. (2005) Promoting apoptosis as a strategy for cancer drug discovery. *Nat Rev Cancer*, **5**, 876-885.
- Fingar, D.C. and Blenis, J. (2004) Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene*, **23**, 3151-3171.
- Fiorelli, G., Picariello, L., Martinetti, V., Tonelli, F. and Brandi, M.L. (1999) Functional estrogen receptor beta in colon cancer cells. *Biochem Biophys Res Commun*, **261**, 521-527.
- Foghsgaard, L., Wissing, D., Mauch, D., Lademann, U., Bastholm, L., Boes, M., Elling, F., Leist, M. and Jaattela, M. (2001) Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. *J Cell Biol*, **153**, 999-1010.
- Fong, W.G., Liston, P., Rajcan-Separovic, E., St Jean, M., Craig, C. and Korneluk, R.G. (2000) Expression and genetic analysis of XIAP-associated factor 1 (XAF1) in cancer cell lines. *Genomics*, **70**, 113-122.
- Fremont, L. (2000) Biological effects of resveratrol. *Life Sci*, **66**, 663-673.
- Fribley, A., Zeng, Q. and Wang, C.Y. (2004) Proteasome inhibitor PS-341 induces apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells. *Mol Cell Biol*, **24**, 9695-9704.
- Fukuda, S. and Pelus, L.M. (2006) Survivin, a cancer target with an emerging role in normal adult tissues. *Mol Cancer Ther*, **5**, 1087-1098.
- Fulda, S. and Debatin, K.M. (2004) Targeting apoptosis pathways in cancer therapy. *Curr Cancer Drug Targets*, **4**, 569-576.
- Galatin, P.S. and Abraham, D.J. (2004) A nonpeptidic sulfonamide inhibits the p53-mdm2 interaction and activates p53-dependent transcription in mdm2-overexpressing cells. *J Med Chem*, **47**, 4163-4165.
- Galderisi, U., Cascino, A. and Giordano, A. (1999) Antisense oligonucleotides as therapeutic agents. *J Cell Physiol*, **181**, 251-257.
- Gelfanov, V.M., Burgess, G.S., Litz-Jackson, S., King, A.J., Marshall, M.S., Nakshatri, H. and Boswell, H.S. (2001) Transformation of interleukin-3-dependent cells without participation of Stat5/bcl-xL: cooperation of akt with raf/erk leads to p65 nuclear factor kappaB-mediated antiapoptosis involving c-IAP2. *Blood*, **98**, 2508-2517.
- Gingras, A.C., Kennedy, S.G., O'Leary, M.A., Sonenberg, N. and Hay, N. (1998) 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev*, **12**, 502-513.

- Gleave, M., Miyake, H., Zangemeister-Wittke, U. and Jansen, B. (2002) Antisense therapy: current status in prostate cancer and other malignancies. *Cancer Metastasis Rev*, **21**, 79-92.
- Golstein, P. (1997) Cell death: TRAIL and its receptors. *Curr Biol*, **7**, R750-753.
- Gonzalez-Polo, R.A., Boya, P., Pauleau, A.L., Jalil, A., Larochette, N., Souquere, S., Eskelinen, E.L., Pierron, G., Saftig, P. and Kroemer, G. (2005) The apoptosis/autophagy paradox: autophagic vacuolization before apoptotic death. *J Cell Sci*, **118**, 3091-3102.
- Granados-Soto, V., Arguelles, C.F. and Ortiz, M.I. (2002) The peripheral antinociceptive effect of resveratrol is associated with activation of potassium channels. *Neuropharmacology*, **43**, 917-923.
- Green, D.R. and Amarante-Mendes, G.P. (1998) The point of no return: mitochondria, caspases, and the commitment to cell death. *Results Probl Cell Differ*, **24**, 45-61.
- Green, D.R. and Kroemer, G. (2004) The pathophysiology of mitochondrial cell death. *Science*, **305**, 626-629.
- Gudkov, A.V. and Komarova, E.A. (2005) Prospective therapeutic applications of p53 inhibitors. *Biochem Biophys Res Commun*, **331**, 726-736.
- Guicciardi, M.E., Deussing, J., Miyoshi, H., Bronk, S.F., Svingen, P.A., Peters, C., Kaufmann, S.H. and Gores, G.J. (2000) Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. *J Clin Invest*, **106**, 1127-1137.
- Guicciardi, M.E., Leist, M. and Gores, G.J. (2004) Lysosomes in cell death. *Oncogene*, **23**, 2881-2890.
- Gusman, J., Malonne, H. and Atassi, G. (2001) A reappraisal of the potential chemopreventive and chemotherapeutic properties of resveratrol. *Carcinogenesis*, **22**, 1111-1117.
- Haider, U.G., Sorescu, D., Griendling, K.K., Vollmar, A.M. and Dirsch, V.M. (2002) Resveratrol suppresses angiotensin II-induced Akt/protein kinase B and p70 S6 kinase phosphorylation and subsequent hypertrophy in rat aortic smooth muscle cells. *Mol Pharmacol*, **62**, 772-777.
- Hantschel, M., Pfister, K., Jordan, A., Scholz, R., Andreesen, R., Schmitz, G., Schmetzer, H., Hiddemann, W. and Multhoff, G. (2000) Hsp70 plasma membrane expression on primary tumor biopsy material and bone marrow of leukemic patients. *Cell Stress Chaperones*, **5**, 438-442.

