

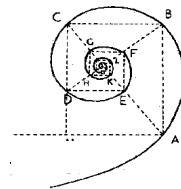


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# ***ROLE OF DIHYDROCERAMIDE IN CELL SIGNALING***

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A Giulia,  
che non mi ha mai  
fatto mancare il  
suo amore.

## SOMMARIO

*Gli sfingolipidi sono una classe di lipidi bioattivi, al cui centro del complicato pathway biosintetico si trova il ceramide: una molecola bioattiva implicata nella regolazione di una varietà di funzioni fisiologiche che includono l'apoptosi, l'arresto della crescita cellulare, la differenziazione, la senescenza, la migrazione e l'adesione delle cellule. Il diidroceramide è l'immediato precursore lungo la via di sintesi de novo del ceramide ed è stato a lungo considerato una molecola inerte e priva di funzione nel sistema di segnalazione intracellulare. Solo recentemente tale molecola è stata associata alla regolazione di processi cellulari quali l'arresto del ciclo cellulare e la morte cellulare programmata. D'altra parte è stata anche dimostrata l'attività anti-apoptotica di questa molecola. Lo scopo del mio lavoro di tesi è stato quello di dimostrare l'attività del diidroceramide nella regolazione di processi implicati nella sopravvivenza cellulare agli stress. A tal fine cellule HGC-27 sono state trattate con resveratrolo, una molecola che stimola l'accumulo di diidroceramide in quanto capace di inibirne la sua desaturazione. Il resveratrolo è un polifenolo con note proprietà antiossidanti e antitumorali, ma è anche in grado di promuovere la sopravvivenza cellulare in quanto, mimando una situazione di ridotto apporto calorico (calorie restriction), attiva le sirtuine. Diversi studi hanno dimostrato che questa molecola modula la via biosintetica de novo del ceramide. Nel mio lavoro viene dimostrato che il trattamento con resveratrolo induce l'accumulo di diidroceramide tramite l'inibizione della sua desaturazione da parte dell'enzima DEGS-1, in modo paragonabile all'inibitore specifico XM462. Tale accumulo si accompagna all'attivazione dell'autofagia non associata a morte cellulare. Inoltre una risposta simile è attivata a seguito di diversi stimoli o trattamenti accomunati dalla loro capacità di indurre accumulo di diidroceramide e in diverse linee cellulari. L'induzione autofagica, pur non incidendo sulla vitalità, ha mostrato avere un effetto di rallentamento della proliferazione. Infatti abbiamo dimostrato che l'accumulo di diidroceramide in cellule HGC-27 induce un arresto temporaneo del ciclo cellulare in fase G0/G1 che si traduce in un rallentamento della progressione delle fasi del ciclo. Sia l'attivazione dell'autofagia che l'arresto del ciclo cellulare sono temporalmente successivi alla risposta a proteine unfolded (UPR) attivata a seguito dello stress del reticolo endoteliale causato dall'accumulo di diidroceramide. Tali risultati offrono un importante contributo alla comprensione del ruolo metabolico dei mediatori sfingolipidici e della loro considerazione nella terapia antitumorale.*

## ABSTRACT

*Sphingolipids are a class of bioactive lipids. Ceramide is the hub molecule of the intricate sphingolipid biosynthetic pathway. It is a bioactive lipid, regulating a number of physiological functions such as apoptosis, cell growth arrest, differentiation, senescence, migration and adhesion. The dihydroceramide is the precursor of ceramide along the de novo biosynthetic pathway. Several groups considered the dihydroceramide an inactive molecule. Recent studies associated dihydroceramide with the induction of cellular processes such as cell cycle arrest and programmed cell death. On the other hand this molecule also showed anti-apoptotic properties. The goal of my doctorate project is to demonstrate the dihydroceramide implication in the regulation of a pro-survival cell response to stress. Hence, the human gastric carcinoma HGC-27 cells were treated with resveratrol, a molecule that induces dihydroceramide accumulation. Resveratrol is a polyphenol with well known anti-oxidant and anti-tumoral properties. On the other hand it is a calorie restriction mimetic, thus it activates sirtuins, promoting cell survival. A number of studies demonstrated that resveratrol modulates the biosynthetic de novo pathway of ceramide. Here we demonstrated that resveratrol, similarly to the specific inhibitor XM462, inhibits DEGS-1 desaturase activity, inducing dihydroceramide accumulation. This accumulation results in autophagy induction without affecting cell viability. We also obtained this response treating different cell lines with several drugs or conditions known to induce dihydroceramide accumulation. Although autophagy did not affect cell viability, however it causes a delay in cell proliferation. In fact we demonstrated that dihydroceramide accumulation in HGC-27 cells induces a temporary arrest in G0/G1 phase resulting in the delayed cell cycle phases progression. Moreover, we demonstrated that both autophagy activation and cell cycle arrest are temporally subsequent to the unfolded protein response (UPR) which is due to the dihydroceramide accumulation-mediated ER stress. All these results give an important contribute to the understanding of the metabolic role of sphingolipid mediators and their targeting in the anti-tumoral therapy.*

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## SIMBOLS LIST

|                                |   |
|--------------------------------|---|
| <i>3K5aR</i>                   | <i>3-ketodihydrosphingosine reductase</i>                 |
| <i>3MA</i>                     | <i>3-methyladenine</i>                                    |
| <i>4-HPR</i>                   | <i>fenretinide</i>  |
| <i>ATF</i>                     | <i>activating transcription factor</i>                    |
| <i>Atg</i>                     | <i>autophagy-related genes</i>                            |
| <i>Bad</i>                     | <i>BCL2 Antagonist of Cell Death</i>                      |
| <i>Bax</i>                     | <i>BCL (B Cell Lymphoma)-Associated X</i>                 |
| <i>Bcl-2</i>                   | <i>B-Cell CLL/Lymphoma 2</i>                              |
| <i>BiP</i>                     | <i>mBinding immunoglobulin protein</i>                    |
| <i>C/EBP<math>\beta</math></i> | <i>CCAAT/enhancer binding protein</i>                     |
| <i>Cdase</i>                   | <i>Ceramidases</i>  |
| <i>CDK</i>                     | <i>cyclin-dependant kinases</i>                           |
| <i>CERS</i>                    | <i>ceramide synthases</i>                                 |
| <i>CHOP</i>                    | <i>C/EBP homologous protein 10</i>                        |
| <i>CK</i>                      | <i>Ceramide kinase</i>                                    |
| <i>CKI</i>                     | <i>CDK inhibitor protein</i>                              |
| <i>CoA</i>                     | <i>coenzyme A</i>   |
| <i>d2-C8-dhCER</i>             | <i>dideuterated C8-dihydroceramide</i>                    |
| <i>(dh)CERS</i>                | <i>(Dihydro)ceramide synthases</i>                        |
| <i>DEGS</i>                    | <i>dihydroceramide desaturase</i>                         |
| <i>dhGalCER</i>                | <i>dihydrogalactosylceramide</i>                          |
| <i>dhGlcCER</i>                | <i>dihydroglucosylceramide</i>                            |
| <i>dhSM</i>                    | <i>dihydrosphingomyelin</i>                               |
| <i>DOC</i>                     | <i>downstream of CHOP</i>                                 |
| <i>eIF2<math>\alpha</math></i> | <i>eukaryotic initiation factor 2 alpha</i>               |
| <i>ER</i>                      | <i>endoplasmic reticulum</i>                              |
| <i>ERAD</i>                    | <i>ER-associated degradation</i>                          |
| <i>ERK</i>                     | <i>Extracellular Signal-Regulated Kinase</i>              |
| <i>Etp</i>                     | <i>etoposide</i>  |
| <i>FIP200</i>                  | <i>family-interacting protein of 200 kDa</i>              |
| <i>GADD153</i>                 | <i>growth arrest and DNA damage genes</i>                 |
| <i>GalCer</i>                  | <i>galactosylceramide</i>                                 |
| <i>GlcCer</i>                  | <i>glycosylceramide</i>                                   |
| <i>GSL</i>                     | <i>glycosphingolipid</i>                                  |
| <i>GNC2</i>                    | <i>general control nondepressible 2 protein kinase</i>    |
| <i>HIF1<math>\alpha</math></i> | <i>hypoxia inducible factor1</i>                          |
| <i>Hsp72</i>                   | <i>Heat Shock Protein 72</i>                              |
| <i>IRE1<math>\alpha</math></i> | <i>Inositol requiring 1 alpha</i>                         |
| <i>lag1</i>                    | <i>longevity assurance gene 1</i>                         |
| <i>LAMP-2</i>                  | <i>lysosomal-associated membrane protein 2</i>            |
| <i>LASS</i>                    | <i>longevity-assurance homologues</i>                     |
| <i>LC3</i>                     | <i>light chain 3</i>                                      |
| <i>LC_MS</i>                   | <i>liquid spectrometry copuled with mass spectrometry</i> |
| <i>LDL</i>                     | <i>low density lipid</i>                                  |
| <i>Leu</i>                     | <i>leupeptin</i>  |
| <i>LP</i>                      | <i>leupepstatin+pepstatin A</i>                           |

|                 |   |
|-----------------|---|
| <i>MAP1LC3B</i> | <i>microtubule-associated protein 1 light chain 3</i>       |
| <i>NADPH</i>    | <i>nicotinamide Adenine Dinucleotide Phosphate Hydrogen</i> |
| <i>NF-κB</i>    | <i>nuclear factor kappa B</i>                               |
| <i>PARP</i>     | <i>poly-adenine-ribose-polymerase</i>                       |
| <i>PAS</i>      | <i>phagore assembly site</i>                                |
| <i>PCNA</i>     | <i>proliferating cell nuclear antigen</i>                   |
| <i>PepA</i>     | <i>pepstatin A</i>  |
| <i>PERK</i>     | <i>RNA-activated protein kinase-like ER kinase</i>          |
| <i>PI</i>       | <i>propidium iodide</i>                                     |
| <i>PKB</i>      | <i>protein kinase B</i>                                     |
| <i>PKC</i>      | <i>protein kinase C</i>                                     |
| <i>PP1</i>      | <i>protein phosphatase 1</i>                                |
| <i>PP2A</i>     | <i>protein phosphatase 2 A</i>                              |
| <i>pRb</i>      | <i>retinoblastoma protein</i>                               |
| <i>Resv</i>     | <i>resveratrol</i>  |
| <i>RHEB</i>     | <i>RAS-homolog enriched in brain</i>                        |
| <i>ROS</i>      | <i>reactive oxygen species</i>                              |
| <i>RPA</i>      | <i>replication factor A</i>                                 |
| <i>S1PR1</i>    | <i>Sphingosine-1-phosphate receptor 1</i>                   |
| <i>Sa</i>       | <i>sphinganine</i>  |
| <i>Sa1P</i>     | <i>sphinganine-1-phosphate</i>                              |
| <i>SK</i>       | <i>sphingosine kinase</i>                                   |
| <i>SM</i>       | <i>Sphingomyelin</i>  |
| <i>Smac</i>     | <i>Second Mitochondria-Derived Activator of Caspases</i>    |
| <i>SMase</i>    | <i>Sphingomyelinase</i>                                     |
| <i>SMS</i>      | <i>Sphingomyelin synthase</i>                               |
| <i>So</i>       | <i>shpingosine</i>  |
| <i>So1P</i>     | <i>sphingosine-1-phosphate</i>                              |
| <i>SPL</i>      | <i>So1Plyase</i>  |
| <i>SPT</i>      | <i>Serine palmitoil transferase</i>                         |
| <i>TLC</i>      | <i>thin layer chromatografy</i>                             |
| <i>TNF-α</i>    | <i>tumor necrosis factor alpha</i>                          |
| <i>TOR</i>      | <i>target of rapamycin</i>                                  |
| <i>TRAF2</i>    | <i>TNF Receptor-Associated Factor 2</i>                     |
| <i>ULK1</i>     | <i>Unc-51-like kinase</i>                                   |
| <i>UPR</i>      | <i>unfolded protein response</i>                            |
| <i>XBP-1</i>    | <i>X-box binding protein 1</i>                              |



## 1. BACKGROUND

Cells are constantly subjected to environment variations that can be dangerous and vulnerability is high during the replicative phase of the cell cycle. Therefore cells evolved a tightly regulated stress survival strategy, to assure their integrity and viability. Altered materials degrading mechanisms, cell cycle arrest, and autophagy are part of this strategy. Their activation is finely orchestrated, sharing several upstream regulative signaling pathway as well as downstream cellular effects. For example nutrient starvation, calorie restriction and low-energy status lead to misfolded protein accumulation into the ER and ER stress: such conditions trigger the UPR (as reviewed in [1]). In parallel the same stimuli also activate autophagy and cell cycle arrest through the inhibition of mTOR (see review [2]). The calorie restriction mimetic, dietary polyphenol, resveratrol, perfectly integrates within the signaling pathways network that regulate cell fate decision, addressing cells either to cell death as well as cell survival [3]. How resveratrol regulates cellular processes it is still poorly understood. We previously demonstrated that resveratrol acts modulating biosynthetic de novo pathway of ceramide inducing apoptosis in several tumoral cell lines [4-6] but also autophagy cell death in caspase 3 deficient cells [7]. Dihydroceramide is the immediate precursor of the pro-apoptotic molecule ceramide. The reaction to obtain ceramide from dihydroceramide is catalyzed by DEGS-1. Recent studies proposed several and controversial bioactive properties for this molecule [8-10]. The aim of the present manuscript is to present all literature evidences that led us to formulate the hypothesis that dihydroceramide-dependant signaling pathway may mediate the pro-survival resveratrol-induced autophagy and to present and discuss our experimental results demonstrating that dihydroceramide is the trigger of a pro-survival response that involves UPR autophagy and cell cycle modulation.

### 1.1 *Sphingolipids*

#### 1.1.1 Structure and classification

Sphingolipids are an important class of ubiquitous lipids present in a variety of organisms including eukaryotes, prokaryotes and some enveloped viruses [11]; [12-15]. This complex family of compounds shares a common structural feature, a sphingoid base backbone 2-aminoalk-(ane or ene)-1,3 diol with stereochemistry (2S,3R), which can be distinguished by the chain length (that in mammalian is comprised mainly of a 18-carbon alkyl chain), the number of double bonds (herein referring to sphinganine (Sa) the saturated form, and sphingosine (So) presenting a 4-5 unsaturated), and the presence or the absence of hydroxyls (4-hydroxysphingosine also referred as phytosphingosine or phytosphinganine). A fatty acid can be amide-linked with the sphingoid bases, it results typically from 16 to 26 carbon atoms in length (but shorter or longer N-acylsphingoids are also present), mostly it is saturated (but also unsaturated e.g. nervonic acid) and in some particular cases it can be  $\alpha$ -hydroxylated. For example, the major sphingolipid, C16-ceramide, derives from palmitoyl N-linked with sphingosine; palmitoyl N-linked with sphinganine is named C16-dihydroceramide; and finally palmitoyl N-linked with the 4-hydroxy- sphingosine or sphingane is named respectively C16-phytoceramide or C16-dihydrophytoceramide. Each one of the above mentioned N-

acyl-sphingoids can be metabolized in more complex sphingolipids attaching different head groups to the hydroxyl group on C1 of the sphingoid base. The most abundant sphingolipid is sphingomyelin, obtained by the linkage of phosphocholine to ceramide. In addition, other head groups can be attached to the dihydro-, phyto-, or N-acylsphingoids via phosphodiester linkages (the phosphosphingolipids), via glycosidic bonds (the simple and complex glycosyl- galactosyl- and lactosyl-sphingolipids, such as cerebrosides and gangliosides), and other groups (such as phosphono- and arseno-sphingolipids) [8, 16].

### 1.1.2 Sphingolipid metabolism

The sphingolipid is a class of bioactive lipids involved in several biological functions. Disorders in its metabolism are associated with several pathologic situations such as genetic diseases [17, 18], cancer [19], cardiovascular diseases [20], diabetes and lipotoxicity [21], neurodegenerative diseases [22], and many other pathologic situations .

The sphingolipid pathway has ceramide as hub molecule, that presents strong bioactive properties and it is used as backbone for the biosynthesis of other metabolites. Many of these metabolites are bioactive as well and take part to a complex and finely regulated network that involves both biosynthetic and catalytic reactions, organized in three major branches: the *de novo* pathway, characterized by the synthesis of ceramide from the condensation of serine with palmitoylCoA; the complex sphingolipids pathway, where ceramide can be metabolized in several complex sphingolipids and from whose hydrolysis ceramide can be obtained; and the salvage pathway, that involves ceramide de-acylation to sphingosine and re-acylation to ceramide.

#### 1.1.2.1 The *de novo* pathway

The sphingoid backbone formation takes place into the endoplasmic reticulum (ER), starting from the condensation of serine and fatty acyl-CoA, ending with the formation of ceramide as the center of sphingolipid metabolism. The *de novo* pathway is the unique metabolic entry point in the sphingolipid pathway. Hence it is tightly regulated by multiple control levels and it is sensible to the environmental stimuli which can require the production of the bioactive molecules included in this pathway. For example, *de novo* pathway is activated during heat shock response both in mammals and in drosophila [23]; it is also induced under hypoxic stress [24]; it can be induced in response to metabolic loading with either serine or palmitate [25]; it is activated by chemotherapeutic agents [26], oxidized LDL [27], cannabinoids [28] and resveratrol [4].

##### 1.1.2.1.1. Serine palmitoyl transferase (SPT)

SPT is a membrane-bound heterodimer [29]. Two different genes produce the different subunits composing the enzyme, SPT-LCB1 and SPT-LCB2, both necessary for the activity. The heterodimer presents only one catalytic active site that catalyzes the condensation of serine with palmitoylCoA obtaining 3-ketoshinganine. SPT activity can be positively or negatively transcriptionally regulated by different stimuli such as endotoxins and cytokines [30], UVB irradiation [31], and other agents [32]. In addition SPT is activated in a post-transcriptional depending manner by retinoic acid [33], 4-HPR [34], resveratrol [4] etoposide [35]. The substrate, serine and palmitoyl are limiting factors for the

reaction and So1P appears to regulate its activity even if its action is not understood [36].

#### 1.1.2.1.2 3-ketodihydrosphingosine reductase (3KSaR)

This enzyme is located in the ER with the active site facing the cytosolic side. This ubiquitous protein catalyzes a rapid reduction of 3-ketosphinganine in Sa [37].