- Heinrich, M., Neumeyer, J., Jakob, M., Hallas, C., Tchikov, V., Winoto-Morbach, S., Wickel, M., Schneider-Brachert, W., Trauzold, A., Hethke, A. and Schutze, S. (2004) Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. *Cell Death Differ*, **11**, 550-563.
- Hess, C.J., Berkhof, J., Denkers, F., Ossenkoppele, G.J., Schouten, J.P., Oudejans, J.J., Waisfisz, Q. and Schuurhuis, G.J. (2007) Activated intrinsic apoptosis pathway is a key related prognostic parameter in acute myeloid leukemia. *J Clin Oncol*, **25**, 1209-1215.
- Hickman, J.A., Potten, C.S., Merritt, A.J. and Fisher, T.C. (1994) Apoptosis and cancer chemotherapy. *Philos Trans R Soc Lond B Biol Sci*, **345**, 319-325.
- Holian, O., Wahid, S., Atten, M.J. and Attar, B.M. (2002) Inhibition of gastric cancer cell proliferation by resveratrol: role of nitric oxide. *Am J Physiol Gastrointest Liver Physiol*, **282**, G809-816.
- Holmes-McNary, M. and Baldwin, A.S., Jr. (2000) Chemopreventive properties of trans-resveratrol are associated with inhibition of activation of the IkappaB kinase. *Cancer Res*, **60**, 3477-3483.
- Hoyvik, H., Gordon, P.B., Berg, T.O., Stromhaug, P.E. and Seglen, P.O. (1991) Inhibition of autophagic-lysosomal delivery and autophagic lactolysis by asparagine. *J Cell Biol*, **113**, 1305-1312.
- Hsieh, T.C., Burfeind, P., Laud, K., Backer, J.M., Traganos, F., Darzynkiewicz, Z. and Wu, J.M. (1999) Cell cycle effects and control of gene expression by resveratrol in human breast carcinoma cell lines with different metastatic potentials. *Int J Oncol*, **15**, 245-252.
- Hsieh, T.C. and Wu, J.M. (1999) Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. *Exp Cell Res*, **249**, 109-115.
- Hsieh, T.C. and Wu, J.M. (2000) Grape-derived chemopreventive agent resveratrol decreases prostate-specific antigen (PSA) expression in LNCaP cells by an androgen receptor (AR)-independent mechanism. *Anticancer Res*, **20**, 225-228.
- Hu, W. and Kavanagh, J.J. (2003) Anticancer therapy targeting the apoptotic pathway. *Lancet Oncol*, **4**, 721-729.
- Huang, C., Ma, W.Y., Goranson, A. and Dong, Z. (1999) Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. *Carcinogenesis*, **20**, 237-242.
- Hwang, J.T., Ha, J., Park, I.J., Lee, S.K., Baik, H.W., Kim, Y.M. and Park, O.J. (2007) Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway. *Cancer Lett*, **247**, 115-121.

- Inoue, H., Jiang, X.F., Katayama, T., Osada, S., Umesono, K. and Namura, S. (2003) Brain protection by resveratrol and fenofibrate against stroke requires peroxisome proliferator-activated receptor alpha in mice. *Neurosci Lett*, **352**, 203-206.
- Issaeva, N., Bozko, P., Enge, M., Protopopova, M., Verhoef, L.G., Masucci, M., Pramanik, A. and Selivanova, G. (2004) Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med*, **10**, 1321-1328.
- Jaattela, M. (2004) Multiple cell death pathways as regulators of tumour initiation and progression. *Oncogene*, **23**, 2746-2756.
- Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W., Fong, H.H., Farnsworth, N.R., Kinghorn, A.D., Mehta, R.G., Moon, R.C. and Pezzuto, J.M. (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, **275**, 218-220.
- Jang, M. and Pezzuto, J.M. (1999) Cancer chemopreventive activity of resveratrol. *Drugs Exp Clin Res*, **25**, 65-77.
- Jannin, B., Menzel, M., Berlot, J.P., Delmas, D., Lancon, A. and Latruffe, N. (2004) Transport of resveratrol, a cancer chemopreventive agent, to cellular targets: plasmatic protein binding and cell uptake. *Biochem Pharmacol*, **68**, 1113-1118.
- Jansen, B. and Zangemeister-Wittke, U. (2002) Antisense therapy for cancer--the time of truth. *Lancet Oncol*, **3**, 672-683.
- Jiang, W., Bell, C.W. and Pisetsky, D.S. (2007) The relationship between apoptosis and high-mobility group protein 1 release from murine macrophages stimulated with lipopolysaccharide or polyinosinic-polycytidylic acid. *J Immunol*, **178**, 6495-6503.
- Jin, S. and White, E. (2007) Role of autophagy in cancer: management of metabolic stress. *Autophagy*, **3**, 28-31.
- Joe, A.K., Liu, H., Suzui, M., Vural, M.E., Xiao, D. and Weinstein, I.B. (2002) Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. *Clin Cancer Res*, **8**, 893-903.
- Johnstone, R.W., Ruefli, A.A. and Lowe, S.W. (2002) Apoptosis: a link between cancer genetics and chemotherapy. *Cell*, **108**, 153-164.
- Jorgensen, P. and Tyers, M. (2004) How cells coordinate growth and division. *Curr Biol*, **14**, R1014-1027.