#### 1.1.2.1.3 (Dihydro)ceramide synthases (dh)CERS)

(Dihydro)Ceramide synthase, first identified in yeast as lag1, is a protein family constituted of six enzymatic isoforms named (dihydro)ceramide synthases (CERS1-6) or also longevity-assurance homologues (LASS1-6). The LASSs are localized on the ER membrane and catalyze the acylation of sphinganine obtaining dihydroceramide (for review [38]). Today it is widely accepted that each CERS has a selected substrate specificity, using preferably a fatty acid with a specific acyl chain length. For example CERS1 uses as preferred substrate a C18:0 acyl chain; CERS2 instead preferably catalyzes the synthesis of dihydroceramide with C24:0 and C24:1 and C26:1. Also LASS3 is specialized in the synthesis of long acyl chain sphingolipids, preferring C24:0, C24:1, C26:0 and C26:1. LASS4 synthesizes C20:0 and C22:0 whereas CERS5 and CERS6 are specialized in short chain dihydroceramide synthesis respectively C14:0 and C16:0. The CERSs specificity in ceramide species synthesis is extremely intriguing since recent studies demonstrate that different ceramide species mediate different responses within cells [39].

#### 1.1.2.1.4 Dihydroceramide desaturase (DEGS)

The final step of ceramide de novo synthesis pathway is the formation of the 4,5-trans-double bond on the sphingoid base of the dihydroceramide, leading to ceramide formation. This reaction is catalyzed by the  $\Delta^4$ -dihydroceramide desaturase (DEGS), an enzyme recently identified and characterized [40]. It belongs to the desaturase/hydroxylase family, that it is characterized by the presence of conserved histidine motifs in the active site [41, 42]. Two different enzymatic isoforms have been identified in human, named DEGS1 and 2; DEGS1 seems to be the most expressed isoform in human, while the second isoform, DEGS-2, whose enzymatic activity seems to catalyze preferably the reaction on phyto- substrate, seems to be expressed preferably in skin, intestine, and kidney cells [43, 44]. DEGS1 is localized on the ER and, in particular, its enzymatic activity is oriented on the cytosolic face of ER [45]. DEGS1, like other desaturase systems, consists in a series of coupled reactions that transport electrons from NADPH to the terminal desaturase which reduces oxygen [46]. The first component of these electron-transport chains is the flavoprotein-cytochrome b5 reductase, which accepts electrons from NADPH transporting them to the cytochrome b5 heme group; the next reaction consists in transferring electrons from cytochrome b5 to the terminal desaturase, using oxygen as co-substrate [47]. For this reason, events that induce variations in oxygen availability, or that induce variations in the NADP<sup>+</sup>/NADPH+H<sup>+</sup> ratio could modulate DEGS-1 activity. Moreover it has been recently found that palmitate accumulation results in an increasing of mRNA encoding DEGS-1, resulting in a positive feedback and in which the starting substrate accumulation results in the activation of the final enzyme of the metabolic pathway [48].

The desaturation reaction catalyzed by DEGS-1 can be reproduced in vitro using either dihydroceramide and dihydrosphingomyelin as substrate [49]. However, in

vivo DEGS-1 preferably uses dihydroceramide as substrate, leading eventually to dihydroSM accumulation rather than SM.

### ***1.1.2.2. The sphingomyelin and complex sphingolipid breakdown pathways***

The ceramide, which is produced by de novo synthesis, is mostly used for the sphingomyelin and monoglycosyl- (glucosyl-, galactosyl-, lactosyl-) ceramide biosynthesis. The first is an important component of plasma membrane, whereas the latter is used as backbone for more complex sphingolipids such as gangliosides. Since a number of stimuli can trigger the breakdown of these sphingolipids, producing a great amount of ceramide, these sphingolipids are considered as a ceramide reservoir (for review see [50]).

#### ***1.1.2.2.1 Sphingomyelin synthase (SMS)***

Sphingomyelin (SM) is synthesized by transferring a phosphocholine from phosphatidylcholine to ceramide, yielding diacylglycerol as a side product [51]. This reaction is catalyzed by a bi-directional lipid choline phosphotransferase, existing in two isoforms, SMS1 and 2, which differing for intracellular localization, since SMS-1 is localized in the lumen of the trans Golgi [52], whereas SMS-2 resides in the plasma membrane [53, 54]. Directionality and activity of these enzymes mainly depend on the substrate availability, but additional levels of control also are known [55].

#### ***1.1.2.2.2 Sphingomyelinase (SMase)***

The cells obtain the major source of ceramide backbone by the breakdown of complex sphingolipids and in particular by the hydrolysis of SM. This reaction is catalyzed by a family of enzymes, named sphingomyelinases (SMases) which differ, for their localization and their optimal pH: the lysosomal/acid SMase (aSMase), secretory/ $Zn^{2+}$ -dependent SMase (sSMase), cytoplasmatic  $Mg^{2+}$ -dependent/neutral SMase (nSMase) and alkaline SMase (bSMase) [56]. The SM hydrolysis catalyzed by SMase is finely regulated and rapidly occurs, within minutes, either under different stimuli such as oxidative stress [57], or in response to cell treatment with TNF- $\alpha$  [58], antibodies directed against functional molecules such as Fas/APO-1 or CD28 proteins [59]. Other agonists of the SM-ceramide cycle include cytokines and growth factors such as interleukin 1b, nerve growth factor,  $\gamma$ -interferon, as well as stress-inducing agents such as ionizing radiation, hydrogen peroxide, UV light, and antileukemic agents [60].

#### ***1.1.2.2.3 Monoglycosyl- ceramide synthase***

An important class of bioactive molecules derived from ceramide is the monoglycosyl-ceramides, produced by the direct transfer of the carbohydrate moiety from a sugar-nucleotide to the ceramide unit. Thus, ceramide is glycosylated to galactosylceramide (GalCer) at the luminal leaflet of the ER by the transfer of galactose from a UDP-galactose donor, reaction catalyzed by a GalCer-synthase [61]. Ceramide can be glycosylated (GlcCer) by a GlcCer- synthase, reaction which unlike glycosylation to GalCer, occurs in the cytosolic leaflet of the Golgi apparatus [62].

#### ***1.1.2.2.4 Ceramide kinase (CK)***

Ceramide can be phosphorylated by ceramide kinase (for review see [63]), a cytosolic enzyme inserting a phosphate group onto C1-hydroxyl of Sa backbone. This enzyme presents wide similarity with So kinase, and it shows a higher affinity for ceramide with acyl chain length longer than C4 [64]. The subcellular localization

of this enzyme is unresolved, even if it has been suggested that it occurs to the plasma membrane [65, 66], Golgi apparatus [67], and the cytoplasm [66].

### **1.1.2.3 The salvage pathway**

Sphingolipids pathway presents an unique exit point: the ceramide de-acylation followed by the irreversible degradation of the sphingoid bases. So can be considered the hub molecule of this catabolic branch of sphingolipids pathway: the salvage pathway (for review see [68]). So derives from direct ceramide de-acylation. Alternatively So can be produced from lyso-SM and lyso-GLS, the N-deacylated derivatives of SM and GSLs [69-71]. In turn, So can be acylated to ceramide, or can be phosphorylated in So1P. In addition, this pathway is implicated in the recycling of exogenous or short-chain ceramides metabolism [72, 73]. Since the similarity of So and Sa, many enzymes of this pathway are shared (and described) within the de novo pathway.

#### 1.1.2.3.1 Ceramide synthases (CERS)

It is a family constituted of six enzymatic isoforms named (dihydro)ceramide synthases ([dh]CERS1-6) or longevity-assurance homologues (LASS1-6), that catalyze the acylation of sphingosine but also sphinganine. Biochemical and functional characteristics were treated above.

#### 1.1.2.3.2 Ceramidases (CDase)

This class of enzyme catalyze the deacylation of ceramide and dihydroceramide in respectively So or Sa. Different CDases were identified and classified as acid (A), neutral (N) and alkaline (B), according to the optimum pH for their activity. The B-CDase exists in three homologue forms: B-CDase1, 2 and 3. They are localized into the Golgi [74] or can be secreted in the extracellular space [75]. Recently Mao et al demonstrate that the B-CDase2 gene expression can be modulated in cancer cells HeLa after treatment with the anti-tumoral drug 4-HPR [76]. Another extensively studied member of the CDase enzyme family is A-CDase, which is localized into the lysosomes [77]. It is directly associated with cancer promotion and resistance to chemotherapy [78].

#### 1.1.2.3.3 Sphingosine Kinases (SK)

One of the most important enzyme in the sphingolipid pathway is SK, as it forms an essential checkpoint that regulates levels of three bioactive lipids such as So, So1P and ceramide [79]. This cytoplasmic enzyme exists in two isoforms, SK1 and SK2, different for spatial and temporal distribution and for substrate affinity. SK1 have a higher affinity for So than for Sa, the opposite for SK2 that can also use phytoSo as substrate [79]. SK activity is finely controlled by numerous regulative levels such as intracellular localization by PKC that can also regulate its transcription and translation [80], by protein-protein interaction (e.g. TRAF2) [81], by proteolysis [82] and by many other mechanisms [79].

#### 1.1.2.3.4 So1P Phosphatases (S1PP)

The mammalian S1PPs are a part of the type 2 lipid phosphate phosphatases that hydrolyze So1P to reconstitute So [83]. Human SPPase1 homologue is localized to the endoplasmic reticulum and shows enzymatic activity toward both So1P and Sa1P [84].

#### 1.1.2.3.5 So1P lyase (SPL)

So1P is irreversibly degraded to ethanolamine phosphate and a long-chain aldehyde [85] by So1P lyase (SPL), a pyridoxal 5'-phosphate dependent enzyme,

highly conserved through the evolution. SPL is required for maintenance levels of So1P and other sphingolipids and for this reason it contributes to the normal cellular biology and function in many species, and its loss of function is correlated to intestinal carcinogenesis [86].

### **1.1.3 Biological function of sphingolipid involved into the *de novo* pathway**

#### **1.1.3.1 Ceramide**

Ceramide is the hub molecule of sphingolipid metabolism; its intracellular amount is finely regulated and a considerable number of diseases are correlated with the deregulation of its metabolism. Ceramide modulation is involved in the regulation of different aspects of cell biology such as inflammatory response, cell proliferation, cell migration, apoptosis, autophagy, senescence, and differentiation [19].

Ceramide intracellular level is modulated in response to different stimuli, such as cytokines, anticancer drugs, heat stress and other stress stimuli [23], which lead to the activation of both the *de novo* and the hydrolysis of complex sphingolipid pathway. For example, several authors reported that treatment with TNF- $\alpha$  triggers apoptosis in different cell lines through the activation of enzymes implicated in *de novo* pathway, such as CERS 5, [87] as well as increasing the SM breakdown through the nSMase2 activation [58]. Ceramide accumulation regulates cell biology using different strategies: by lipid-protein interaction and by the biophysical property modification of biological membranes. Recent attracting results indicate that ceramide acts as a second messenger by a lipid-protein interaction. An excellent example is the direct ceramide interaction with phosphoprotein phosphatase 2A (PP2A), resulting in PP2A enzymatic activation [88, 89]. Once that PP2A is activated by ceramide, it dephosphorylates either protein with pro- and anti-apoptotic function. PP2A activation results in the inhibition of proteins that show anti-apoptotic function such as the protein kinase B (PKB also a.k.a. Akt) and Bcl-2 [90], and on the other hand it activated pro-apoptotic proteins, such as Bax and Bad [91]. Another important protein that seems subjected to both direct and indirect ceramide-mediated PP2A activation, is the kinase suppressor of Ras [92, 93], that would mediate the activation of the axis ERK1/ERK2. Furthermore, ceramide also regulates apoptosis and cell cycle arrest by the activation of a phosphorylative cascade that involves the direct and indirect activation of PKC proteins [94]. Moreover, ceramide induces apoptosis increasing the permeability of mitochondria membrane through the formation of channels that facilitate the transmigration of cytochrome C from mitochondria to the cytoplasm and thus activating the caspase cleavage cascade [95]. In addition, ceramide directly binds and activates cathepsin D [96], resulting in Bax conformational change leading to mitochondrial release of pro-apoptotic factors like cytochrome c and Smac [97].

#### **1.1.3.2 The sphinganine axis**

Sa, obtained by reduction of 3-ketoSa or by deacylation of dihydroceramide, can be phosphorylated in C1 by SK (see above) obtaining Sa1P. Both Sa and Sa1P show opposite bioactive properties. Several studies demonstrated that the cytotoxic effect of the mycotoxin fumonisin B1 is mediated by the sphinganine accumulation. On the other hand, Sa1P shows pro-survival and anti-apoptotic properties, through So1P receptor 1 (S1PR1) activation with subsequent activation of ERK and PKB [98]. It has been recently proposed that the cytotoxic effect of 4-

HPR, an anticancer drug, currently tested in phase I clinical trials [99], is mediated by the balance perturbation of pro-death/pro-survival effect of these two molecules [76, 100].

### **1.1.3.3 Dihydroceramide**

Dihydroceramide, synthesized from sphinganine by CERs, can be desaturated in ceramide or alternatively, metabolized in dihydrosphingomyelin (dhSM), dihydroglucosyl-, galactosyl-, ceramide (respectively dhGlcCER, dhGalCER). Since C2-dihydroceramide did not show biological activity, for a long time it has been used as negative control for C2-ceramide studies [101]. Moreover, the classical TLC methods cannot distinguish saturated from unsaturated ceramide; this guided several groups towards wrong solutions that dihydroceramide is a biological inactive biochemical precursor of the bioactive lipid ceramide [101]. Thereafter, the introduction of more accurate methods in sphingolipid analysis, based on mass spectrometry, led to reconsider the biological properties of dihydroceramide starting from the observation that several molecules, thought to induce their biological effect through ceramide accumulation, were instead found to induce accumulation of their saturated form, dihydroceramide. An interesting example is 4-HPR, whose anticancer properties were thought for a long time to be due to a large increase in ceramide [34, 102, 103], whereas thereafter it was found to induce DEGS-1 inhibition and accumulation of dihydroceramide as well as other precursors [8, 9, 100, 104].

Recently, an increasing number of publications have described the dihydroceramide signaling activity in different, and sometime contrasting, regulative processes of cellular life and death. Krevetka et al., showed that the silencing of DEGS-1 gene induced an increase in dihydroceramides, but not ceramides, concomitantly to cell cycle arrest in G0/G1 phase and pRb hypophosphorylation in human neuroblastoma cells. The effect was mediated by the activation of PP1. 4-HPR showed a comparable anti-proliferative effect associated with DEGS-1 inhibition in cultured cells [9]. The implication of dihydroceramide in cell cycle regulation was also confirmed by Cabot group that reported how the over-expression of DEGS-1 in three different cancer cell lines resulted in an increase of cells in S/G2/M phase, meaning an increase of cell proliferation rate, due to the concomitant up-regulation of cyclin D1 through the action of NF- $\kappa$ B. Moreover in this manuscript it was also reported that esophageal carcinoma cells, Eca109 cells, reduce cell migration when DEGS-1 gene expression was silenced using specific siRNA. Furthermore in the same study, it was also reported that nude mice, in which Eca109 cells expressing the luciferase gene and silenced for DEGS-1 were injected into the heart, developed tumor metastases with a markedly lower severity than control mice [105]. This suggests DEGS-1 as a negative prognostic marker in patients with esophageal carcinoma. Recently, autophagy has been suggested as a cellular process modulated by dihydroceramide accumulation. In fact Merrill's group first proposed the association of the 4-HPR –mediated dihydroceramide accumulation with autophagy cell death in DU145 breast cancer cells [8]. They justified the different cell function observed after dihydroceramide or ceramide accumulation, with the higher dipole potential of dihydroceramides compared to ceramides. This biophysical difference could result in a decrease of the packing density [106] and with the lower tendency of dihydroceramide to promote flip-flop [107]. Moreover, since the transporting protein CERT shows a lower affinity for

dihydroceramide than ceramide, dihydroceramide retains longer at the ER membrane than ceramide [108]. Interestingly Stiban et al proposed an anti-apoptotic action of dihydroceramide, non based on enzymatic activity. In fact, they reported that pre-incubation of isolated mitochondria with dihydroceramide inhibits ceramide channel formation indicating that it interferes with the initiation or nucleation of ceramide channel rather than disassembling them. They suggested that dihydroceramide action is to ascribe at the perturbation of ceramide/dihydroceramide inside the cells [10].

## **1.2 Cell survival**

A cellular “stress” can be defined as whatever condition that perturbs the cellular homeostasis and that results in a cellular response. The conditions can be distinguished in physics, such as UV exposition, temperature and radiation; chemical, such as pH, ion concentration, oxygen tension, redox potentials, and metabolite, concentrations; biological, such as contact-dependent signals, hormones, cytokines, and neurotransmitters, and microbial pathogens. Eukaryotic cells are constantly exposed to stresses, which could potentially damage them. If the stimuli are above a certain threshold, they could lead cells to death. Hence, cells evolved several strategies to maintain their homeostasis, constituted of a complex network of signaling pathways that regulate cellular processes and assure cell survival to stress. Nevertheless, these processes are also highly integrated with the programmed cell death pathways to guarantee a clearance of those cells whose integrity is irremediably compromised. Unfolded protein response (UPR) to endoplasmic reticulum (ER) stress, cell cycle arrest and autophagy are three main actors in this integrated strategy.

### **1.2.1 ER stress and UPR in cell life/death regulation**

Eukaryotic cells are provided of endoplasmic reticulum (ER) an intracellular organelle composed of a membranous tubular network whose integrity is essential for cell life. In fact ER works as an intracellular Ca<sup>2+</sup> reservoir [109], and it is involved in biosynthetic processes. Neo-synthesized secretory or membrane proteins are folded in more complex structures by numerous chaperons proteins residing inside the ER lumen. Moreover, the ER membrane is the site involved in the de novo biosynthesis of ceramide [45], and other lipids and sterols. The correct protein folding is essential for cell surviving. Therefore this process is guaranteed by a quality control system, that recognizes misfolded proteins and addresses them either to a chaperones mediated refolding or to the sorting out of ER to the ER-associated degradation (ERAD) (for review see [110]). Several physiological and pathological conditions such as glucose deprivation, hypoxia and oxidative stress, but also drugs that affect protein folding such as tunicamycin or DTT, can result in the accumulation of misfolded proteins which exceed ER folding capacity: a situation called ER stress. This condition activates a high regulated cellular response, defined as unfolded protein response (UPR), whose goal is to reduce the pressure into the ER and reestablish homeostasis by protein folding efficiency enhancement. Thus UPR results in up-regulation of molecular chaperones and protein processing enzymes; in the attenuation of global protein translation, and in the clearance of unfolded protein. Hence, ER stress induces UPR to promote survival, or only if the stimuli is too prolonged or too strong, to commit cells to



programmed cell death strategy [111]. UPR effectors work inducing cell cycle arrest, senescence, differentiation and activate autophagy.