- Joyce, J.A. and Hanahan, D. (2004) Multiple roles for cysteine cathepsins in cancer. *Cell Cycle*, **3**, 1516-1619.
- Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y. and Yoshimori, T. (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosomal membranes after processing. *Embo J*, **19**, 5720-5728.
- Kannan, K. and Jain, S.K. (2000) Oxidative stress and apoptosis. *Pathophysiology*, **7**, 153-163.
- Kanzawa, T., Germano, I.M., Komata, T., Ito, H., Kondo, Y. and Kondo, S. (2004) Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ*, **11**, 448-457.
- Kanzawa, T., Kondo, Y., Ito, H., Kondo, S. and Germano, I. (2003) Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. *Cancer Res*, **63**, 2103-2108.
- Karin, M. and Ben-Neriah, Y. (2000) Phosphorylation meets ubiquitination: the control of NF- $\kappa$ B activity. *Annu Rev Immunol*, **18**, 621-663.
- Kataoka, T. (2005) The caspase-8 modulator c-FLIP. *Crit Rev Immunol*, **25**, 31-58.
- Katayama, M., Kawaguchi, T., Berger, M.S. and Pieper, R.O. (2007) DNA damaging agent-induced autophagy produces a cytoprotective adenosine triphosphate surge in malignant glioma cells. *Cell Death Differ*, **14**, 548-558.
- Kaushik, S. and Cuervo, A.M. (2006) Autophagy as a cell-repair mechanism: activation of chaperone-mediated autophagy during oxidative stress. *Mol Aspects Med*, **27**, 444-454.
- Kennedy, S.G., Wagner, A.J., Conzen, S.D., Jordan, J., Bellacosa, A., Tschlis, P.N. and Hay, N. (1997) The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev*, **11**, 701-713.
- Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*, **26**, 239-257.
- Kessel, D. and Reiners, J.J., Jr. (2007) Initiation of apoptosis and autophagy by the Bcl-2 antagonist HA14-1. *Cancer Lett*, **249**, 294-299.
- Khan, N., Afaq, F. and Mukhtar, H. (2008) Cancer chemoprevention through dietary antioxidants: progress and promise. *Antioxid Redox Signal*, **10**, 475-510.
- Kihara, A., Kabeya, Y., Ohsumi, Y. and Yoshimori, T. (2001) Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Rep*, **2**, 330-335.

- Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H. and Peter, M.E. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J*, **14**, 5579-5588.
- Klionsky, D.J. (2007) Autophagy: from phenomenology to molecular understanding in less than a decade. *Nat Rev Mol Cell Biol*, **8**, 931-937.
- Kobayashi, K., Hatano, M., Otaki, M., Ogasawara, T. and Tokuhisa, T. (1999) Expression of a murine homologue of the inhibitor of apoptosis protein is related to cell proliferation. *Proc Natl Acad Sci U S A*, **96**, 1457-1462.
- Komarov, P.G., Komarova, E.A., Kondratov, R.V., Christov-Tselkov, K., Coon, J.S., Chernov, M.V. and Gudkov, A.V. (1999) A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science*, **285**, 1733-1737.
- Komata, T., Kanzawa, T., Takeuchi, H., Germano, I.M., Schreiber, M., Kondo, Y. and Kondo, S. (2003) Antitumour effect of cyclin-dependent kinase inhibitors (p16(INK4A), p18(INK4C), p19(INK4D), p21(WAF1/CIP1) and p27(KIP1)) on malignant glioma cells. *Br J Cancer*, **88**, 1277-1280.
- Koneri, K., Goi, T., Hirono, Y., Katayama, K. and Yamaguchi, A. (2007) Beclin 1 gene inhibits tumor growth in colon cancer cell lines. *Anticancer Res*, **27**, 1453-1457.
- Kops, G.J. and Burgering, B.M. (1999) Forkhead transcription factors: new insights into protein kinase B (c-akt) signaling. *J Mol Med*, **77**, 656-665.
- Kotha, A., Sekharam, M., Cilenti, L., Siddiquee, K., Khaled, A., Zervos, A.S., Carter, B., Turkson, J. and Jove, R. (2006) Resveratrol inhibits Src and Stat3 signaling and induces the apoptosis of malignant cells containing activated Stat3 protein. *Mol Cancer Ther*, **5**, 621-629.
- Kuma, A., Hatano, M., Matsui, M., Yamamoto, A., Nakaya, H., Yoshimori, T., Ohsumi, Y., Tokuhisa, T. and Mizushima, N. (2004) The role of autophagy during the early neonatal starvation period. *Nature*, **432**, 1032-1036.
- Kussie, P.H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A.J. and Pavletich, N.P. (1996) Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science*, **274**, 948-953.
- Lane, D.P. and Lain, S. (2002) Therapeutic exploitation of the p53 pathway. *Trends Mol Med*, **8**, S38-42.
- Le Beau, M.M., Ito, C., Cogswell, P., Espinosa, R., 3rd, Fernald, A.A. and Baldwin, A.S., Jr. (1992) Chromosomal localization of the genes encoding the p50/p105 subunits of NF-kappa B (NFKB2) and the I kappa B/MAD-3 (NFKBI) inhibitor of NF-kappa B to 4q24 and 14q13, respectively. *Genomics*, **14**, 529-531.