### **1.2.1.1 UPR sensor proteins**

UPR is mediated by the activation of three major trans-membrane signal transducers: IRE1, PERK and ATF6. In normal conditions they bound Binding immunoglobulin protein (BiP), which inhibits their activity [112]. The accumulation of unfolded protein induces a switch of BiP from the trans-membrane signal transducers to the unfolded protein, inducing the UPR activation.

#### 1.2.1.1.1 Inositol requiring 1 $\alpha$ (IRE1 $\alpha$ )

IRE1 $\alpha$  is a type-I trans-membrane receptor. In normal condition it binds BiP, resulting inactive. During ER stress BiP-free IRE1 $\alpha$  homodimerizes, it induces its autophosphorylation and becoming active [113]. Activated IRE1 $\alpha$  activates JNK transcription, a transcription factor involved in pro-death signal pathways. On the other hand, IRE1 $\alpha$  expresses endoribonuclease activity, inducing the splicing of a mRNA encoding for the transcription factor X-box binding protein 1 (XBP-1) that induces the transcription of several UPR target genes, including ER chaperones (BiP, ERdj4, ERdj5, HEDJ, GRP58, and PDIP5), ERAD components (darlin-1, EDEM and HRD-1 HERP, and p58IPK), transcription factors (C/EBP homologous protein 10 [CHOP] and the same XBP-1 gene), and other proteins related to the secretory pathway [114]. XBP-1 gene expression is associated with pro-survival strategies, in fact it has been recently demonstrated that silencing of C/EBP $\beta$  gene, implicated in regulation of cell proliferation and cell survival, leads to down-regulation of XBP-1 gene accompanied by a strong inhibition of proliferation [115]. Spliced XBP-1 correlates with a pro-survival ER stress response. Solid tumor cells are subjected to chronic hypoxia, and it results essential for cancer development to resist hypoxic condition. Solid tumor cells subjected to hypoxia induced XBP-1 over-expression and activated splicing of its mRNA, resulting in cell survival. Loss of XBP-1 severely inhibited tumor growth due to a reduced capacity for these transplanted tumor cells to survive in a hypoxic microenvironment [116]. In addition, the knockdown of XBP-1 induced apoptosis in BaF3 cells, whereas its constitutive expression protected BaF3 from apoptosis during IL-3 depletion [117]. In addition, Gupta et al. reported that an increased production of active XBP-1 was necessary for heat shock protein 72 (Hsp72) to exert its pro-survival effect under conditions of ER stress [118]. On the contrary, it has been reported that sustained XBP-1 activation leads to endothelial cells apoptosis [119]. The dualism of spliced XBP-1 can be explained with the observation that the binding of anti- and pro-apoptotic proteins to IRE1 $\alpha$  controls the amplitude of its signaling and determines cell fate during conditions of ER stress.

#### 1.2.1.1.2 RNA-activated protein kinase-like ER kinase (PERK)

PERK is a type-I trans-membrane receptor that shows high similarity with IRE1 [120]. The disassociation of BiP from PERK induces its diimerization and its activation by autophosphorylation. Active PERK directly phosphorylates the eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) which, once activated, reduces the formation of initiation complex and the recognition of AUG initiation codons. This results in a general reduction in protein translation [121]. However, this condition does not affect ATF4 mRNA translation, a protein that strongly activates the expression of CHOP. The activation of eIF2 $\alpha$  PERK-mediated seems to play an essential role in cell survival during UPR, since PERK mutated cells are impaired to survive under

ER stress [122]. In addition, it has been reported that eIF2 $\alpha$  phosphorylation by PERK mediated a pro-survival response, whereas its activation by GCN2 promoted pro-apoptotic effect. Therefore, eIF2 $\alpha$  phosphorylation is a key step to maintain a balance between the life and death of a glucose-deficient cell [123]. The PERK/eIF2 $\alpha$ /ATF4 arm of the UPR is implicated in regulation of autophagy through the up-regulation of MAP1LC3B and ATG5 genes in solid tumor cells and results an important mediator of hypoxia tolerance [124].

#### 1.2.1.1.3 Activating transcription factor 6 (ATF6)

ATF6 is a type-II ER trans-membrane transcription factor. BiP-free ATF6 is transported to the Golgi apparatus where it is cleaved. The cleaved form of ATF-6 shows a N-terminal site necessary for transportation inside the nucleus, where it activates the transcription of chaperone genes as well as the transcription of CHOP. ATF-6 $\alpha$ , seems to absolve a double role in cell fate regulation, ATF6 $\alpha$  is essential for CHOP expression early in UPR, whereas it is not for its late expression in UPR. On the other hand, ATF-6 $\alpha$ -deficient cells are less able to survive to prolonged stress, due to lower chaperone expression and increased apoptosis [125].

#### **1.2.1.2 UPR effector proteins: the CHOP example**

C/EBP homologous protein 10 (CHOP) is a transcription factor, it is part of the growth arrest and DNA damage genes (GADD153) family, whose transcription is induced by genotoxic stress and growth arrest signals. CHOP over-expression is downstream of either PERK, ATF-4 and ATF-6 signals and is mostly involved in apoptosis through the over-expression of three downstream of CHOP (DOC) genes. Moreover it has been reported that CHOP over-expression leads to the down-regulation of the anti-apoptotic Bcl-2 protein [126] and the translocation from cytosol to mitochondria of Bax [127]. The tight relationship between CHOP and cell death is also demonstrated by the observation that silencing of CHOP protects against apoptosis [128]. CHOP is also induce gene expression suppression of the anti-apoptotic p21 in a p53 independent manner [129].

### **1.2.2 Cell Cycle**

Cell cycle is defined as the biochemical and morphological succession of events necessary to cell proliferation. It can be divided in interphase and mitosis. Cells spend the majority of their life within the interphase, which is organized in G1, S and G2 phases. During G1 phase the anabolic and catabolic processes necessary for cells life occur. Cells remain within this cell cycle phase for an extremely variable time that greatly depends by cell types. Based on the duration of this phase cells could be divided in actively proliferating, such as cells from hematopoietic tissue, non proliferating cells, that are terminally differentiated, and cells temporary quiescent. If the requirements of genetic and cellular integrity are fulfilled, cells progress to S phase, which is characterized by the replication of genetic material and that it is followed by G2 phase. During this cell cycle phase, cells set the conditions for the division in two daughter cells, event that occurs during mitosis, also defined M phase. Under particular stressor conditions, such as, nutrient starvation, cell confluence, cytostatic drugs, DNA damage, cells can temporary suspend their proliferation, becoming quiescent (G0 phase). Thus quiescence could be viewed as a form of self prevention when proliferation in not desirable.

The progression along the different phases is extremely important for organogenesis and for a well orchestrated tissue architecture. Therefore, the transition from a phase to the following is finely regulated by a cascade of events that initiates with the formation of heterodimeric complex composed of: cyclins, the regulative subunit, whose expression is cell-phase specific; cyclin-dependant kinases (CDKs), the catalytic subunit with kinase activity. Cyclin/CDK heterodimer phosphorylates downstream targets necessary for cell cycle progression. The progression from a phase to another is subjected and regulated by checkpoints that verify if the optimal conditions for cell progression exist. DNA damage, but also nutrient starvation hypoxic condition and other stresses can activate checkpoints and result in cell cycle arrest.

Cyclins are proteins that accumulate inside the cells in a cell-phase specific manner, and that are quickly degraded by the ubiquitine-proteosome system once overcame their specific phase. Nine different cyclins, named with letters from A to I, were identified and schematically grouped in two major classes: G1/S phase cyclins, that drive the cell cycle progression from G1 to the complete genetic material replication are Cyclin D, E and A; cyclin B is instead involved in the progression through the G2 phase and mitosis.

CDKs are a class of proteins that show kinase activity only after association with a cyclin. These protein transfer a phosphate on serine or threonine activating or inhibiting target proteins such as Rb, E2F, B-myb, other CDK, the Cdc25A phosphatase, p27 [130]. To date nine CDKs were identified, whose activity is finely regulated by several strategies: they can be activated through the binding with the proper cyclin [131], they can be both inactivate or inactivated respectively by phosphorylative or dephosphorylative events on different aminoacids [132]; alternatively they can be inactivated by the binding with specific CDK inhibitor proteins (CKI) that work inhibiting the interaction cyclin/CDK [133].

An essential moment for the life of a cell is the decision to progress from G1 to S phase, duplicating its genetic material. Proliferation signals, such as a growth factors, trigger a signal transduction cascade Ras-mediated, that leads to the expression of cyclin D [134], which plays a pivotal role in cell cycle progression. Cyclin D family is constituted of three closely related cyclins, D1, 2 and 3. It forms a holoenzyme with CDK 4 or 6 that phosphorylates and inactivates the retinoblastoma gene product, pRb [135]. Phosphorylated pRb is no longer able to bind the transcription factor E2F, who becomes free to induces the overexpression of enzymes and regulators essential for cell cycle progression and DNA replication [136]. A target gene of E2F is cyclin E, a protein that specifically binds CDK2 and cooperates with cyclin D for pRb phosphorylation [137]. Cyclin E/CDK2-mediated phosphorylation of pRb is a limiting factor for the G1-to-S phase progression [138, 139]. One of the last events characterizing the G1-to-S cell cycle phase progression is the expression of cyclin A, and its interaction with CDK2, that drives the cell to S phase [140, 141]. Cyclin A/CDK2 complex cooperates for pRb phosphorylation, and in addition collaborates with cyclin E/CDK2 to activate proteins that are essential for pre-replicative complex formation such as the DNA replication factor A (RPA) or the proliferating cell nuclear antigen (PCNA) [131]. Moreover both cyclin E and A/CDK2 are essential to prevent re-replication events.

### **1.2.2.1 Cell Cycle in stress response**

Under undesirable or potentially dangerous environmental conditions, such as limited nutrient provision and growth factor depletion, hypoxia and redox homeostasis unbalance, or DNA damage, cycling cells can take the decision to temporarily arrest their proliferation, until the optimal growth conditions are re-established. The decision to continue through the cell cycle after mitosis is taken during G2. During the restriction point (or R point) within the G1 phase, cells takes the decision whether proceeding to S phase and replicating their genomic material. In this sense a pivotal role is played by the CKIs, that on one hand inhibit CDKs activity, preventing pRb phosphorylation, and activating phosphatases (PP1 and PP2) that remove phosphate group and activate pRb. Two families of CKI were described depending on structural similarity INK4 (p16INK4A, p14ARF, p15INK4B, p18INK4C, p19INK4D) and CIP/KIP (p21Cip1, p27Kip1, p57Kip2). The CIP/KIP p21 and p27 protein play an important role preventing the CDK activation inhibitors [142], and their loss of expression or function has been implicated in the genesis or progression of many human malignancies and can mediate a drug-resistance phenotype. However, due to their ability to interact with cyclin D1 increasing its transportation into the nucleus, p21 and p27 were also associated with tumors promotion, indicating a paradoxical effect [143]. Their regulation is modulated by either gene expression regulation or post-transductional modifications or proteolytic degradation [144].

Cell cycle arrest in G0/G1 phase is part of an adaptive response to stress. Genotoxic stress is probably the best studied inducer of checkpoint activation. Briefly, several condition such as UV light, radiation, ROS can induces single or double strand DNA damages. During G1/S checkpoint a class of proteins, RADs, specialized to check DNA integrity, senses DNA damages, and triggers a signal cascade that activates the transcription factor p53. Active nuclear p53 in turns transactivates pro-apoptosis, pro-autophagy and cycle arresting genes. p21 is directly regulated by p53, inhibits the association of cyclin D, E and A with the respective CDKs. The cyclin/CDK absence causes the hypophosphorylation of pRb and the consequent cell cycle arrest.

Cell cycle arrest in G0/G1 phase is also the central part of a survival strategy used by solid cancer cells to survive prolonged state of starvation and hypoxia. Gardner and coworker elegantly demonstrated that cells, growing under hypoxic condition, reduce their proliferative rate by regulation of p27 expression [145].

ER stress induces cell cycle arrest in G0/G1 providing the cell an opportunity to restore cellular homeostasis before committing to apoptosis [146]. In particular PERK and GCN2 mediated eIF2 $\alpha$  phosphorylation arrests cell cycle in G0/G1 phase by preventing cyclin D1 expression [147].

Autophagy and cell cycle arrest are two strategies used by cells to survive stress. During nutrient deprivation, condition known to activate both processes, cell cycle is activated as a mechanism of cellular integrity preservation whereas autophagy maintains viability by furnishing cells with nutrients and building blocks for biosynthetic processes until conditions become favorable for reentry into cell cycle. Since these cellular responses share that same goal, the cell survival to stress, cell cycle and autophagy also shared common regulative pathways, and can be inversely regulated by the same stimuli. In particular the regulatory mechanism of these processes converges on mTOR regulation, that on one hand negatively regulate autophagy, on the other and positively modulate cell growth and

proliferation. In *Drosophila* the loss of TOR results in a cellular phenotype typical of quiescent and autophagic cells such as reduced nucleolar size and autophagosome formation in the larval fat body [148]. Other studies show that treatment with mTORC-1/2 inhibitors affected T-acute lymphoblastic leukemia (T-ALL) cell viability by inducing cell-cycle arrest in G0/G1 phase, apoptosis and autophagy [149]. Moreover, Crighton et al. demonstrated that activation of p53 and its nuclear translocation induces the expression of the pro-autophagic protein DRAM [150], whereas Komata et al reported that p27 overexpression, known to induce quiescence, is sufficient to induce autophagy [151]. On the other hand cell arrest was also associated with cell death [152, 153], and p53 activation results in the activation of cell cycle arrest before apoptosis induction [154].

### 1.2.3 Autophagy

Autophagy is a physiological ubiquitous degradation process in eukaryotic cells, conserved from yeast to human. It is defined as a process involving the degradative delivery of a portion of the cytoplasm to the lysosome that does not involve direct transport through the endocytic or vacuolar protein sorting (Vps) pathways (for review see [155]).

Different types of autophagy have been described, depending on the mechanism that mediates the delivery of cytosolic cargo to lysosomes for degradation: chaperone-mediated autophagy, microautophagy and macroautophagy. Chaperone-mediated autophagy is the selective targeting to lysosomes of cytosolic proteins by their direct translocation across the lysosomal membrane into the lysosomal lumen (for review see [156]). Microautophagy is the direct uptake of soluble or particulate cellular constituents into lysosomes; it involves direct invagination and fission of the vacuolar/lysosomal membrane under nutrient deprivation. This type of autophagy is implicated in micronucleophagy in *S.cerevisiae* during starvation. Macroautophagy (here referred as “autophagy”) is the most prevalent type of autophagy. It involves double-membrane vesicles to sequester portions of cytoplasm that are delivered to lysosomes for degradation. Autophagy is responsible of degradation of long-lived proteins and damaged and/or surplus intracellular organelles and structures. Depending on cargo, it is possible to distinguish mitophagy, as a selective removal of mitochondria (for review see [157]); reticulophagy, as a degradation of parts of the endoplasmic reticulum (for review see [158]); ribophagy, as a selective degradation of ribosomes [159]; macro- and micropexophagy, responsible of rapid decrease of peroxisomes within cells (for review see [160]). Autophagy is also implicated in processes such as protection during infection through lysosomal degradation of invading pathogens, also named xenophagy (for review see [161, 162]); hormone secretion regulation by crinophagic process comprising fusions of secretory granules with lysosomes [163]. Recently it has been described a function of autophagy in regulating intracellular lipid stores, named macrolipophagy [164].

#### 1.2.3.1 Activation of autophagy

Under physiological conditions cells undergo autophagy to get rid of aberrant or long-lived proteins or to degrade damaged organelles. However, autophagy flux can be activated under particular conditions. Nutrient deprivation, low energy state and insulin activate autophagy in almost every different human cell type [164, 165]

recycling basic building block to support the survival and to maintain cellular homeostasis. The sensor of the cellular energetic state is target of rapamycin (TOR) protein, an evolutionarily conserved protein kinase that regulates cell growth, cell cycle progression, nutrient import and protein synthesis in all eukaryotic cells. This protein was first described in yeast and it is organized in functional complex, in mammals mTORC1 is formed by mLST8 and raptor [166]. Increasing amino acids, insulin-mediated activation of Akt pathway and increase of ATP/AMP ratio following high energy status, induce the binding of Rheb and Rag GTPase with mTORC1 and its activation [167]. When activated, mTORC1 phosphorylates a class of autophagy-related genes (Atg), ULK1, mAtg13 and FIP200, mammalian homolog of Atg1, Atg13 and Atg17 in yeast. The hyperphosphorylated state of these proteins inhibits their interaction, thus resulting inactive. Nutrient deprivation and low energy state inhibit mTORC1, thus ULK1, mATG13 and FIP200 are hypophosphorylated and can interact. The formation of the complex ULK1/mAtg13/FIP200 results necessary for autophagy activation [168].

#### 1.2.3.1.1 Autophagosome nucleation

The autophagosome nucleation begins with the isolation of a lipid bilayer structure, named phagofore. Its formation originates from a phagofore assembly site (PAS), localized in the peri-vacuolar region [169], PAS origin is still unknown. It is known that several Atg proteins take contact with PAS to facilitate phagofore formation. Central role in Atg recruitment is played by Beclin1 complex. This complex is constituted by Beclin1 and class III phosphatidylinositol-3 kinase (class III PI3K or hVps34); the first one does not show any enzymatic activity but it works as an assembly platform; the second one is responsible of the phosphorylation of PtdIns to PtdIns3P, whose levels seem to be important for recruitment and assembly of other Atg proteins, such as Atg2 and WIPI-1, on phagofore. Regulative proteins of this complex, that directly binds Beclin1 are UVRAG, which increases the interaction of Beclin1 with Vps34 and activates autophagy, and the anti-apoptotic Bcl-2, that negatively regulates autophagy. The mechanism that links Beclin1 complex to ULK1 complex is still unknown.

#### 1.2.3.1.2 Autophagosome elongation

After its formation, phagofore extends its edges and wraps cytoplasmic portion designated to lysosomal degradation. While the cargo selection is unknown, the elongation process is well understood [170]. Elongation depends on two ubiquitin-like protein: Atg12 and light chain 3 protein (LC3). Atg12 interacts with Atg5, in a Atg7 and Atg10, and it results important for LC3 conjugation reaction. Autophagosome elongation is tightly dependent on LC3. LC3 was identified originally as a protein co-purified with microtubule-associated proteins (MAPs) [171]. This protein, synthesized as a Pro-LC3, is cleaved by proteolysis from Atg4 [172] that exposes the carboxyl terminal Gly, obtaining LC3-I. LC3-I is divided into N- and C-terminal subdomains. The N-terminal subdomain of LC3 is essential for its binding of tubulin and microtubules, the C-terminal is conjugated with phosphatidylethanolamine (PE). This reaction is depending on Atg7 (an E1-like enzyme) and Atg3 (an E2-like enzyme) [173]. The PE-conjugated form of LC3 (LC3-II), driven by Atg12/Atg5 complex, is incorporated both on the internal and external face of the autophagosome. LC3 seems to be directly involved in the elongation process as well as in the autophagosome closure [174]. Before the autophagosome completion, all the Atg proteins are recycled. Atg-4 delipidates the

portion of LC3-II that is present on the external face of the autophagosome. Only the LC3-II present on the internal layer remains associated with the autophagosome.

#### 1.2.3.1.3 Maturation and degradation of autophagosome.

After formation, autophagosome delivers its cargo to lysosome for degradation. Autophagosome can directly fuse with the lysosome (autolysosome) or merge with endosome (anphisome) with a subsequent fusion with lysosome. The fusion of autophagosome with endosome is under control of proteins involved in controlling membrane fusion in the intracellular transport such as Rab GTPase, SNARE and ESCRT proteins [175, 176]). Successively lysosomal transmembrane proteins, such as lysosomal-associated membrane protein 2 (LAMP-2) [177], are responsible of autophagosome fusion with lysosome. Finally, the autophagosomal content is degraded by lysosomal proteases [178].

#### **1.2.3.2 Autophagy and cell fate regulation.**

Autophagy is an essential cellular strategy for cell survival. Under physiological conditions, autophagy occurs in cells with different magnitudes depending on different tissues contributing to maintain cell homeostasis through lysosomal clearance of damaged organelles or long-lived proteins. Autophagy is thus involved in cell survival by apoptosis inhibition and cells protection from stresses [179]. Autophagy is mainly involved in lysosomal nutrient recycle during starvation or calorie restriction, providing cells with substrates for oxidation and for ATP synthesis in mitochondria [180]. Indeed low energy status is a condition that strongly triggers autophagy both in vitro and in vivo, and furnishes material for energy production and guarantees cell survival. New born mice rapidly activate autophagy in almost all the tissues of their organism to counteract the starvation following the birth [181]. Fasting condition before cesarean section induces autophagy activation in human placenta [182].

Autophagy is implicated in the calorie restriction- mediated anti-aging effect and in lifespan increase observed in several animal models. In fact, in vivo studies show that autophagy is activated in life-long calorie restricted mammals [183]. This effect is mediated by the activation of SIRT-1 protein, whose overexpression increases lifespan of yeast and *C. elegans* [184]. In fact, calorie restriction induces the intracellular NAD<sup>+</sup> accumulation, which activates the NAD<sup>+</sup> -dependant deacetylase SIRT-1. Activated SIRT-1 deacetylates the autophagy related proteins Atg5 and Atg7, resulting in an autophagy increase, as reported in human colorectal (HCT-116) and cervical (HeLa) cancer cell [185].

Autophagy is not just a mechanism for nutrients recycle, rather it is a highly regulated strategy to adapt the cell to stresses that perturb its homeostasis. It is activated during oxidative stress, hypoxia and pathogen. Formation of reactive oxygen species (ROS) strongly induces autophagy [186]; autophagy in fact degrades damaged mitochondria, thus reducing ROS production and supporting cell survival [187]. Several evidences suggest that ROS directly sustain autophagy flux by oxidation of a cystein of Atg-4, resulting in its activation. Active Atg-4 promotes LC3-I conversion and autophagosome elongation [188].

Low level of oxygen (<1%) is known as hypoxia. Hypoxia exists not only in both physiologic and pathologic condition such as in placenta [182, 189], during embryogenesis [190], but also during ischemic events [183] or in the inner part of rapid growth solid cancer [191]. Cells subjected to hypoxic stress activate

autophagy as protective mechanism. Experimental evidences suggest that hypoxia activates autophagy through three strategies. The first one is correlated to ROS production and mitochondrial degradation [192]; the second one depends directly on HIF1 $\alpha$ . HIF1 $\alpha$  in fact upregulates BNIP3 gene expression that competes with Beclin-1 for Bcl-2 interaction increasing Beclin-1-free cellular level and autophagosome initiation [193]. Finally, hypoxia seems to induce autophagy in a TOR dependent fashion, by TOR inhibition and block of eukaryotic initiation factor, eIF4F [194].

Accumulation of long lived or damaged proteins as well as accumulation of misfolded proteins inside ER (ER stress) activates autophagy. UPR is a strong activator of autophagy that results in an elective strategy to degrade proteins accumulated inside ER. Moreover, UPR-related autophagy is mainly correlated with a pro-survival strategy to counteract ER stress dependent cell death. Rheumatoid and osteoarthritis synovial fibroblasts transfected with beclin siRNA can not undergo autophagy and thus are more susceptible to ER stress-induced cell death. On the other hand, silencing CHOP gene expression increases autophagy and improves cell survival [195]. Several evidences suggest that all the three signaling branches involved in UPR can regulate autophagy. Cells that carry non-phosphorylatable eIF2 $\alpha$  fail to activate ER stress induced autophagy, suggesting an important role for PERK/eIF2 $\alpha$  axis in autophagy activation. Cytoplasmic poly-Q aggregates induce accumulation of unfolded proteins and subsequent ER stress cell death [196]. Autophagy is protective against poly-Q72-induced ER stress cell death, because it can degrade poly-Q aggregate. Recently it has been demonstrated that poly-Q72 induces ER stress mediated activation of PERK/eIF2 $\alpha$ . eIF2 $\alpha$  selective translation of transcription factors upregulates the transcription of Atg-12, resulting in autophagy stimulation and the LC3-I conjugation with PE. In addition, the upregulation of ATF-4 and CHOP, following the activation of PERK/eIF2 $\alpha$  by severe hypoxia induced ER stress, results in their direct binding with the promoter and following upregulation of MAP1LC3B and Atg-5 gene [124, 197]. Despite the autophagy promoting action, PERK/eIF2 $\alpha$  has been reported as a pro-survival mechanism inducer. Kim et al. have recently demonstrated how the autophagy PERK/eIF2 $\alpha$  axis mediate radiosensitivity in caspase 3/7 deficient MCF-7 cells [198]. The control of autophagy by the UPR sensor IRE-1 $\alpha$  is controversial. Ogata et al., demonstrated that IRE-1 $\alpha$ /JNK is necessary for pro-survival autophagy activation in human neuroblastoma cell line [199]. Moreover IRE-1 $\alpha$  and the downstream signal of XBP-1 are involved in cell survival. Nevertheless, XBP-1 deficiency in the nervous system protects against amyotrophic lateral sclerosis by increasing autophagy [200], and its accumulation is associated with degenerative motoneurons after severe proximal axonal lesions, thus showing a inhibitory action on autophagy [201]. Kroemer justifies these observations hypothesizing that IRE/XBP1 dependent signal has a function to dampen excessive autophagy triggered via the PERK/eIF2 $\alpha$  pathway [179].

### **1.2.3.3 Autophagy and cell death**

Autophagy can protect cells from cell death and can inhibit apoptosis; on the other hand autophagy presents a pro-death face that can work independently or in cooperation with apoptosis leading to cell death known as type II programmed cell death [202]. Indeed, autophagy and apoptosis shared several regulative elements, for example BNIP3, DAP kinase and ceramide activate both autophagy and



apoptosis [203, 204], while the anti-apoptotic Bcl-2 also inhibits autophagy [205]. On the contrary, a number of authors demonstrated that cells undergo autophagy programmed cell death when apoptosis is inhibited, thus describing the type II programmed cell death as a substitutive and alternative cell death process [7, 206]. Particularly important in autophagic/apoptotic cell death crosstalk is Beclin-1 primarily because of its ability to directly interact with Bcl-2 (for review see [207]).

#### **1.2.3.4 Autophagy and sphingolipids**

Sphingolipids are intimately linked with cell fate regulation, therefore their implication in autophagy regulation is not surprising. The heterogeneity of the different sphingolipids species regarding the regulation of pro-death or pro-life mechanism is reflected in the ability to activate a pro-death or pro-life autophagy. For example ceramide was described as a pro-apoptotic sphingolipid. It coherently shows activation of pro-death autophagy [208]. Our group described tamoxifen as a drug that is able to induce ceramide mediated autophagic cell death in MCF-7 cells [6]. Ceramide activates autophagy relieving the class I PI3K/Akt/PKB pathway and provoking the accumulation of the gene product Atg6/Beclin-1. Moreover ceramide induces upregulation of the mitochondrial bound BH3-only BNIP3 protein [208]. Recently Zheng et al proposed dihydroceramide as pro-death autophagy inductor in DU145 prostate cancer cells [8].

Ceramide and So show a markedly anti-growth and pro apoptosis effect whereas So1P is associated with a pro-growth, pro-survival activity [82]. Our group demonstrated that SK1 overexpression and relative So1P increase induced pro-survival autophagy in MCF-7 cells, inhibiting mTORC-1 and protecting cell from death during starvation [6].

### **1.3 Resveratrol**

Resveratrol is a dietary polyphenol with extensively studied antioxidant and antitumoral activity [4, 209]. Resveratrol main action, in most of the cancer cell lines tested, is to block proliferation, arresting cell cycle at the G0/S boundaries with final apoptotic cell death upon prolonged treatment. One decade after the pioneering study of Pezzuto's group, in which resveratrol was shown to block tumor initiation promotion and progression in mice [210] we are now witnessing the beginning of resveratrol clinical trials recruiting patients with colon cancer and melanoma (<http://www.clinicaltrials.gov>). In contrast to its cytotoxicity, resveratrol was also shown to activate pro survival targets such as sirtuins [211, 212]. Indeed, resveratrol was recently reported as a calorie restriction mimetic [213, 214]. Since calorie restriction was suggested to exert antiaging effects [212] and autophagy is part of the program activated by calorie restriction in cells [215], particular attention has been paid to a phyto-calorie restriction mimetic such as resveratrol and its apoptotic or autophagic potential. The apparent dual role of this compound may be reconciled taking into account that resveratrol was shown to induce autophagy in a variety of cell lines [7, 216, 217] and to modulate the activity of Akt and AMPK, two important regulators of the autophagic process, although its direct targets, activated pathways and outcomes are still debated [218, 219]. By virtue of its autophagy promoting activity, resveratrol treatment may possibly trigger a first (autophagic) salvage response followed by cell death with high doses and/or

sustained administrations or depending on the specific cell ability to resist the autophagic process.

We previously demonstrated that resveratrol induces apoptotic cell death via accumulation and signalling of the known pro-apoptotic sphingolipid mediator ceramide in breast and prostate cancer cells [4, 5]. We proved that ceramide increased via the *de novo* synthesis pathway, but not from the hydrolysis of more complex sphingolipids such as sphingomyelins [4]. Moreover, we showed that tamoxifen induced autophagy is mediated by ceramide derived from increased rate of the *de novo* synthesis and that treatment with exogenous short chain analogues of ceramide, known to stimulate sphingolipid synthesis *de novo*, triggers autophagy [6]. Similarly, in yeast model, it was successively reported that mTor inactivation induces *de novo* sphingolipid synthesis [220]. Recently, we reported that in apoptosis resistant cells by caspase 3 deficiency, resveratrol induces a caspase-independent type II cell death by stimulating autophagy via Akt dephosphorylation [7]. Thus autophagy may occur in response to treatment and, depending on the produced mediators, it may be overcome by apoptosis through caspase activation. In support of this concept, we showed that sphingosine-1-phosphate, a sphingolipid mediator derived from ceramide catabolism with signalling roles opposite to ceramide, triggers a ceramide independent autophagy of survival [221].

## 2. MATERIALS AND METHODS

### 2.1 Reagents and Antibodies

XM462, Jaspin B, Dideuterated-C<sub>8</sub>-dihydroceramide (d2-C8-dhCER), NBD-C<sub>6</sub>-ceramide (NBD-CER) and NBD-C<sub>6</sub>-dihydroceramide (NBD-dhCER) were kindly provided by Dr. G. Fabrias. Resveratrol (Resv) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). 3-methyladenine (3MA), etoposide (Etp), fenretinide (4-HPR), rapamycin, CoCl<sub>2</sub>, nocodazole (NOC), leupeptin, pepstatin A, SRB dye, propidium iodide, trypan blue dye, acrylamide, polyacrylamide were purchased from Sigma–Aldrich (St. Louis, MO USA).

### 2.2 Cell Culture

The human gastric cancer cell line HGC- 27 was purchased from Health Protection Agency Culture Collections (Salisbury, UK), and maintained at 37 °C in 5% CO<sub>2</sub> in MEM (Euroclone) supplemented with 10% fetal bovine serum (FBS) (Euroclone), 1% non essential aminoacids (NEAA) (Euroclone), 1% glutamine (Euroclone) and 100 ng/ml each of penicillin and streptomycin (P/S) (Sigma). The breast carcinoma MDA-MB 231 and MCF-7 were purchased from ATCC (USA) cells were cultured in RPMI (Euroclone) added with 10% FBS and 100 ng/ml P/S.

### 2.3 Cell viability

HGC-27 cell viability was examined by the Trypan blue dye exclusion test. Cells were seeded in 12-well tissue culture plates at 6 x 10<sup>4</sup> cells/well allowed to adhere for 24 hours. Cells were counted on hemocytometer the day of treatment (T=0) and 16, 24, 48 or 72 hours after treatment (see figure legends for treatment conditions). The experiment was performed in triplicate. Cells were harvested using trypsin 0.25%. Samples were resuspended in 1 ml of PBS solution: Tripan blue dye (1:1). Values, evaluated as percentage of alive versus dead cells, were reported as ratio over control. Statistical significance versus control values was calculate using the two tails T-test.

### 2.4 Sulforhodamine B (SRB) assay

HGC-27 cells were seeded in a 96-wells tissue culture plate at 1 x 10<sup>3</sup> cell/well allowed to adhere for 24 hours before treatment. Cells were grown in media added vehicle only or in presence of the drug (see figure legends for treatment conditions), using five wells replicates for each treatment condition. Cell proliferation was analyzed 0, 2, 3 or 6 days after treatment by Sulforhodamine B (SRB) assay. Protein were precipitated by a 10% final concentration trichloroacetic acid in PBS for 1 hour and 30 min at 4°C, then stained with SRB dye, 0.4% final concentration in PBS/1% acetic acid for 30 min. Finally, precipitated proteins were washed and solubilised in 10mM Tris buffer, pH 10.6. Absorbance, optical density (OD), was measured at 540 nm using a microplate reader. For each treatment condition, proliferation index was calculated as ratio of OD of single treated over OD of untreated cell obtained at T=0.

## 2.5 Western blotting

Cell treatment and collection. Cells ( $2.5 \times 10^5$  cells/plate) were seeded in 100 mm petri dishes, and medium without antibiotic was replaced after 24 hours. When cells confluence reached about 60%, media was replaced with fresh complete medium containing the different drugs (see figure legend for treatment condition). Starvation was provoked by culturing cells in the absence of both FBS and non NEAA media. Real hypoxic condition was obtained by blowing into the flask a gas mixture containing 94% N<sub>2</sub>, 1% O<sub>2</sub> and 5% CO<sub>2</sub>. for 30 min. The flask was sealed using a silicon cap and cells grew under this condition for 16 or 24 hours. Cell harvesting, by scraping in cold PBS on ice, was performed into a specially designed gas chambers in which N<sub>2</sub> was constantly blown in, maintaining an atmosphere that never exceeded 1% O<sub>2</sub>. Next cells were spun at  $1,200 \times g$  for 5 min at 4°C. An aliquot of pelleted cells was used for protein quantification [222]; the remaining cells were resuspended in Laemmli buffer [223], boiled for 5 min and stored at -20°C.

Electrophoresis and blotting. Equal amount of proteins (10 µg) were separated by SDS-electrophoresis using the vertical electrophoresis apparatus Mini-Protean 3 Cell (Bio-Rad). The acrylamide gels had a concentration depending on the protein target: 8% for PARP, 12% for Cyclin D1 and E and 15% for LC3 detection. After the separation, proteins were transferred onto nitrocellulose membranes using an immersion Mini Trans-Blot apparatus (Bio-Rad). After blocking unspecific binding sites with 5% dry milk in PBST, the membranes were incubated overnight at 4°C with primary antibodies anti-LC3 (Novus), diluted 1:1000 in PBST-3% BSA, anti-PARP diluted 1:500 (Cell Signaling Technology Inc), anti-Cyclin D1 and anti-Cyclin E both diluted 1:250 (Santa Cruz Biotechnology). Anti-β-Actin 1:5000 dilution (Sigma) was used as house keeping gene for data normalization. Membranes were then incubated for 2 hours at room temperature with the appropriate HRP-secondary antibody (from Jackson Laboratories) diluted 1:10000 in PBST- 5% dry milk. Proteins were detected using chemiluminescence system LiteAbLot (EuroClone Life Science Division) and bands intensity quantified by Gel Doc 2000, using Quantity One Software (Bio-Rad).

## 2.6 LC-MS analysis

Total sphingolipid were extracted from  $1.5 \times 10^6$  treated or untreated HGC-27 cell pellet (see figure legend for treatment conditions). Cells were harvested by scraping in cold PBS on ice, collected in a 1.5 ml tube, spun at  $10000 \times rpm$  for 3 min. Cellular pellet was washed twice with ice cold PBS and dissolved in 100 µl PBS. An aliquot was used for protein quantification. Cells, added with 500 µl MetOH, 250 µl chloroform, 100 µl water and fortified with 10 µl of internal standards mix containing N dodecanoylsphingosine, N dodecanoylglucosylsphingosine, and N dodecanoylsphingosyl phosphorylcholine, 0.2 nmol each. Next they were lysed by 1-2 min sonication until organic phase separated from aqueous phase Cell lysate was incubated overnight a 48°C then, once cooled down to 37°C it was added with 75 µl KOH in MetOH and sonicated for 1-2 min. Finally it was incubated for 2 hours at 37°C, added with 3 µl glacial acetic acid and dried by N<sub>2</sub>.