- LeBlanc, H.N. and Ashkenazi, A. (2003) Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ*, **10**, 66-75.
- Leu, J.I., Dumont, P., Hafey, M., Murphy, M.E. and George, D.L. (2004) Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. *Nat Cell Biol*, **6**, 443-450.
- Levenson, A.S., Gehm, B.D., Pearce, S.T., Horiguchi, J., Simons, L.A., Ward, J.E., 3rd, Jameson, J.L. and Jordan, V.C. (2003) Resveratrol acts as an estrogen receptor (ER) agonist in breast cancer cells stably transfected with ER alpha. *Int J Cancer*, **104**, 587-596.
- Levine, B. and Klionsky, D.J. (2004) Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*, **6**, 463-477.
- Li, H., Wang, X., Li, N., Qiu, J., Zhang, Y. and Cao, X. (2007) hPEBP4 resists TRAIL-induced apoptosis of human prostate cancer cells by activating Akt and deactivating ERK1/2 pathways. *J Biol Chem*, **282**, 4943-4950.
- Li, Y.Q., Li, Z.L., Zhao, W.J., Wen, R.X., Meng, Q.W. and Zeng, Y. (2006) Synthesis of stilbene derivatives with inhibition of SARS coronavirus replication. *Eur J Med Chem*, **41**, 1084-1089.
- Li, Z.G., Hong, T., Shimada, Y., Komoto, I., Kawabe, A., Ding, Y., Kaganoi, J., Hashimoto, Y. and Imamura, M. (2002) Suppression of N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol. *Carcinogenesis*, **23**, 1531-1536.
- Liang, C., Feng, P., Ku, B., Dotan, I., Canaani, D., Oh, B.H. and Jung, J.U. (2006) Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat Cell Biol*, **8**, 688-699.
- Liang, X.H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H. and Levine, B. (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature*, **402**, 672-676.
- Liang, Y.C., Tsai, S.H., Chen, L., Lin-Shiau, S.Y. and Lin, J.K. (2003) Resveratrol-induced G2 arrest through the inhibition of CDK7 and p34CDC2 kinases in colon carcinoma HT29 cells. *Biochem Pharmacol*, **65**, 1053-1060.
- Lima, R.T., Martins, L.M., Guimaraes, J.E., Sambade, C. and Vasconcelos, M.H. (2004) Specific downregulation of bcl-2 and xIAP by RNAi enhances the effects of chemotherapeutic agents in MCF-7 human breast cancer cells. *Cancer Gene Ther*, **11**, 309-316.
- Lindsten, T., Ross, A.J., King, A., Zong, W.X., Rathmell, J.C., Shiels, H.A., Ulrich, E., Waymire, K.G., Mahar, P., Frauwirth, K., Chen, Y., Wei, M., Eng, V.M., Adelman, D.M., Simon, M.C., Ma, A., Golden, J.A., Evan, G., Korsmeyer, S.J., MacGregor, G.R. and Thompson, C.B. (2000) The combined functions of

- proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell*, **6**, 1389-1399.
- Liu, S.S., Tsang, B.K., Cheung, A.N., Xue, W.C., Cheng, D.K., Ng, T.Y., Wong, L.C. and Ngan, H.Y. (2001) Anti-apoptotic proteins, apoptotic and proliferative parameters and their prognostic significance in cervical carcinoma. *Eur J Cancer*, **37**, 1104-1110.
- Locksley, R.M., Killeen, N. and Lenardo, M.J. (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*, **104**, 487-501.
- Lu, R. and Serrero, G. (1999) Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. *J Cell Physiol*, **179**, 297-304.
- Luciani, F., Spada, M., De Milito, A., Molinari, A., Rivoltini, L., Montinaro, A., Marra, M., Lugini, L., Logozzi, M., Lozupone, F., Federici, C., Iessi, E., Parmiani, G., Arancia, G., Belardelli, F. and Fais, S. (2004) Effect of proton pump inhibitor pretreatment on resistance of solid tumors to cytotoxic drugs. *J Natl Cancer Inst*, **96**, 1702-1713.
- Lum, J.J., Bauer, D.E., Kong, M., Harris, M.H., Li, C., Lindsten, T. and Thompson, C.B. (2005) Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell*, **120**, 237-248.
- Lum, J.J., Schnepfle, D.J., Nie, Z., Sanchez-Dardon, J., Mbisa, G.L., Mihowich, J., Hawley, N., Narayan, S., Kim, J.E., Lynch, D.H. and Badley, A.D. (2004) Differential effects of interleukin-7 and interleukin-15 on NK cell anti-human immunodeficiency virus activity. *J Virol*, **78**, 6033-6042.
- Lunemann, J.D., Schmidt, J., Schmid, D., Barthel, K., Wrede, A., Dalakas, M.C. and Munz, C. (2007) Beta-amyloid is a substrate of autophagy in sporadic inclusion body myositis. *Ann Neurol*, **61**, 476-483.
- Madar, Z., Kalet-Litman, S. and Stark, A.H. (2005) Inducible nitric oxide synthase activity and expression in liver and hepatocytes of diabetic rats. *Pharmacology*, **73**, 106-112.
- Mahyar-Roemer, M., Katsen, A., Mestres, P. and Roemer, K. (2001) Resveratrol induces colon tumor cell apoptosis independently of p53 and precede by epithelial differentiation, mitochondrial proliferation and membrane potential collapse. *Int J Cancer*, **94**, 615-622.
- Mahyar-Roemer, M., Kohler, H. and Roemer, K. (2002) Role of Bax in resveratrol-induced apoptosis of colorectal carcinoma cells. *BMC Cancer*, **2**, 27.
- Maiuri, M.C., Le Toumelin, G., Criollo, A., Rain, J.C., Gautier, F., Juin, P., Tasdemir, E., Pierron, G., Troulinaki, K., Tavernarakis, N., Hickman, J.A., Geneste, O. and Kroemer, G. (2007a) Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. *Embo J*, **26**, 2527-2539.