The liquid chromatography-mass spectrometer consists of a Waters Aquity UPLC system connected to a Waters LCT Premier orthogonal accelerated time of flight mass spectrometer (Waters, Millford, MA), operated in positive electrospray ionisation mode. Full scan spectra from 50 to 1500 Da were acquired and individual spectra were summed to produce data points each 0.2 seconds. Mass accuracy and reproducibility were maintained by using an independent reference spray by the LockSpray interference. The analytical column was a 100 mm×2.1 mm i.d., 1.7  $\mu$ m C8 Acquity UPLC BEH (Waters). The two mobile phases were phase A: methanol/water/formic acid (74/25/1 v/v/v); phase B: methanol/formic acid (99/1 v/v), both also contained 5 mM ammonium formate. A linear gradient was programmed - 0.0 min: 80 % B; 3 min: 90 % B; 6 min: 90 % B; 15 min: 99 % B; 18 min: 99 % B; 20 min: 80 % B. The flow rate was 0.3 mL min<sup>-1</sup>. The column was held at 30 °C. Quantification was carried out using the extracted ion chromatogram of each compound, using 50 mDa windows. The linear dynamic range was determined by injecting standard mixtures. Positive identification of compounds was based on the accurate mass measurement with an error <5 ppm and its LC retention time, compared to that of a standard ( $\pm 2$  %). The identification of XM462 metabolites was performed in the same samples, using the extracted ion chromatogram of the expected compound, using a 50 mDa window, and were confirmed by checking that the accurate mass measurement had an error <5 ppm.

## **2.7 Dihydroceramide desaturase activity measurements**

HGC-27 Cells ( $1 \times 10^6$ ) were homogenated by sonication (30 sec sonication followed by 30 sec in ice, then 5 cycle of 15 sec sonication followed by 15 sec in ice, and finally 30 sec sonication then in ice) in 100  $\mu$ l of phosphate buffer 0.2M pH 7.4 (Na<sub>2</sub>PO<sub>4</sub> 0.2M, NaH<sub>2</sub>PO<sub>4</sub> 0.2M). Cell homogenates were added of vector (N/T), 50  $\mu$ M resveratrol or 8  $\mu$ M XM462 for 16 or 24 hours, then DEGS-1 enzymatic activity was assessed incubating samples for 4 hours at 37°C in a final reaction volume of 300  $\mu$ l of phosphate buffer added with 85  $\mu$ l BSA:Substrate Mix 35  $\mu$ M (BSA 3.3mg/ml in phosphate buffer, and 35  $\mu$ M of 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-C<sub>6</sub>-dihydroceramide, sonicated for 3 min) final concentration of 10  $\mu$ M, and with 30  $\mu$ l 1  $\mu$ mol of NADH in phosphate buffer. Reaction was stopped adding 700  $\mu$ l MeOH, then samples were mixed by vortexing and incubated over night at 4°C. Samples were centrifuged at 20000 x rpm for 3 min and supernatant was transferred in HPLC vials. HPLC analysis were performed with an Alliance apparatus coupled to a fluorescence detector using a CROMACIL-100 reverse-phase C18 column eluted with water : acetonitrile (25:75), both with a 0.1 % of trifluoroacetic acid, flowing at 1 mL mL<sup>-1</sup>. A total of 100  $\mu$ l each sample was injected. The detector was set at an excitation wavelength of 465 nm and measure the emission wavelength at 530 nm. Each sample was run for up to 15 min.

## **2.8 Fluorescence microscopy**

HGC-27 cells were transfected with GFP-LC3 containing vector (Addgene Inc., Cambridge MA, USA) (Jackson, PLoS Biol, 2005). Transient transfection was performed by using FuGENE 6 transfection reagents (Roche Diagnostic Products) and following the procedures recommended by the manufacturer. Briefly, cells ( $0.5 \times 10^5$  cells/well) were plated in 8-well chamber slides (IWAKI), allowed to adhere for 24 hours and then transfected with the plasmid containing either the GFP-LC3 conjugate or GFP (control). 24 hours later cells were briefly examined to evaluate fluorescent protein expression. Then the medium was supplemented with new medium containing the treatment molecules (resveratrol, XM462) at the final above specified concentrations. Starved cells received addition of medium without FCS and non essential aminoacids and were submitted to the same procedures of the other samples. Fluorescence images were obtained at a magnification of 40X in an inverted fluorescence microscope provided with digital camera (Zeiss, Germany).

## **2.9 Confocal Microscopy**

HGC-27 cells were seeded into in 8-well chamber slides (IWAKI), at final concentration of  $3.5 \times 10^4$  cells/well, allowed to adhere for 24 hours. Cells were then pre-treated for 15 min with  $0.5 \mu\text{M}$  ER-Tracker™ Red (glibenclamide BODIPY® TR) (Invitrogen) dissolved in HBSS (Euroclone). After the pre-treatment cells were washed with HEPES, they were treated with  $5 \mu\text{M}$  of either NBD-C8-ceramide or NBD-C8-dihydroceramide dissolved in NBS solution (HBSS+ FBS 1%+NEAA 1%+ L-glutamine 1%+ HEPES 1.25M). The NBD-CER/dhCER with RE tracker co-localization was analyzed 6 or 16 hours after cell treatment using a Leica TCS SP2 confocal microscope. Images were viewed at 162x magnification.

## **2.10 Cell synchronization and cell cycle analysis**

Cells HGC-27 were seeded at  $2.5 \times 10^5$  cells/plate into 60 mm plates. Cells were allowed to adhere for 24 hours, then they were synchronized inducing cell cycle arrest in G2/M phase with  $0.2 \mu\text{M}$  nocodazole (Sigma). After 10 hours from nocodazole treatment, cell were released from block by replacing media with fresh media added of vector (0.1% ethanol) alone (NOC) or added with XM462 or d2-C8-dhCER (see figure legends for treatment conditions). The cells were harvested and fixed with ethanol immediately after media replacing (T=0) or 4, 8, 16, 24, 32, 48, or 72 hours after release. Briefly, cell growth media is taken apart, and cells were trypsinized for 2 min with 0.4 ml trypsin, whose action is stopped using the same cell growth media. About  $1 \times 10^6$  cells were pulled down by centrifuging 5 min. at 1500 rpm, cell pellet was washed once with 1ml PBS ice cold and again centrifuged with same conditions. Cells were fixed at  $-20^\circ\text{C}$  overnight incubation with a 70% ethanol in PBS+FBS solution. Fixed cells were pulled washed once with ice cold PBS and stained at RT 1 hour with propidium iodide (PI) in  $50 \mu\text{g/ml}$  in 1ml PBS. Stained cells were analyzed by flow cytometry using a FACSort (Becton Dickinson); data analysis was performed using the software Cyflogic™ (CyFlo Ltd, Finland)

### **2.11 Reverse transcription PCR (RT-PCR)**

Cells HGC-27 were seeded at  $1.5 \times 10^5$  cells/well into 6 well plate. Cells were allowed to adhere for 24 hours, then were treated for 6, 16 and 24 hours with vehicle alone (N/T) or with XM462, d2-C8-dhCER or Revs (see figure legends for treatment conditions). Total RNA was extracted with RNA extraction column kit (Promega), and 1  $\mu$ g of RNA was reverse transcribed in cDNA using M-MLV Retrotranscriptase using oligo dT primers (Promega). The reaction was performed in a final reaction volume of 20  $\mu$ l incubating samples for 2 min at RT, followed by an incubation at 37°C for 45 min, then heated up at 42°C for 45 min. From the resulting cDNA, 25ng were amplified using GoTaq Poymerase (Promega) with primer pair that recognizes both unspliced and spliced XBP-1: forward primer 5'-GTTGAGAACCAGGAGTTAAGA -3' and reverse primer 5'-CATTAAATGGCTTCCAGCTTGG-3'. The PCR reaction occurred in a final volume of 25  $\mu$ l and it was performed using a GeneAmp PCR system 2770 (Applied Biosystem) with thermal cycler protocol: 1 cycle at 94°C for 2 min; 25 cycles at 94°C for 30 sec, 55°C for 1 min, 72°C for 30 sec; 1 cycle 72°C for 5 min. Amplification products were separated by electrophoresis in a 1.25% acrylamide gel subjected to 90 volts for 2 hours and 30 min.

### **2.12 Statistical analysis**

Data significance was evaluated by either *t* student test at  $p < 0.05$  or one-way ANOVA followed by the Bonferroni multiple comparisons if ANOVA significant ( $p < 0.05$ ). The western blottings presented are the most representative of at least three independent experiments. For densitometry quantification, data were acquired from three independent experiments and the medium value  $\pm$  standard deviation was reported.

### 3. RESULTS

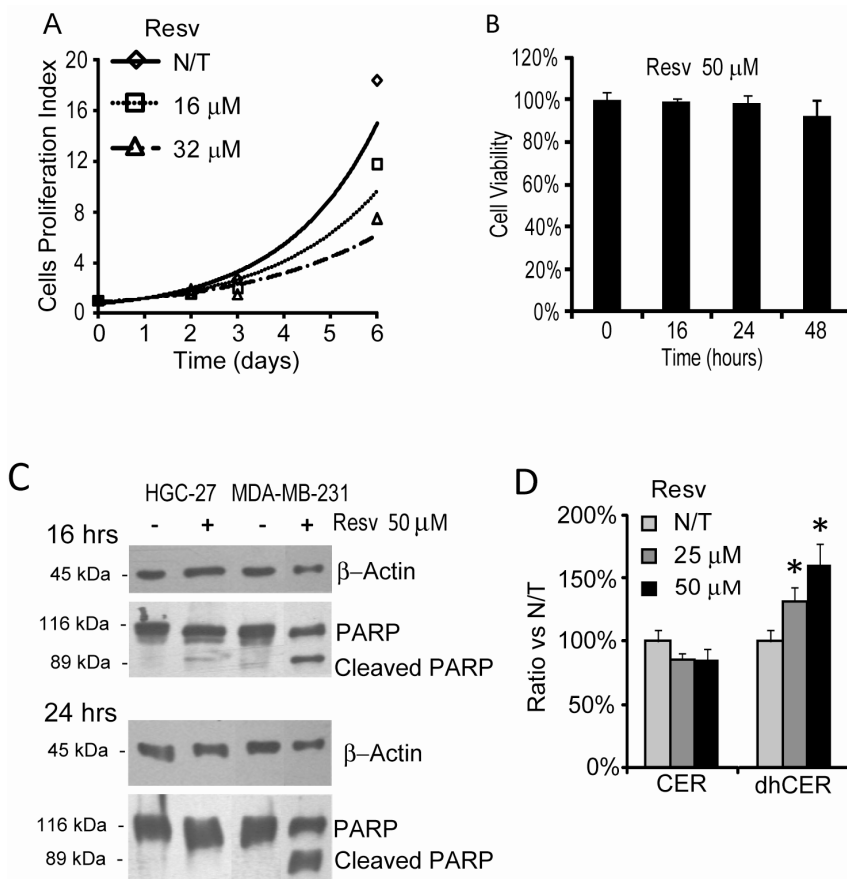
#### ***3.1 Resveratrol does not induce apoptosis and death in HGC-27 cells***

Resveratrol exhibits a double face on cell life/death regulation; from one hand it is able to induce cell death, from the other hand, simulating a calorie restriction state, it triggers cell viability (for review see [224]). In order to test resveratrol effect on HGC-27 cell line, we measured proliferation in compound treated cells by SRB assay (Fig. 1, panel A). HGC-27 cells were treated with resveratrol, at 16 and 32  $\mu\text{M}$ , and its effect on cell proliferation was detected 2, 3, or 6 days after treatment. The resveratrol treatment resulted in HGC-27 cell proliferation reduction within 6 days in a dose depending manner. Resveratrol did not affect HGC-27 cell survival within 48 hours after treatment, as revealed by trypan blue exclusion test (Fig. 1, panel B). To confirm the absence of resveratrol cytotoxic effect on HGC-27 cells, we evaluated, by western blotting analysis, the absence of cleaved poly-adenine-ribose polymerase (PARP), the terminal caspases target (Fig 1, panel C). PARP cleavage was not evident in HGC-27 cells treated for 16 and 24 hours with 50  $\mu\text{M}$  resveratrol. Using the same treatment condition for the MDA-MB 231 breast carcinoma cells, which were used as positive controls [4], PARP cleavage revealed that apoptosis was already induced 16 hours after resveratrol treatment (Fig. 1, panel C). All these data suggest that resveratrol had an effect on HGC-27 cell proliferation that, at least within 48 hours after treatment, is not associated with cell death. Previous studies from our laboratory showed that resveratrol-induced cell death is mediated by ceramide accumulation and signaling [4, 225]. From liquid-chromatography coupled to mass spectrometry (LC-MS) analysis we observed that treatment of HGC-27 cell line with resveratrol did not induce ceramide accumulation, but a high and dose dependant increase of the ceramide precursor, dihydroceramide was observed (Fig. 1, panel D).

#### ***3.2 Resveratrol induces a dihydroceramide accumulation comparable to that induced by XM462***

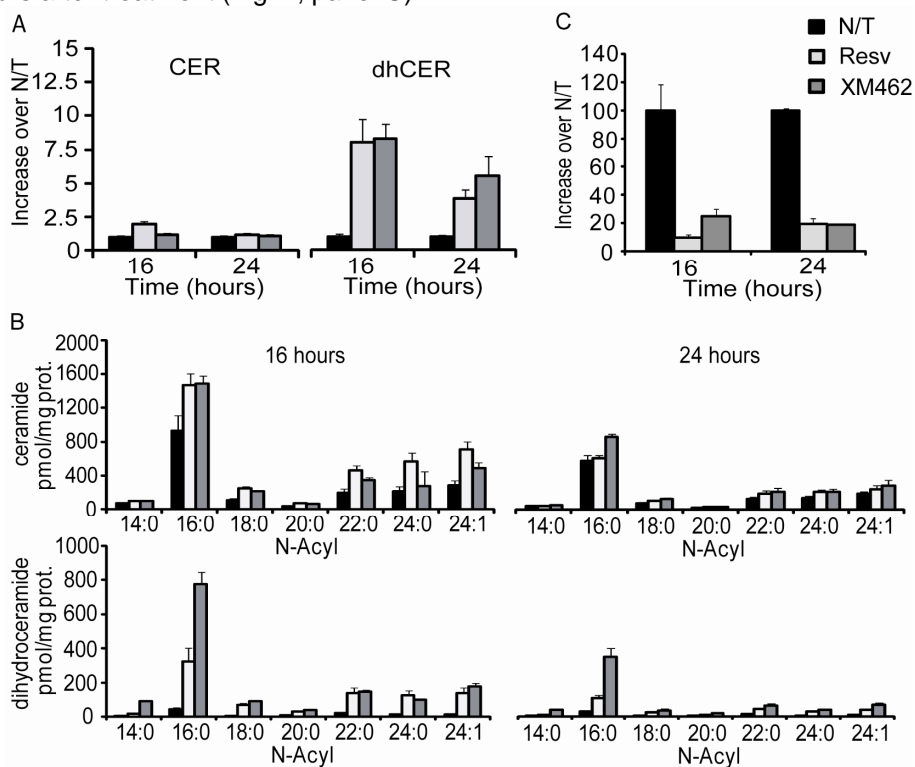
We next compared the effect of resveratrol with that of the specific inhibitor of DEGS-1 enzymatic activity (XM462) on intracellular concentration of dihydroceramide and ceramide species. Liquid-chromatography coupled to mass spectrometry (LC-MS) was performed to analyze total sphingolipids extracts from HGC-27 cells treated with resveratrol (50  $\mu\text{M}$ ) or XM462 (8  $\mu\text{M}$ ) for 16 and 24 hours. Treatment with resveratrol for 16 hours was able to induce an 8 fold increase over untreated cells in total dihydroceramides. The resveratrol effect on dihydroceramides accumulation was comparable with XM462 (Fig. 2, panel A). The resveratrol and XM462 treatment did not affected total ceramides amount. Interestingly, N-C<sub>16</sub> acyl chain specie exhibited higher increase compared with the other dihydroceramides under both resveratrol and XM462 (Fig. 2, panel B, bottom). The dihydroceramides accumulation persisted 24 hours after treatment, even if its magnitude was lower than that reported at 16 hours





**Fig. 1 Resv did not induce HGC-27 cell death.** A) HGC-27 cell proliferation was assessed by SRB assay. Cells were analyzed after 2, 3, or 6 days of treatment with the vector only (0.1% ethanol) (N/T), 25 or 50  $\mu$ M Resv, dissolved in ethanol. Cell proliferation was described using an exponential trend line. B) HGC-27 cells viability was assessed, by Trypan blue exclusion test, immediately before treatment (time 0) or after 16, 24 or 48 hours of treatment with 50  $\mu$ M Resv. Values from three samples, reported as average  $\pm$  SD, were calculated as ratio of a live cells over total counted cells. C) HGC-27 and MDA-MD-231 cells were treated for 16 or 24 hours with the vector only (0.1% ethanol) (-) or with 50  $\mu$ M Resv (+). The cleaved form of PARP was assessed as apoptotic marker. The western blottings presented are the most representative of three independent experiments. D) HGC-27 cells were treated for 24 hours with the vector only (0.1% ethanol) (N/T) or with 25 or 50  $\mu$ M Resv. Total ceramide and dihydroceramide were extracted and analyzed by LC-MS. Fold increase over N/T are reported as average  $\pm$  SD, calculated from three samples. Statistical significance was calculated using a two-tail Student T-test ( $p < 0.05$ ).

We speculated that resveratrol increases dihydroceramides endogenous level by inhibiting DEGS-1 enzymatic activity. To prove this hypothesis, desaturase activity was measured in homogenates of HGC-27 cells previously treated with resveratrol for 16 and 24 hours. Resveratrol dramatically reduced the DEGS-1 activity, showing an inhibitory action on desaturase comparable with the effect of XM462. The inhibition of both resveratrol and XM462 on DEGS-1 action persisted up to 24 hours after treatment (Fig. 2, panel C).