- Maiuri, M.C., Zalckvar, E., Kimchi, A. and Kroemer, G. (2007b) Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol*, **8**, 741-752.
- Majno, G. and Joris, I. (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol*, **146**, 3-15.
- Marsters, S.A., Sheridan, J.P., Pitti, R.M., Brush, J., Goddard, A. and Ashkenazi, A. (1998) Identification of a ligand for the death-domain-containing receptor Apo3. *Curr Biol*, **8**, 525-528.
- Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M. and Green, D.R. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med*, **182**, 1545-1556.
- Martinez-Vicente, M. and Cuervo, A.M. (2007) Autophagy and neurodegeneration: when the cleaning crew goes on strike. *Lancet Neurol*, **6**, 352-361.
- Mathew, R., Karantza-Wadsworth, V. and White, E. (2007) Role of autophagy in cancer. *Nat Rev Cancer*, **7**, 961-967.
- McManus, D.C., Lefebvre, C.A., Cherton-Horvat, G., St-Jean, M., Kandimalla, E.R., Agrawal, S., Morris, S.J., Durkin, J.P. and Lacasse, E.C. (2004) Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics. *Oncogene*, **23**, 8105-8117.
- Meijer, A.J. and Codogno, P. (2004) Regulation and role of autophagy in mammalian cells. *Int J Biochem Cell Biol*, **36**, 2445-2462.
- Mendrysa, S.M., McElwee, M.K., Michalowski, J., O'Leary, K.A., Young, K.M. and Perry, M.E. (2003) mdm2 Is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. *Mol Cell Biol*, **23**, 462-472.
- Meurette, O., Lefevre-Orfila, L., Rebillard, A., Lagadic-Gossmann, D. and Dimanche-Boitrel, M.T. (2005) Role of intracellular glutathione in cell sensitivity to the apoptosis induced by tumor necrosis factor {alpha}-related apoptosis-inducing ligand/anticancer drug combinations. *Clin Cancer Res*, **11**, 3075-3083.
- Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P. and Moll, U.M. (2003) p53 has a direct apoptogenic role at the mitochondria. *Mol Cell*, **11**, 577-590.
- Mitchell, K.O. and El-Deiry, W.S. (1999) Overexpression of c-Myc inhibits p21WAF1/CIP1 expression and induces S-phase entry in 12-O-tetradecanoylphorbol-13-acetate (TPA)-sensitive human cancer cells. *Cell Growth Differ*, **10**, 223-230.

- Mitsiades, C.S., Mitsiades, N. and Koutsilieris, M. (2004) The Akt pathway: molecular targets for anti-cancer drug development. *Curr Cancer Drug Targets*, **4**, 235-256.
- Mizushima, N. and Klionsky, D.J. (2007) Protein turnover via autophagy: implications for metabolism. *Annu Rev Nutr*, **27**, 19-40.
- Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhiya, T., Ohsumi, Y. and Yoshimori, T. (2001) Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol*, **152**, 657-668.
- Mohan, J., Gandhi, A.A., Bhavya, B.C., Rashmi, R., Karunagaran, D., Indu, R. and Santhoshkumar, T.R. (2006) Caspase-2 triggers Bax-Bak-dependent and -independent cell death in colon cancer cells treated with resveratrol. *J Biol Chem*, **281**, 17599-17611.
- Momand, J., Wu, H.H. and Dasgupta, G. (2000) MDM2--master regulator of the p53 tumor suppressor protein. *Gene*, **242**, 15-29.
- Munafo, D.B. and Colombo, M.I. (2001) A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. *J Cell Sci*, **114**, 3619-3629.
- Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S. and Dixit, V.M. (1998) An induced proximity model for caspase-8 activation. *J Biol Chem*, **273**, 2926-2930.
- Nishino, I. (2006) Autophagic vacuolar myopathy. *Semin Pediatr Neurol*, **13**, 90-95.
- Nylandsted, J., Gyrd-Hansen, M., Danielewicz, A., Fehrenbacher, N., Lademann, U., Hoyer-Hansen, M., Weber, E., Multhoff, G., Rohde, M. and Jaattela, M. (2004) Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. *J Exp Med*, **200**, 425-435.
- Nylandsted, J., Rohde, M., Brand, K., Bastholm, L., Elling, F. and Jaattela, M. (2000) Selective depletion of heat shock protein 70 (Hsp70) activates a tumor-specific death program that is independent of caspases and bypasses Bcl-2. *Proc Natl Acad Sci U S A*, **97**, 7871-7876.
- Nylandsted, J., Wick, W., Hirt, U.A., Brand, K., Rohde, M., Leist, M., Weller, M. and Jaattela, M. (2002) Eradication of glioblastoma, and breast and colon carcinoma xenografts by Hsp70 depletion. *Cancer Res*, **62**, 7139-7142.
- Ogier-Denis, E. and Codogno, P. (2003) Autophagy: a barrier or an adaptive response to cancer. *Biochim Biophys Acta*, **1603**, 113-128.
- Olsson, A., Diaz, T., Aguilar-Santelises, M., Osterborg, A., Celsing, F., Jondal, M. and Osorio, L.M. (2001) Sensitization to TRAIL-induced apoptosis and