**Fig. 2 Resv DEGS-1 inhibition and dihydroceramide accumulation was comparable with XM462.** HGC-27 cells were treated for 16 and 24 hours with the vector only (0.1% ethanol) (N/T), with 50  $\mu$ M Resv or 8  $\mu$ M XM462. Total lipids were extracted and both ceramide and dihydroceramide species were quantified by LC-MS. A) The fold increase of total ceramides and dihydroceramides calculated as ratio of treated cells versus N/T is reported. B) The amount (picomoles) of the single species of ceramides and dihydroceramide, normalized over the mg of proteins, is reported. C) The dihydroceramide desaturase activity was evaluated in cells homogenates. In all cases, data were obtained from three experiments and values were expressed as the average  $\pm$  SD. Data significance was evaluated by either *t* student test at  $p < 0.05$  or one-way ANOVA followed by the Bonferroni multiple comparisons if ANOVA significant ( $p < 0.05$ )

### **3.3 Dihydroceramide accumulation does not induce cell death.**

Recently dihydroceramide has been proposed as a molecule involved in cell signaling.

In order to understand if dihydroceramide accumulation and survival observed in resveratrol treated cells was due to a resveratrol side effect or to the direct signaling induced by dihydroceramide, we investigated the effect of DEGS-1 inhibitor XM462 on cell survival. Cells viability, assessed using trypan blue exclusion test, was not affected after 48 hours treatment with any of the XM462 concentration used. (Fig. 3, panel A). Moreover the absence of cleaved PARP, analyzed by western blotting on HGC-27 cells treated for 24 hours with XM462, confirmed that the dihydroceramide accumulation is not cytotoxic. We excluded the possibility of apoptosis deficiency in our cell line by detecting PARP cleavage after 50  $\mu$ M etoposide treatment for 24 hours (Fig.3, Panels B). Interestingly, proliferation studies by SRB assay (Fig. 3, panel C) showed a dose dependant proliferation delay upon XM462 treatment, similarly to resveratrol treatment.

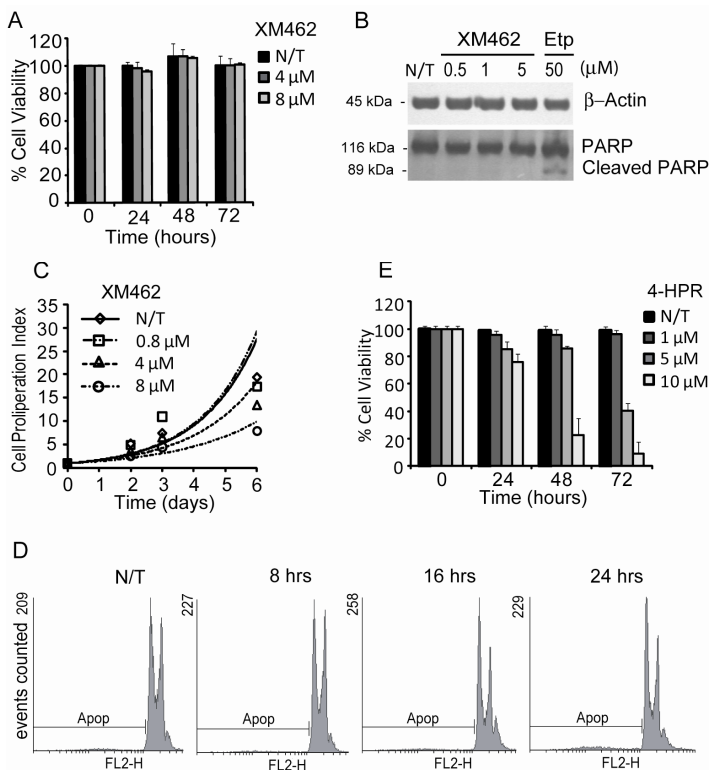
Therefore, we ought to investigate XM462 action by cell cycle analysis using propidium iodide (PI) DNA staining (Fig. 3, Panels D). HGC-27 cells, treated with 5  $\mu$ M XM462 for 8, 16 and 24 hours, did not show a significant apoptosis induction compared to the control population (N/T) (Fig. 3, panel D). These data suggested that dihydroceramide accumulation, obtained treating cells with a specific DEGS-1 inhibitor, was not able to trigger an apoptosis mediated cell death and did not influence cell viability.

Merrill and co-workers proposed that the antitumoral drug fenretinide (4-HPR) is able to induce dihydroceramide accumulation in MCF-7 cells associated with autophagy cell death [8].

Moreover dihydroceramide accumulation obtained in human neuroblastoma cell line either after transient DEGS-1 silencing or 4-HPR treatment inhibited cell growth through cell cycle arrest in G0/G1 phase [9]. On the other hand, dihydroceramide was reported to have an anti-apoptotic role by preventing ceramide-mediated channel formation in isolated mitochondria [10].

Thus dihydroceramide signaling is still unknown and might induce a different effect according to cellular settings.

In order to tight the link between resveratrol and dihydroceramide induced effects, we compared the treatment with XM462 and the non specific dihydroceramide inducer 4-HPR in HGC-27 cells. Knowing from previous studies reported in the literature that treatment in the micromolar range of tumoral cell line with 4-HPR are able to trigger dihydroceramide accumulation [8, 9, 100], HGC-27 cells were treated with 4-HPR (1, 5, 10  $\mu$ M) and XM462, viability was investigated, using Trypan blue exclusion test. Treatment with 4-HPR at 1  $\mu$ M did not affect HGC-27 cell viability (Fig. 3, panel E). Increasing 4-HPR concentration at 5  $\mu$ M, we observed a reduction in cell viability between 48 and 72 hours after treatment.

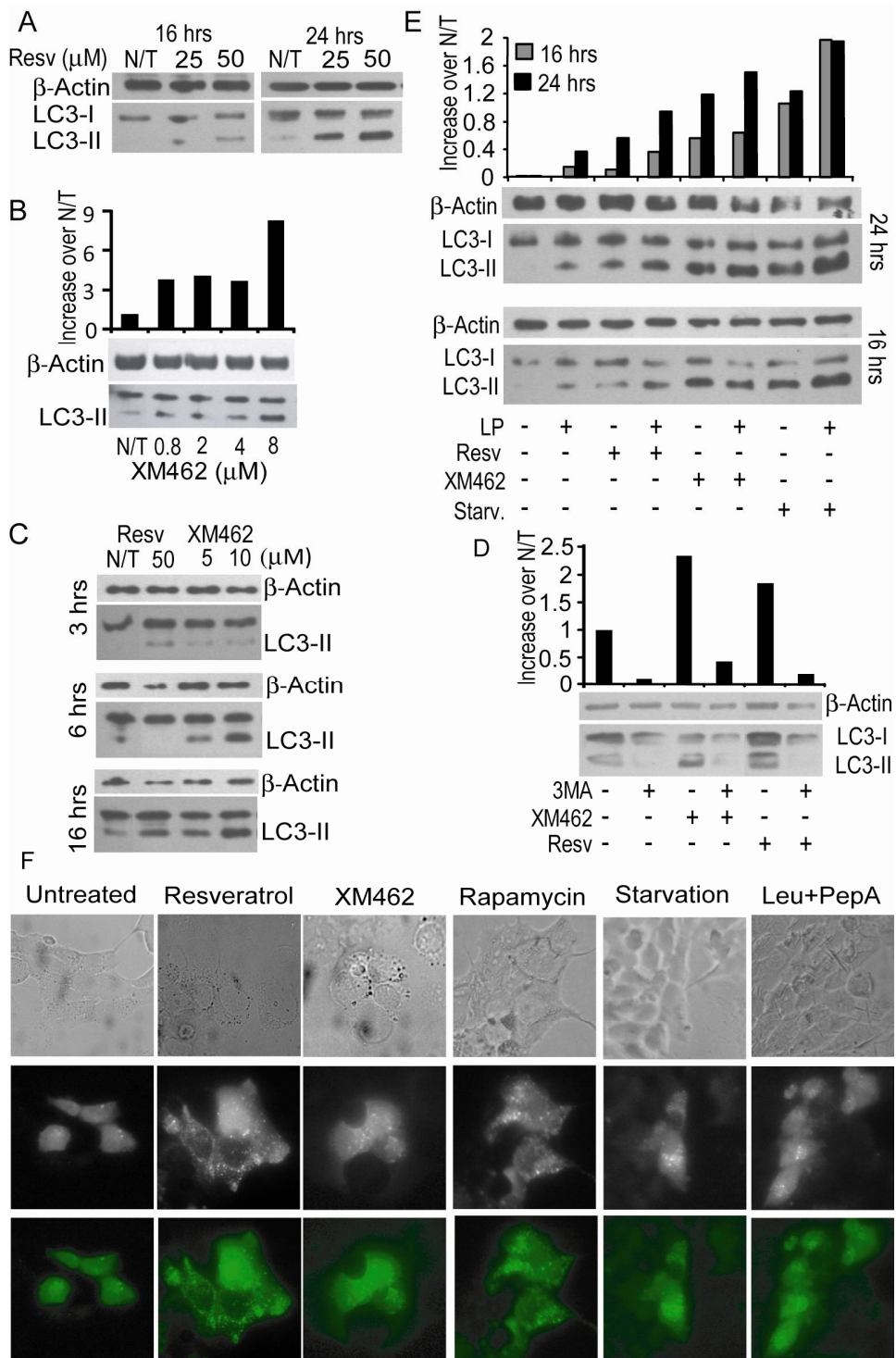


**Fig. 3 The effect of dihydroceramide accumulation on cell viability and cell death.** A) HGC-27 cells viability was assessed, by Trypan blue exclusion test, immediately before treatment (time 0) or after 24, 48, or 72 hours of treatment the vehicle only (0.1% ethanol) (N/T), with 4 or 8 μM XM462. Values from three samples, reported as average ± SD, were calculated as ratio of live cells over total counted cells. B) HGC-27 cells were treated for 16 or 24 hours with the vehicle only (0.1% ethanol) (N/T), with 0.5, 1 or 5 μM XM462, or 50 μM etoposide (Etp). The presence of the cleaved form of PARP was assessed as apoptotic marker. To verify the loading homogeneity, the housekeeping protein β-actin was also detected. The western blottings presented are the most representative of at three independent experiments. C) HGC-27 cell proliferation was assessed, by SRB assay, after 2, 3, or 6 days of treatment with the vector only (0.1% ethanol) (N/T), 4 or 8 μM XM462. Cell proliferation index values are reported as average ± SD and proliferation is described using an exponential trend line. D) HGC-27 cell cycle was analyzed immediately before treatment (time 0), 8, 16 and 24 hours after treatment with 5 μM XM462 using PI staining (PI) DNA staining. Events detected for FL2 signal was reported in logarithm scale (FL2-H). Experiment reported was the most representative of three. E) HGC-27 cells viability was assessed, by Trypan blue exclusion test, immediately before treatment (time 0) or after 24, 48, or 72 hours of treatment the vector only (0.1% ethanol) (N/T), with 1, 5 or 10 μM 4-HPR. Values from three samples, reported as average ± SD, were calculated as ratio of live cells over total counted cells. Data significance was evaluated by either *t* student test at  $p < 0.05$ .

The cytotoxic effect on HGC-27 cells was exacerbated increasing 4-HPR concentration up to 10  $\mu$ M, concentration significantly reduced cell viability between 24 and 48 hours after treatment (Fig. 3, panel E). Such dose dependant toxicity of 4-HPR is in agreement with the observation that 4-HPR acts on sphingolipid pathway inhibiting DEGS-1 activity but also inducing a concomitant SPT and CERSs activation as well as B-CDase2 overexpression [76, 100]. Treatment with this drug leads to the accumulation of dihydroceramide but also of Sa, a molecule with cytotoxic properties, and its phosphorylated form, Sa1P, molecule with putative pro-survival properties. Wang and coworker proposed a compensatory effect between Sa and Sa1P, and suggested that the 4-HPR cytotoxic effect could be explained by the increase ratio between Sa and Sa1P [100]. Our hypothesis is that dihydroceramide immediate accumulation may not trigger cell death but the accumulation of Sa byproducts may do that at higher doses.

### **3.4 Resveratrol and XM462 induce autophagy in HGC-27 cells**

Preliminary data reported that resveratrol is associated with autophagy induction in MCF-7 cells, followed by cell death [7]. Autophagy has a dual outcome: from one hand it is implicated in cell protection and survival; on the other hand its massive and prolonged induction leads to cell death, also known as Type II cell death [202]. Autophagy induction was evaluated in HGC-27 cells treated with different concentration of resveratrol, detecting the accumulation of the autophagosome membrane component LC3-II (the conjugated product of LC3-I to phosphatidylethanolamine). Resveratrol was able to induce autophagy in a dose dependant manner (Fig. 4 panel A). To verify the role of dihydroceramide in autophagy induction, we treated HGC-27 cells with increasing concentration of XM462. It resulted in a dose dependent manner LC3-II accumulation, suggesting that dihydroceramide is implicated in autophagy induction (Fig. 4 panel B). To understand the timing in autophagy induction, HGC-27 cells were treated in parallel with resveratrol and XM462 and LC3-II accumulation was analyzed 3, 6 and 16 hours after treatment. The specific DEGS-1 inhibitor rapidly induced autophagy in HGC-27 cells, leading to the accumulation of LC3-II within 6 hours after treatment at 5 or 10  $\mu$ M XM462. Resveratrol induced autophagy only after 16 hours of treatment (Fig. 4 panel C). In addition the co-treatment of either resveratrol and XM462, with 3-methyladenine (3MA), an PI3K type III complex inhibitor [226], abrogated LC3 conjugation with PE confirming the data above reported (Fig. 4, panel D). LC3-II could accumulate in two circumstances: an increase of autophagy or a reduction of its lysosomal clearance [227]. Hence, LC3-II was analyzed after co-treatment of resveratrol or XM462 with the lysosomal protease inhibitor leupeptin and pepstatin A (LP). Co-treatment with LP increased LC3-II accumulation mediated by resveratrol and XM462, confirming that the two compounds are able to enhance the autophagy flux (Fig.4 panel E).

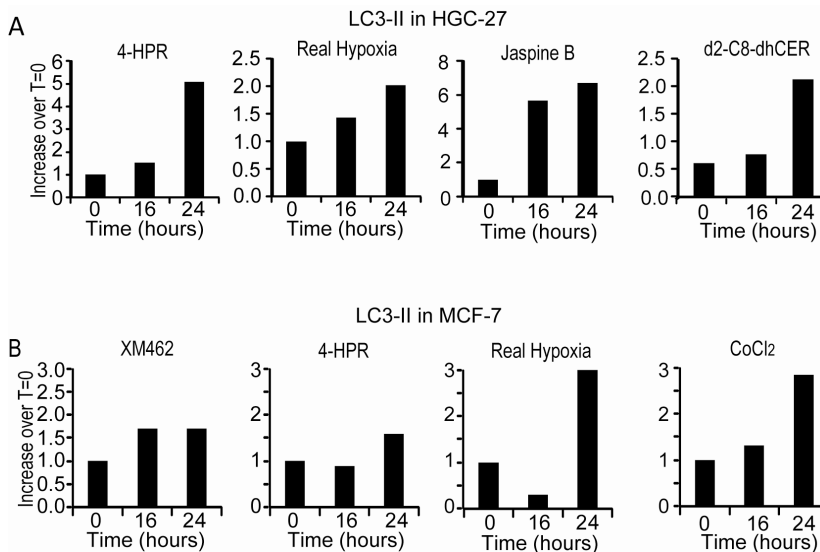


**Fig. 4 Resveratrol and XM462 promoted autophagy.** Autophagy induction evaluation of LC3-II by western blotting from HGC-27 cells. To verify the loading homogeneity the housekeeping protein  $\beta$ -actin was also detected. A) Cells were treated for 16 or 24 hours the vector only (0.1% ethanol) (N/T), with 25 or 50  $\mu$ M Resv. B) Cells were treated for 24 hours with the vector only (0.1% ethanol) (N/T) or with 0.8, 2, 4 or 8  $\mu$ M XM462. The band density was measured and increase of N/T was evaluated. C) Cells were treated for 3, 6 or 16 hours with the vector only (0.1% ethanol) (N/T), with 5 or 10  $\mu$ M XM462 or with 50  $\mu$ M Resv. D) Cells were treated for 24 hours with the vector only (0.1% ethanol) (N/T), with 8  $\mu$ M XM462 or with 50  $\mu$ M Resv, alone or in co-treatment with 5  $\mu$ M 3MA. E) Autophagy flux induction was evaluated treating cells for 16 or 24 hours with 50  $\mu$ M Resv, 8  $\mu$ M XM462 or starved in serum and non essential aminoacids free medium (Starv), in presence or absence of 10  $\mu$ g/ml of each protease inhibitors Leu and PepA (LP). F) GFP-LC3 localization in HGC-27 cells transfected with GFP-LC3 containing plasmid, rested for 24 hours and then treated with 10 $\mu$ g/ml of protease inhibitors (Leu+PepA), 50  $\mu$ M Resv, 8  $\mu$ M XM462. Treatment with 1  $\mu$ M rapamycin, or starvation were used as autophagy positive controls (40x magnification).

To further confirm that these molecules were able to induce autophagy, the subcellular localization of GFP-LC3 protein after transient transfection and its accumulation in autophagosomes upon treatment was also analyzed. HGC-27 cells were transiently transfected with a LC3-GFP conjugate containing vector and 24 hours after transfection, cells were treated for 24 hours with resveratrol or XM462. As a positive control of autophagy induction we used the direct mTOR inhibitor rapamycin or serum and non essential aminoacids starvation. As a further positive control, we treated cells with LP that induces intra cytoplasmic GFP-LC3 dots due to the inhibition of lysosomal clearance of autophagosomes. Fluorescence microscopy revealed that in negative control the fluorescence was diffused in the cytoplasm. Both resveratrol and XM462 were able to induce the formation of intra cytoplasmic GFP-LC3 dots in a fashion similar to the positive controls (Fig.4 panel F). All these data demonstrate that both resveratrol and XM462 induced autophagy, and strongly suggest that their action is due to the dihydroceramide increase.

Finally we evaluated autophagy induction in HGC-27 in response to other compounds previously known to trigger dihydroceramide accumulation (Fig. 5, panel A). Treatment with 5  $\mu\text{M}$  4-HPR, a concentration known to induce dihydroceramide accumulation in several cell lines [8, 9, 112], induced LC3-II accumulation within 24 hours of treatment. HGC-27 growth under hypoxic condition, that have been shown to induce dihydroceramide accumulation in tumoral cells [228], showed autophagy activation 24 hours after treatment. Moreover, treatment with 2.5  $\mu\text{M}$  of the natural marine compound, jaspine B, that induces dihydroceramide accumulation in A549 human lung carcinoma cell line [229], strongly activated autophagy already 16 hours after treatment. In order to prove that dihydroceramide exerts a direct effect on autophagy induction, we treated cells with a short chain dihydroceramide analogue that can not be metabolized into complex sphingolipids neither hydrolyzed. Treatment with 2.5  $\mu\text{M}$  of d2-C8-dhCER revealed LC3-II accumulation after 24 hours. All together these data reinforce the finding that dihydroceramide accumulation is tightly connected to autophagy activation and that dihydroceramide maybe a direct activator of autophagy.