- modulation of FLICE-inhibitory protein in B chronic lymphocytic leukemia by actinomycin D. *Leukemia*, **15**, 1868-1877.
- Ono, K., Kim, S.O. and Han, J. (2003) Susceptibility of lysosomes to rupture is a determinant for plasma membrane disruption in tumor necrosis factor alpha-induced cell death. *Mol Cell Biol*, **23**, 665-676.
- Opipari, A.W., Jr., Tan, L., Boitano, A.E., Sorenson, D.R., Aurora, A. and Liu, J.R. (2004) Resveratrol-induced autophagocytosis in ovarian cancer cells. *Cancer Res*, **64**, 696-703.
- Paglin, S., Hollister, T., Delohery, T., Hackett, N., McMahill, M., Sphicas, E., Domingo, D. and Yahalom, J. (2001) A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res*, **61**, 439-444.
- Pahl, H.L. (1999) Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene*, **18**, 6853-6866.
- Pannone, G., Bufo, P., Serpico, R., Rubini, C., Zamparese, R., Corsi, F., Pedicillo, M.C., Pannone, G., Staibano, S., De Rosa, G. and Lo Muzio, L. (2007) Survivin phosphorylation and M-phase promoting factor in oral carcinogenesis. *Histol Histopathol*, **22**, 1241-1249.
- Park, J.W., Choi, Y.J., Suh, S.I., Baek, W.K., Suh, M.H., Jin, I.N., Min, D.S., Woo, J.H., Chang, J.S., Passaniti, A., Lee, Y.H. and Kwon, T.K. (2001) Bcl-2 overexpression attenuates resveratrol-induced apoptosis in U937 cells by inhibition of caspase-3 activity. *Carcinogenesis*, **22**, 1633-1639.
- Parks, D.J., Lafrance, L.V., Calvo, R.R., Milkiewicz, K.L., Gupta, V., Lattanze, J., Ramachandren, K., Carver, T.E., Petrella, E.C., Cummings, M.D., Maguire, D., Grasberger, B.L. and Lu, T. (2005) 1,4-Benzodiazepine-2,5-diones as small molecule antagonists of the HDM2-p53 interaction: discovery and SAR. *Bioorg Med Chem Lett*, **15**, 765-770.
- Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D. and Levine, B. (2005) Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell*, **122**, 927-939.
- Perlmutter, D.H. (2006) The role of autophagy in alpha-1-antitrypsin deficiency: a specific cellular response in genetic diseases associated with aggregation-prone proteins. *Autophagy*, **2**, 258-263.
- Peter, M.E. and Krammer, P.H. (2003) The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ*, **10**, 26-35.
- Pozo-Guisado, E., Alvarez-Barrientos, A., Mulero-Navarro, S., Santiago-Josefat, B. and Fernandez-Salguero, P.M. (2002) The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. *Biochem Pharmacol*, **64**, 1375-1386.

- Qiu, Y., Waters, C.E., Lewis, A.E., Langman, M.J. and Eggo, M.C. (2002) Oestrogen-induced apoptosis in colonocytes expressing oestrogen receptor beta. *J Endocrinol*, **174**, 369-377.
- Qu, X., Yu, J., Bhagat, G., Furuya, N., Hibshoosh, H., Troxel, A., Rosen, J., Eskelinen, E.L., Mizushima, N., Ohsumi, Y., Cattoretti, G. and Levine, B. (2003) Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest*, **112**, 1809-1820.
- Ramp, U., Krieg, T., Caliskan, E., Mahotka, C., Ebert, T., Willers, R., Gabbert, H.E. and Gerharz, C.D. (2004) XIAP expression is an independent prognostic marker in clear-cell renal carcinomas. *Hum Pathol*, **35**, 1022-1028.
- Regev-Shoshani, G., Shoseyov, O., Bilkis, I. and Kerem, Z. (2003) Glycosylation of resveratrol protects it from enzymic oxidation. *Biochem J*, **374**, 157-163.
- Rohde, M., Dugaard, M., Jensen, M.H., Helin, K., Nylandsted, J. and Jaattela, M. (2005) Members of the heat-shock protein 70 family promote cancer cell growth by distinct mechanisms. *Genes Dev*, **19**, 570-582.
- Roshy, S., Sloane, B.F. and Moin, K. (2003) Pericellular cathepsin B and malignant progression. *Cancer Metastasis Rev*, **22**, 271-286.
- Rowinsky, E.K. (2004) Targeting the molecular target of rapamycin (mTOR). *Curr Opin Oncol*, **16**, 564-575.
- Rubinsztein, D.C., Gestwicki, J.E., Murphy, L.O. and Klionsky, D.J. (2007) Potential therapeutic applications of autophagy. *Nat Rev Drug Discov*, **6**, 304-312.
- Russo, V.C., Kobayashi, K., Najdovska, S., Baker, N.L. and Werther, G.A. (2004) Neuronal protection from glucose deprivation via modulation of glucose transport and inhibition of apoptosis: a role for the insulin-like growth factor system. *Brain Res*, **1009**, 40-53.
- Rusten, T.E., Lindmo, K., Juhasz, G., Sass, M., Seglen, P.O., Brech, A. and Stenmark, H. (2004) Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. *Dev Cell*, **7**, 179-192.
- Sale, S., Verschoyle, R.D., Boocock, D., Jones, D.J., Wilsher, N., Ruparelia, K.C., Potter, G.A., Farmer, P.B., Steward, W.P. and Gescher, A.J. (2004) Pharmacokinetics in mice and growth-inhibitory properties of the putative cancer chemopreventive agent resveratrol and the synthetic analogue trans 3,4,5,4'-tetramethoxystilbene. *Br J Cancer*, **90**, 736-744.
- Sarkar, S., Perlstein, E.O., Imarisio, S., Pineau, S., Cordenier, A., Maglathlin, R.L., Webster, J.A., Lewis, T.A., O'Kane, C.J., Schreiber, S.L. and Rubinsztein, D.C. (2007) Small molecules enhance autophagy and reduce toxicity in Huntington's disease models. *Nat Chem Biol*, **3**, 331-338.