In order to demonstrate that the effect of dihydroceramide on autophagy activation is not exclusive of HGC-27 cell line, we evaluated its effect also in the breast cancer cell line MCF-7. This cell line was treated for 16 and 24 hours with 5  $\mu\text{M}$  XM462 or 5  $\mu\text{M}$  4-HPR or with the hypoxic mimetic cobalt chloride ( $\text{CoCl}_2$ ) 0.4  $\mu\text{M}$ . All these treatments showed autophagy activation (Fig. 5, panel B).



**Fig. 5 Drugs inducers of dihydroceramide accumulation are also autophagy inducers.** A) HGC-27 cells were treated with for 16 or 24 hours with 5  $\mu\text{M}$  4-HPR, 2.5  $\mu\text{M}$  jaspine B, 2.5  $\mu\text{M}$  d2-C8-dhCER or grew under hypoxic condition. B) MCF-7 cells were analyzed 16 and 24 hours after treatment with 5  $\mu\text{M}$  XM462, 5  $\mu\text{M}$  4-HPR, 0.4  $\mu\text{M}$   $\text{CoCl}_2$  or grew in hypoxic condition.

Values were reported as increase over time 0, cells analyzed immediately before treatment.



### **3.5 Dihydroceramide accumulation induces a transient cell cycle arrest**

Cells respond to stress, such as nutrient depletion, hypoxia and unfolded protein accumulation, activating a plethora of cellular mechanisms necessary to the adaptation to the new environment. Autophagy is a major strategy in this response and several evidences demonstrate that its activation is accompanied by cell cycle arrest [230, 231].

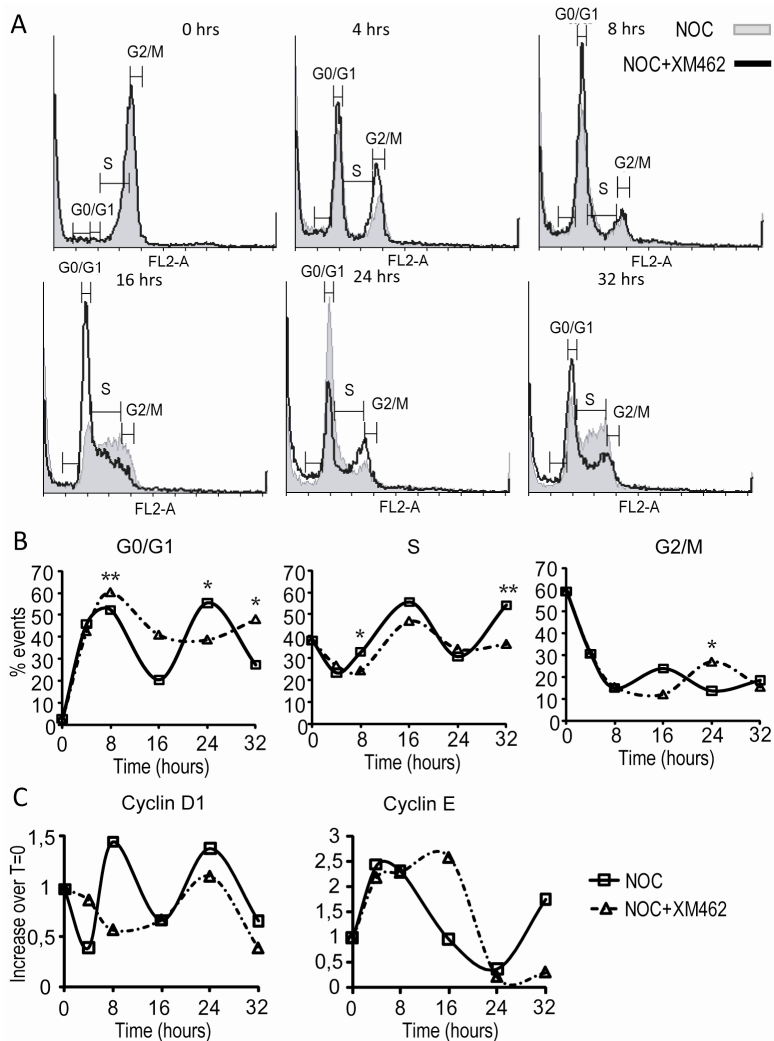
Silencing of DEGS-1 gene expression in neuroblastoma cell line induces cell cycle arrest in G0/G1 phase within 16 hours [9], suggesting that dihydroceramide could be involved in cell cycle regulation.

Therefore we analyzed the modulation of sphingolipids intracellular amount following XM462 treatment in HGC-27 cells. This modulation was correlated with variations in cell cycle progression. For such studies we synchronized cells using nocodazole (a pharmacological agent that blocks microtubules polymerization, arresting cells at the G2/M phase entry).

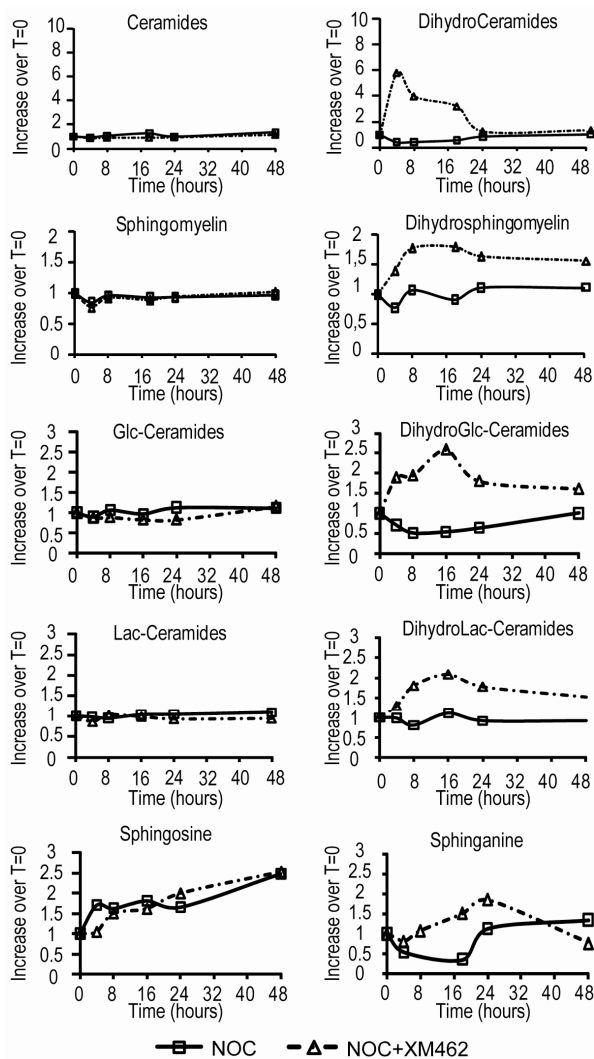
Once released from synchronization, by nocodazole removal, HGC-27 cells proceeded along cell cycle. 4 hours after release, cells moved from G2/M to G0/G1 phase, peaking in this next phase 8 hours after release. Untreated cells, peaked in S and G2/M phases within 16 hours after release, and newly in G0/G1 phase at 24 hours (Fig. 6 panel A and B). The synchronization was maintained by the cells for 72 hours (data not shown). XM462, treatment immediately at the release from nocodazole, induced a dihydroceramide accumulation that was able to perturb HGC-27 cell cycle progression with an anti-proliferative effect inducing a stop-and-go effect on cell cycle progression. The anti-proliferative effect was detected between 4 and 16 hours, when XM462 treated cells accumulated and remained in G0/G1 phase, whereas untreated cells started to flow through S phase (after 8 hours from release). This effect was only transient, causing a delay in cell cycle progression, because treated cells moved to S phase from 16 hours onwards. The progression from S and G2/M required the same time of untreated cells, peaking again in G1 phase after 32 hours (with an approximately 8 hours delay in respect to untreated cells). Thus dihydroceramide prolonged the permanence of cells in the G0/S boundaries and this delayed synchronization was maintained until 72 hours (data not shown).

The XM462 action between 4 and 16 hours was confirmed by the cyclin D1 and E analysis by western blotting (Fig. 6 panel C). Cyclin D1 conjugates with CDK4 and induces pRb phosphorylation and triggers cell cycle progression from G1 to S phase [134]. Cyclin D1 accumulation begins the cascade of events necessary to proceed with DNA replication during S phase. Untreated cells accumulated this protein at 8 and 24 hours after treatment, right at the time in which they passed from one G1 peak through the following cycle phases. On the contrary, cells did not accumulate cyclin D1 within the first 16 hours of XM462 treatment, resulting in a prolonged peaking at G0/G1. Later on, we detected an increase of cyclin D1, reaching a maximum at 24 hours, demonstrating that cells were still effectively able of progression along cell cycle. Cyclin E is a late G1 phase protein whose activity depends on its conjugation with CDK2 and it is degraded during S phase progression. Protein analysis revealed that in untreated control cells it accumulated within 8 hours after release, together with cells exit from G0, flowing to S. Cells

treated with XM462 exhibited a cyclin E peak at 16 hours delayed of approximately 8 hours from control cells, mirroring the 8 hours delay of cycle phases progression. To investigate whether dihydroceramide rather than one of its metabolites accumulation was able to induce the effect on cell cycle progression, the amount of sphingolipids species in nocodazole synchronized HGC-27 cells were measured using LC-MS at 0, 4, 8, 16, 24 and 32 hours after release with or without XM462. Analysis revealed that dihydroceramides accumulated in HGC-27 cells treated with XM462, having a peak 4 hours after release; then dihydroceramides level slightly decreased, returning to the original concentration at 24 hours after treatment (Fig.7 panel A). Ceramides did not increase between 8 and 48 hours after release (Fig.7 panel A) confirming that the desaturation block forced dihydroceramides metabolism in other sphingoid species starting from 4 hours after treatment onward. By analyzing different dihydroceramides metabolites, we found out that dihydrosphingomyelin species rapidly accumulated, reaching a peak 8 hours after release, remaining almost stable at all the analyzed times (Fig.7 panel B). The other two dihydroceramide metabolites dihydro-lactosyl-ceramide and dihydro-glucosyl-ceramide slightly accumulated after XM462 treatment, peaking 16 hours after release and then slowly decreased (Fig.7 panel C and D). Finally, 24 hours after release, sphinganine accumulated in HGC-27 cells (Fig.7 panel E). From such evidences we can conclude that dihydroceramide was at first metabolized in dihydrosphingomyelin that accumulated already 8 hours after release, only successively dihydro lactosyl- and glucosyl- ceramides grew up, peaking at 16 hours. The last sphingolipid to peak was sphinganine. We speculate that the delay of the cell cycle, focused within 16 hours after XM462 treatment, was due to the dihydroceramide accumulation (already present at 4 hours of treatment) rather than to the action of other metabolites.

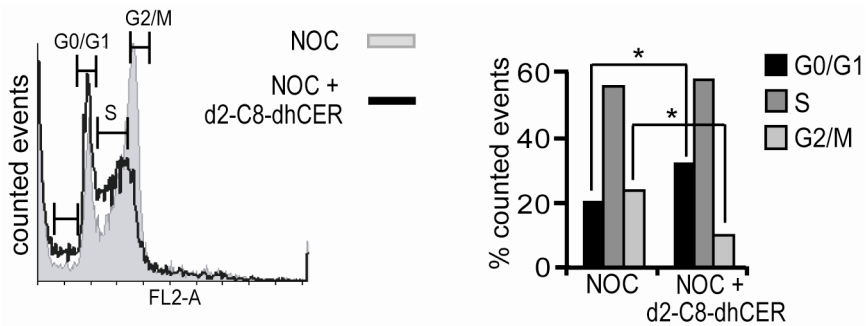


**Fig. 6 Dihydroceramide induce a transient cell cycle arrest.** HGC-27 cells were synchronized in G2/M phase treating for 10 hours with nocodazole 0.2  $\mu$ M. Synchronized cells were released from NOC-mediated synchronization replaced in fresh media in presence (NOC+XM) or absence (NOC) of 8  $\mu$ M XM462. DNA was stained with PI and cell cycle was analyzed by flow cytometry immediately before the release (time 0), 4, 8, 16, 24 and 32 hours after release. A) Overlay histogram from NOC (gray) and NOC+XM (black line) samples. Histogram was reported as counted events detected and FL2 signal reported in linear scale (FL2-A). The experiment reported was the most representative of three. B) Events quantified for each phase and calculated from three different experiments, were reported as percentage over total analyzed events. C) Cyclin D1 and cyclin E amount, reported as increase over time 0, were detected by western blotting and reported as bands intensity value normalized over  $\beta$ -actin. Data significance was evaluated by t student test at  $p < 0.05$ .



**Fig. 7 Spingolipids metabolism after XM462 treatment.** Total lipid extract from HGC-27 cells synchronized in G2/M phase by a 10 hours treatment with 0.2  $\mu$ M nocodazole. Synchronized cells were released from NOC-mediated synchronization by replacing fresh media in the presence (NOC+XM) or absence (NOC) of 8  $\mu$ M XM462. Total amount of different species of ceramide, dihydroceramide, sphingomyelin, dihydrosphingomyelin, glucosyl- (Glc) ceramide, Dihydro-Glc-ceramide, galactosyl- (Gal) ceramide, Dihydro-Gal-ceramide, lactosyl- (Lac) ceramide, Dihydro-Lac-ceramide, sphingosine and sphinganine were analyzed by LC-MS immediately before the release (time 0) or 4, 8, 16, 24 and 48 hours after release. Values were reported as spingolipids fold increase over time 0.

This finding was confirmed by the observation that treatment with 20  $\mu\text{M}$  d2-C8-dhCER, resulted in HGC-27 cell cycle arrest in G0/G1 phase. In fact the amount of treated cells in G0/G1 phase increase of 60%, compared with untreated cells. The presence of a discrete amount of cells in S phase concomitantly with a sensible reduction of cells in G2/M phase after treatment suggest that cells were transiently arrested in G0/G1 phase, and proceeded throughout cell cycle within 16 hours, probably as soon as dihydroceramide was cleared by sphingolipid metabolism. (Fig. 8)



**Fig. 8 Non-metabolized deuterated-dihydroceramide analogue induces cell cycle delay.** HGC-27 cells were synchronized in G2/M phase by a 10 hours treatment with 0.2  $\mu\text{M}$  nocodazole. Synchronized cells were released from NOC-mediated synchronization by replacing cells in fresh media in the presence (NOC+d2-C8-dhCER) or absence (NOC) of 20  $\mu\text{M}$  d2-C8-dhCER. Cells were stained with PI and cell cycle was analyzed by flow cytometry 16 hours after release. A) The counted events, detected as FL2 signal in linear scale (FL2-A), are reported as overlay histogram from NOC (gray) and NOC+d2-C8-dhCER (black line) samples. B) Events were quantified for each phase and reported as percentage over total analyzed events.

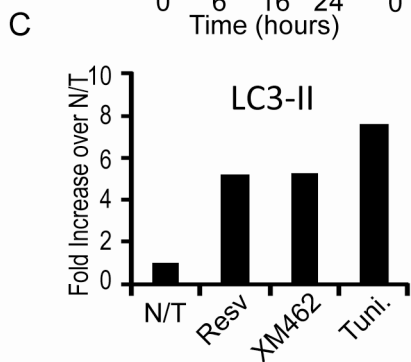
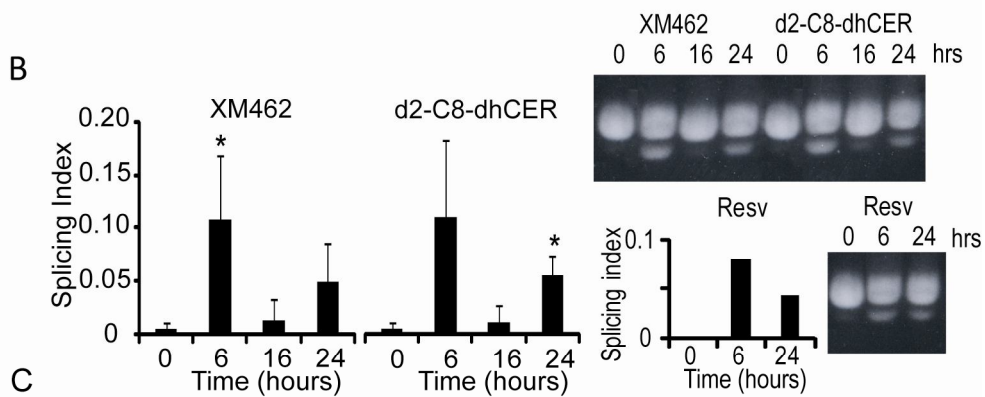
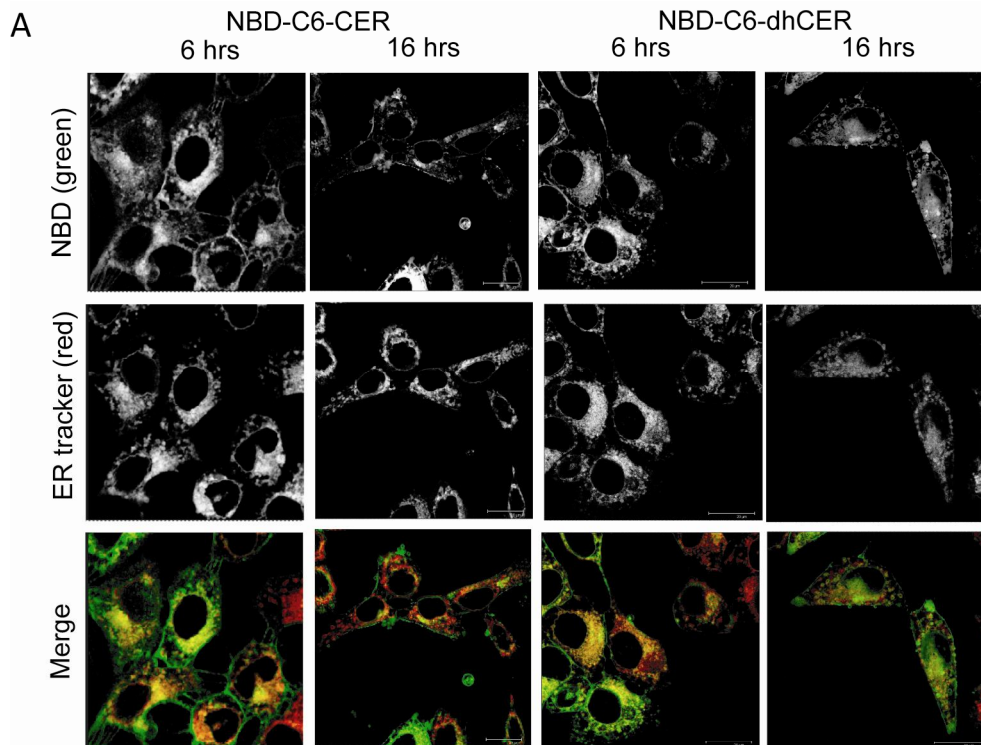
### **3.6 Dihydroceramide-induced autophagy is associated an ER stress response activation.**

Stress stimuli, such as glucose deprivation, hypoxia and oxidative stress lead to the accumulation of unfolded proteins in the endoplasmic reticulum (ER). Cell responds to ER engulfment activating a transcriptional and translational response, known as unfolded proteins response (UPR), an adaptive mechanism that regulates both survival and death effectors. An increasing number of studies indicate that autophagy and cell cycle arrest are induced by ER stress. Since the de novo synthesis of ceramide is located in ER [45], and that dihydroceramide transport from ER is about 40% slower than ceramide [108], we assumed that dihydroceramide accumulation into ER leads to an ER stress response.