- Savill, J. and Fadok, V. (2000) Corpse clearance defines the meaning of cell death. *Nature*, **407**, 784-788.
- Scaffidi, P., Misteli, T. and Bianchi, M.E. (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*, **418**, 191-195.
- Schatzkin, A. and Kelloff, G. (1995) Chemo- and dietary prevention of colorectal cancer. *Eur J Cancer*, **31A**, 1198-1204.
- Schmitt, E., Parcellier, A., Gurbuxani, S., Cande, C., Hammann, A., Morales, M.C., Hunt, C.R., Dix, D.J., Kroemer, R.T., Giordanetto, F., Jaattela, M., Penninger, J.M., Pance, A., Kroemer, G. and Garrido, C. (2003) Chemosensitization by a non-apoptogenic heat shock protein 70-binding apoptosis-inducing factor mutant. *Cancer Res*, **63**, 8233-8240.
- Schneider, Y., Durantou, B., Gosse, F., Schleiffer, R., Seiler, N. and Raul, F. (2001) Resveratrol inhibits intestinal tumorigenesis and modulates host-defense-related gene expression in an animal model of human familial adenomatous polyposis. *Nutr Cancer*, **39**, 102-107.
- Schneider, Y., Vincent, F., Durantou, B., Badolo, L., Gosse, F., Bergmann, C., Seiler, N. and Raul, F. (2000) Anti-proliferative effect of resveratrol, a natural component of grapes and wine, on human colonic cancer cells. *Cancer Lett*, **158**, 85-91.
- Schotte, P., Declercq, W., Van Huffel, S., Vandenabeele, P. and Beyaert, R. (1999) Non-specific effects of methyl ketone peptide inhibitors of caspases. *FEBS Lett*, **442**, 117-121.
- Scorrano, L. and Korsmeyer, S.J. (2003) Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. *Biochem Biophys Res Commun*, **304**, 437-444.
- Scorrano, L., Oakes, S.A., Opferman, J.T., Cheng, E.H., Sorcinelli, M.D., Pozzan, T. and Korsmeyer, S.J. (2003) BAX and BAK regulation of endoplasmic reticulum Ca<sup>2+</sup>: a control point for apoptosis. *Science*, **300**, 135-139.
- Scott, R.C., Schuldiner, O. and Neufeld, T.P. (2004) Role and regulation of starvation-induced autophagy in the *Drosophila* fat body. *Dev Cell*, **7**, 167-178.
- Seglen, P.O. and Gordon, P.B. (1982) 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc Natl Acad Sci U S A*, **79**, 1889-1892.
- Seglen, P.O., Gordon, P.B. and Poli, A. (1980) Amino acid inhibition of the autophagic/lysosomal pathway of protein degradation in isolated rat hepatocytes. *Biochim Biophys Acta*, **630**, 103-118.
- Seligson, D.B., Hongo, F., Huerta-Yepez, S., Mizutani, Y., Miki, T., Yu, H., Horvath, S., Chia, D., Goodglick, L. and Bonavida, B. (2007) Expression of X-linked

- inhibitor of apoptosis protein is a strong predictor of human prostate cancer recurrence. *Clin Cancer Res*, **13**, 6056-6063.
- Seoane, J., Le, H.V. and Massague, J. (2002) Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature*, **419**, 729-734.
- Shankar, S. and Srivastava, R.K. (2007) Bax and Bak genes are essential for maximum apoptotic response by curcumin, a polyphenolic compound and cancer chemopreventive agent derived from turmeric, *Curcuma longa*. *Carcinogenesis*, **28**, 1277-1286.
- Sharma, S., Kulkarni, S.K. and Chopra, K. (2006) Resveratrol, a polyphenolic phytoalexin attenuates thermal hyperalgesia and cold allodynia in STZ-induced diabetic rats. *Indian J Exp Biol*, **44**, 566-569.
- She, Q.B., Bode, A.M., Ma, W.Y., Chen, N.Y. and Dong, Z. (2001) Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase. *Cancer Res*, **61**, 1604-1610.
- Shih, A., Davis, F.B., Lin, H.Y. and Davis, P.J. (2002) Resveratrol induces apoptosis in thyroid cancer cell lines via a MAPK- and p53-dependent mechanism. *J Clin Endocrinol Metab*, **87**, 1223-1232.
- Shimizu, S., Kanaseki, T., Mizushima, N., Mizuta, T., Arakawa-Kobayashi, S., Thompson, C.B. and Tsujimoto, Y. (2004) Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nat Cell Biol*, **6**, 1221-1228.
- Shintani, T. and Klionsky, D.J. (2004) Autophagy in health and disease: a double-edged sword. *Science*, **306**, 990-995.
- Srivastava, R.K., Mi, Q.S., Hardwick, J.M. and Longo, D.L. (1999) Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis. *Proc Natl Acad Sci U S A*, **96**, 3775-3780.
- Stefani, M. and Dobson, C.M. (2003) Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. *J Mol Med*, **81**, 678-699.
- Stoka, V., Turk, B. and Turk, V. (2005) Lysosomal cysteine proteases: structural features and their role in apoptosis. *IUBMB Life*, **57**, 347-353.
- Subbaramaiah, K., Chung, W.J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J.M. and Dannenberg, A.J. (1998) Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *J Biol Chem*, **273**, 21875-21882.
- Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T. and Ohsumi, Y. (2001) The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *Embo J*, **20**, 5971-5981.



- Suzuki, M., Youle, R.J. and Tjandra, N. (2000) Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell*, **103**, 645-654.
- Tamm, I., Kornblau, S.M., Segall, H., Krajewski, S., Welsh, K., Kitada, S., Scudiero, D.A., Tudor, G., Qui, Y.H., Monks, A., Andreeff, M. and Reed, J.C. (2000) Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. *Clin Cancer Res*, **6**, 1796-1803.
- Terman, A. and Brunk, U.T. (2005) Autophagy in cardiac myocyte homeostasis, aging, and pathology. *Cardiovasc Res*, **68**, 355-365.
- Tessitore, L., Davit, A., Sarotto, I. and Caderni, G. (2000) Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and p21(CIP) expression. *Carcinogenesis*, **21**, 1619-1622.
- Tinhofer, I., Bernhard, D., Senfter, M., Anether, G., Loeffler, M., Kroemer, G., Kofler, R., Csordas, A. and Greil, R. (2001) Resveratrol, a tumor-suppressive compound from grapes, induces apoptosis via a novel mitochondrial pathway controlled by Bcl-2. *Faseb J*, **15**, 1613-1615.
- Trincheri, N.F., Nicotra, G., Follo, C., Castino, R. and Isidoro, C. (2007) Resveratrol induces cell death in colorectal cancer cells by a novel pathway involving lysosomal cathepsin D. *Carcinogenesis*, **28**, 922-931.
- Tseng, S.H., Lin, S.M., Chen, J.C., Su, Y.H., Huang, H.Y., Chen, C.K., Lin, P.Y. and Chen, Y. (2004) Resveratrol suppresses the angiogenesis and tumor growth of gliomas in rats. *Clin Cancer Res*, **10**, 2190-2202.
- Tsuneoka, M., Umata, T., Kimura, H., Koda, Y., Nakajima, M., Kosai, K., Takahashi, T., Takahashi, Y. and Yamamoto, A. (2003) c-myc induces autophagy in rat 3Y1 fibroblast cells. *Cell Struct Funct*, **28**, 195-204.
- Ulbrich, K. and Subr, V. (2004) Polymeric anticancer drugs with pH-controlled activation. *Adv Drug Deliv Rev*, **56**, 1023-1050.
- Ullenhag, G.J., Frodin, J.E., Strigard, K., Mellstedt, H. and Magnusson, C.G. (2002) Induction of IgG subclass responses in colorectal carcinoma patients vaccinated with recombinant carcinoembryonic antigen. *Cancer Res*, **62**, 1364-1369.
- Valente, G., Manfroi, F., Peracchio, C., Nicotra, G., Castino, R., Nicosia, G., Kerim, S. and Isidoro, C. (2006) cFLIP expression correlates with tumour progression and patient outcome in non-Hodgkin lymphomas of low grade of malignancy. *Br J Haematol*, **132**, 560-570.
- Varambally, S., Yu, J., Laxman, B., Rhodes, D.R., Mehra, R., Tomlins, S.A., Shah, R.B., Chandran, U., Monzon, F.A., Becich, M.J., Wei, J.T., Pienta, K.J., Ghosh, D., Rubin, M.A. and Chinnaiyan, A.M. (2005) Integrative genomic

- and proteomic analysis of prostate cancer reveals signatures of metastatic progression. *Cancer Cell*, **8**, 393-406.
- Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, R.L., Simpson, R.J. and Vaux, D.L. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell*, **102**, 43-53.
- Vyas, S., Asmerom, Y. and De Leon, D.D. (2006) Insulin-like growth factor II mediates resveratrol stimulatory effect on cathepsin D in breast cancer cells. *Growth Factors*, **24**, 79-87.
- Walczak, H., Miller, R.E., Ariail, K., Gliniak, B., Griffith, T.S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R.G., Rauch, C.T., Schuh, J.C. and Lynch, D.H. (1999) Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med*, **5**, 157-163.
- Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B. and Korsmeyer, S.J. (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science*, **292**, 727-730.
- Williams, A., Jahreiss, L., Sarkar, S., Saiki, S., Menzies, F.M., Ravikumar, B. and Rubinsztein, D.C. (2006) Aggregate-prone proteins are cleared from the cytosol by autophagy: therapeutic implications. *Curr Top Dev Biol*, **76**, 89-101.
- Willis, A.C. and Chen, X. (2002) The promise and obstacle of p53 as a cancer therapeutic agent. *Curr Mol Med*, **2**, 329-345.
- Willis, S.N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J.I., Adams, J.M. and Huang, D.C. (2005) Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev*, **19**, 1294-1305.
- Wolter, F., Akoglu, B., Clausnitzer, A. and Stein, J. (2001) Downregulation of the cyclin D1/Cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines. *J Nutr*, **131**, 2197-2203.
- Wolter, F. and Stein, J. (2002) Resveratrol enhances the differentiation induced by butyrate in caco-2 colon cancer cells. *J Nutr*, **132**, 2082-2086.
- Workman, P. (2004) Inhibiting the phosphoinositide 3-kinase pathway for cancer treatment. *Biochem Soc Trans*, **32**, 393-396.
- Wu, M., Yuan, S., Szporn, A.H., Gan, L., Shtilbans, V. and Burstein, D.E. (2005) Immunocytochemical detection of XIAP in body cavity effusions and washes. *Mod Pathol*, **18**, 1618-1622.
- Yamamoto, K., Abe, S., Nakagawa, Y., Suzuki, K., Hasegawa, M., Inoue, M., Kurata, M., Hirokawa, K. and Kitagawa, M. (2004) Expression of IAP family proteins

- in myelodysplastic syndromes transforming to overt leukemia. *Leuk Res*, **28**, 1203-1211.
- Yorimitsu, T. and Klionsky, D.J. (2005) Autophagy: molecular machinery for self-eating. *Cell Death Differ*, **12 Suppl 2**, 1542-1552.
- Yousefi, S., Perozzo, R., Schmid, I., Ziemiecki, A., Schaffner, T., Scapozza, L., Brunner, T. and Simon, H.U. (2006) Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat Cell Biol*, **8**, 1124-1132.
- Yue, Z., Horton, A., Bravin, M., DeJager, P.L., Selimi, F. and Heintz, N. (2002) A novel protein complex linking the delta 2 glutamate receptor and autophagy: implications for neurodegeneration in lurcher mice. *Neuron*, **35**, 921-933.
- Zhang, L., Yu, J., Park, B.H., Kinzler, K.W. and Vogelstein, B. (2000) Role of BAX in the apoptotic response to anticancer agents. *Science*, **290**, 989-992.
- Zhang, S., Lin, Y., Kim, Y.S., Hande, M.P., Liu, Z.G. and Shen, H.M. (2007) c-Jun N-terminal kinase mediates hydrogen peroxide-induced cell death via sustained poly(ADP-ribose) polymerase-1 activation. *Cell Death Differ*, **14**, 1001-1010.