To demonstrate this hypothesis, intracellular trafficking of C<sub>6</sub>-dihydroceramide and ceramide conjugated with a fluorophore (7-nitrobenz-2-oxa-1,3-diazole; respectively NBD-dhCER and NBD-CER) were assessed by confocal microscopy 6 and 16 hours after treatment. NBD fluorescence for both NBD-CER and NBD-dhCER (green signal), resulted localized into the ER, labeled with a specific tracker (red signal) after 6 hours treatment. Difference in NBD-CER and NBD-dhCER intracellular localization was reported 16 hours after treatment. While NBD-CER fluorescence signal moved into Golgi apparatus and plasma membrane, NBD-dhCER fluorescence signal was retained inside the ER compartment (Fig 9 panel A). These data confirm that also in our experimental model, the transport from ER to Golgi is more efficient for ceramide then dihydroceramide which, at list until 16 hours, accumulated inside ER.

To test whether dihydroceramide accumulation induced ER stress triggering UPR, HGC-27 cells were treated with XM462 and ER stress was investigated 8, 16 and 24 hours after treatment analyzing the XBP-1 splicing.

Treatment with the positive control tunicamycin induced UPR and XBP-1 splicing in HGC-27 at all the time point analyzed (data not shown). XM462, leading dihydroceramide accumulation within 4 hours after treatment, induced a significant XBP-1 splicing at the earliest time point. Splicing index in fact resulted statistically different from untreated cells only in samples analyzed 8 hours after treatment, while no significant difference was reported 16 and 24 hours after treatment. This evidence is in agreement with the hypothesis that dihydroceramide induced autophagic response is able to overcome a stress and to warren cell survival.



**Fig. 9 Dihydroceramide accumulated at the ER and induces ER stress.** A) HGC-27 cells, treated with 5  $\mu$ M of NBD-C6-ceramide (NBD-CER) or NBD-C6-dihydroceramide (NBD-dhCER) (green) in co-treatment with 0.5  $\mu$ M ER tracker (red), were analyzed 6 or 16 hours after treatment by confocal microscopy. NBD-dhCER, but not NBD-CER, co-localized (yellow) with ER tracker until 16 hours after treatment. B) XBP-1 splicing from HGC-27 cells analyzed immediately before treatment (time 0), 6, 16 or 24 hours after treatment with 8  $\mu$ M XM462, or 25  $\mu$ M d2-C8-dhCER; cells treated with 50  $\mu$ M Resv were analyzed after 6 or 24 hours after treatment. Unspliced XBP-1, upper band, were resolved from spliced XBP-1, lower band, by a 1.25% acrylamide gel. Splicing index, calculated as ratio of spliced versus unspliced XBP-1 values, were reported as average  $\pm$  SD from three different experiments. Statistical significance by 2-tails T-test with  $p \leq 0.05$ . C) Autophagy during ER stress was investigated analyzing LC3-II in HGC-27 cells treated for 24 hours with 50  $\mu$ M Resv, 8  $\mu$ M XM462 and 1  $\mu$ g/ml tunicamycin (Tuni). Values of LC3-II, normalized over  $\beta$ -actin, were reported as fold increase over untreated cells (N/T).

To confirm the dihydroceramide effect as ER stress inducer, HGC-27 cells were treated with the deuterated dihydroceramide analogue d2-C8-dihydroceramide (d2-C8-dhCER), a dihydroceramide analogue that can not be metabolized in other sphingolipids. Treatment with this molecule also induced XBP-1 splicing in 24 hours. The different time frame of ER stress induction may be related to the difference of the molecular pools (natural dihydroceramides versus short chain exogenous dihydroceramides) and to the required time for cellular uptake of the analogue. These data were also enforced by results obtained treating HGC-27 with resveratrol, that induced a XBP-1 splicing at 8 and 24 hours with a trend comparable to XM462 and d2-C8-dhCER (Fig. 9 panel B). All these data suggested that dihydroceramide accumulation is associated with ER stress induction.

To confirm that UPR activation is able to induce autophagy in our cell model, we treated HGC-27 cells for 24 hours with tunicamycin, a drug known to induce ER stress. This treatment resulted in an autophagy induction comparable with that observed under XM462 and resveratrol treatment (Fig. 9 panel C).



## 4. DISCUSSION

Objective of my doctorate project was to investigate on the biological role and the potential signaling activity of dihydroceramide.

Data from our laboratory in the gastric cancer cell line HGC-27 suggest that resveratrol, a polyphenol known for its anti-tumoral and antioxidant properties, is a good inducer of dihydroceramide. The results of my doctorate research have been partially published on Cancer Letters [232] and are included in a second manuscript in preparation.

Gastric epithelial cancer represents a suitable model to study the anticancer properties of resveratrol, being the gastrointestinal tract directly exposed to the native compound at the highest dietary concentration prior to its rapid glycosylation in the blood for further elimination. Surprisingly, treatment of the gastric epithelial cancer cell line HGC-27 with resveratrol did not affect cell viability (Fig.1, panel B and C), even when it was used at higher concentration than those that induced death in other cancer cells. Several authors, including us, described resveratrol anti-proliferative and pro-apoptotic activity in a variety of cancer cell lines as a consequence of the induced accumulation of ceramide and its signaling to either apoptosis- [4, 225] or autophagy- [7].mediated cell death.

Indeed resveratrol was not able to induce ceramide accumulation in HGC-27 cells, addressing us to hypothesize that these cells may switch on a putative resistance mechanism.

From the other hand HGC-27 maintained a sensitivity to resveratrol, which was able to exert anti-proliferative effect as shown by the reduction of cellular proliferation by SRB test (Fig. 1, panel A). This cell line responds to resveratrol treatment by increasing significantly the spontaneous base line level of autophagy. Autophagy increase was dose dependant to resveratrol treatment, as demonstrated by LC3-II accumulation and GFP-LC3 autophagosome-lysosome location (Fig. 4). Again, autophagy was not associated with cell viability decrease.

Several authors described resveratrol as a cell survival inducer, working as a calorie restriction mimetic molecule that enhances SIRT-1 activity, increasing life span in metazoans as well as in other organisms [213, 233, 234]. Any nutrient restriction, glucose and aminoacids, together with oxygen restriction, is tightly linked to enhanced autophagy in order to reduce cell demand and proliferation. Thus we investigated on resveratrol induced autophagy and sphingolipid metabolism in HGC27.

Peculiar of HGC-27 response to resveratrol is the effect on sphingolipid metabolic pattern. HGC-27 treated with resveratrol, in fact, did not accumulate ceramide, instead they accumulated its saturated form, dihydroceramide, which reached a 10 fold increase over control in the C<sub>16</sub>-dihydroceramide species (Fig. 2 panel A and B). This form derives from sphingolipid *de novo* synthesis and can be next metabolised in the other forms with longer and/or desaturated acyl chains. Thus resveratrol is able to block sphingolipid metabolism in a specific step of their *de novo* synthesis.

We found that resveratrol blocked the dihydroceramide desaturation into ceramide, reaction catalyzed by DEGS-1. The action of resveratrol was evident at 16 hours and increased its potency at 24 hours of treatment. Resveratrol effect is comparable with that induced by a specific DEGS-1 inhibitor XM462 (Fig. 2 panel

C). The resveratrol-mediated DEGS-1 activity inhibition may be linked to the cellular balance of  $\text{NAD(P)}^+/\text{NAD(P)H}+\text{H}^+$ . Desaturation reaction, catalyzed by DEGS-1, requires  $\text{NADPH}+\text{H}^+$  oxidation and oxygen reduction. Although the rate limiting step in the *de novo* sphingolipid synthesis is the serine-palmitate condensation, the desaturation of dihydroceramides may offer another important checkpoint governed by cell metabolism and energy state changes, which would depend on the of  $\text{NAD(P)}^+/\text{NAD(P)H}+\text{H}^+$  ratio and oxygen levels. We hypothesized that resveratrol, either directly or indirectly, mimicking a calorie restriction condition and changing the energy and redox cell state, could down regulate desaturase activity. This hypothesis is also corroborated by the observation that oxidative stress induced by  $\text{H}_2\text{O}_2$ , tert-butylhydroperoxide (an hypoxia mimicking agent) and menadione (an intracellular ROS inducer) inhibit DEGS-1 activity [235], and that depletion of cellular level of thiols result in a DEGS-1 activity inhibition [49].

Recent data reported from several groups described a contradictory role for dihydroceramide: from one hand it seems to be involved in pro-death mechanism [8, 9], on the other hand it induces pro-survival effect [10].

A few studies used 4-HPR, a drug known for its pro-apoptotic [236] and growth inhibitory [237] effect on tumoral cells, as a strategy for inducing dihydroceramide accumulation. Merrill and co-worker proposed that dihydroceramide accumulation after 4-HPR treatment of DU145 cell was associated with autophagic cell death [8]. In addition, the dihydroceramide accumulation following the transient gene expression silencing of DEGS-1 in a neuroblastoma cell line was recapitulated by treatment with low concentration of 4-HPR. Both treatments resulted in the arrest of cell cycle at the G0/G1 phase and consequently to growth inhibition [9].

From the other hand the anti-apoptotic role of dihydroceramide was also proposed, describing dihydroceramide action in prevention of ceramide-mediated channel formation in isolated mitochondria [10].

In the gastric cancer cell line HGC-27, resveratrol induced dihydroceramide accumulation instead of ceramide (Fig. 1, panel D). Similarly to resveratrol, treatment with DEGS-1 specific inhibitor XM462, in order to accumulate dihydroceramide, was not associated with any cell viability reduction, cell death or apoptosis induction (Fig. 3 panel B).

Dihydroceramide accumulation following the treatment of HGC-27 cells with XM462, resulted in autophagy induction 6 hours after treatment. Autophagic flux increased in a time and a dose dependent manner until 24 hours of treatment (Fig. 4). Taking into account that XM462 was able to induce a dihydroceramide peak 4 hours after treatment, earlier than autophagy, we can conclude that autophagy induction is downstream of dihydroceramide signalling.

When we treated cells with a non specific dihydroceramide inducer, 4-HPR we obtained the same enhancement of autophagy (Fig. 5 panel A), confirming that the common mediator dihydroceramide is linked to autophagy induction. We also noticed a dose dependent effect on cell viability (Fig. 3 panel A). 4-HPR in fact acts on sphingolipid pathway inhibiting DEGS-1 activity but also inducing a concomitant activation of the SPT, of CERSs and B-CDase2 [76]. For this reason treatment with this drug leads not only to the accumulation of dihydroceramide but also of sphinganine (Sa), which is a sphingolipid catabolism byproduct with cytotoxic properties. Cabot and co-workers recently proposed a balanced effect in cell fate regulation for the pro-death Sa and the pro-survival Sa-1-P, that is perturbed after 4-HPR treatment. Therefore they suggested that the 4-HPR cytotoxic effect is due

to the increased ratio between Sa and Sa1P [100]. These evidences suggest that the reduction of HGC-27 cells viability that we observed by 4-HPR treatment can be induced by the accumulation of sphingoid bases exacerbated in a time and dose dependent manner.

A further confirmation that dihydroceramide is involved in autophagy activation in HGC-27 cells resulted from the observation that stimuli or molecule, other than 4-HPR, known to induce dihydroceramide accumulation such as hypoxia and jaspine B [228, 229] were similarly able to induce autophagy. We detected autophagy increase after real hypoxia or jaspine B (Fig. 5 panel A) within 24 hours of treatment. Moreover this dihydroceramide action is not only prerogative of HGC-27 but it was also observed in breast tumoral cells treated with the metabolic inhibitor XM462 (Fig. 5 panel B).

When we treated HGC-27 cells with the metabolic inhibitor XM462 we observed that the dihydroceramide, accumulated in 4 hours, started to decline after 8 hours and returned back to basal level within 24 hours. Its clearance involved the metabolism in more complex sphingolipids, such as dihydrosphingomyelins first, followed by dihydrocerebrosides and the catabolism with the release of dihydrosphinganine. Dihydrosphingomyelins accumulation peaked already at 8 hours, whereas dihydro lactosyl- and dihydro glucosyl ceramides peaked at 16 hours after treatment. Catabolism by products such as dihydrosphingosine peaked at 24 hours after treatment (Fig. 7).

To confirm the direct effect of dihydroceramide on autophagy, we used a non metabolizable dihydroceramide analogue, d2-C8-dhCER, bearing the substitution of two hydrogen with two deuterium atoms, that accumulates within the cell after treatment. Western blotting analysis on HGC-27 treated with low concentration (2.5  $\mu$ M) of d2-C8-dhCER revealed that the analogue intracellular accumulation is able to induce LC3-II increase after 24 hours treatment (Fig. 5 panel A) with no sign of viability reduction (data not shown).

Autophagy is an essential mechanism for cell survival during stress. In nutrient starved MEF cells autophagy is activated within minutes [238]. Kuma et al. demonstrated that new born mice rapidly activate autophagy in several tissues in response to neonatal starvation [181]. Moreover autophagy is activated in vivo and in vitro under hypoxic conditions as a critical adaptive mechanism to maintain homeostasis, leading to cell survival [183, 239]. Thus under stress condition, autophagy can rescue survival impairing unnecessary cell effort such as proliferation. Potentially dangerous stimuli such as DNA damage, hypoxia, nutrient starvation, or ER stress, trigger a signal cascade that, acting on check points that are present in particular phases of the cell cycle, addresses cell to temporary growth arrest. Autophagy is tightly connected with cell cycle arrest, and their cooperation seems part of a survival strategy that maintains cell viability while cell proliferation is not desirable; in addition, cell cycle arrest and autophagy share common upstream signal transducer, such as mTOR inhibition. Recently Kravka et al. reported human neuroblastoma cells accumulate in G0 phase after 48 hours transient silencing of DEGS-1 and that was associated with reduction in cell viability [9], moreover the up-regulation of DEGS-1 gene expression was reported to up-regulate cyclin D1, thus triggering the progression from G1 to S phase.

Our data suggest that dihydroceramide, accumulating either in response to the nutrient stress mimicker resveratrol or to the sphingolipid metabolism inhibitor XM462, plays a role as a signalling inducer both in the regulation of cell cycle and autophagy, orchestrating the survival response to stressor stimuli. HGC-27 cells treated with XM462 delayed their progression along cycle, transiently arresting in G0/G1 phase, and this effect was observed since 8 hours after treatment, immediately after dihydroceramide accumulation. Such an effect is really peculiar in that we did not observe a cell cycle block that may lead to cell death, but a delayed ability to overcome the G0/S check point. Such a delay may actively enhance cell resistance since cells are known to increase their sensitivity to stress during the S phase. The XM462 effect on cell cycle progression, detected both by cell cycle analysis and protein quantification of cyclin D1 and E, was also limited at the gap between 4 and 24 hours, which represents the same time frame of dihydroceramide increase.

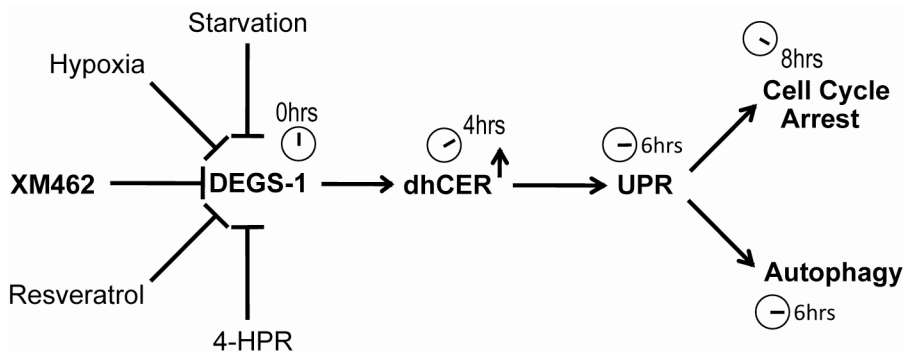
Several stresses can be sensed by ER, where the activation of the UPR can provide cells with the opportunity to adapt to stress and survive or to commit to programmed cell death. Treatment of HGC-27 with fluorescent labelled short chain analogues of dihydroceramide and ceramide (NBD-C<sub>6</sub>- dhCer or Cer) in co-treatment with specific ER tracker, reveals that the dihydroceramide analogue co-localize with ER for a longer time than the ceramide analogue (Fig. 9, panel A). Dihydroceramide may have a role in ER membrane composition. DEGS-1 is in fact an ER membrane enzyme and its activity is orientated toward the cytosolic face of the ER [45]. In addition dihydroceramide shows a reduced affinity to the carrier CERT than ceramide, resulting in a slow transportation from ER to Golgi [108]. Dihydroceramide intracellular accumulation is rapidly (within 2 hours from peaking) felt by ER sensors that initiate the UPR, as demonstrated by spliced XBP-1 increase in cells treated with XM462, resveratrol and the deuterated dihydroceramide (Fig. 9, panel B). The UPR is a potent autophagy inducer. The three signaling branches of this response, PERK which phosphorylates eIF2 $\alpha$ , IRE1 causing XBP-1 splicing, and ATF6 transcriptional activation, activate autophagy. Therefore we demonstrated that tunicamycin, a known ER stress inducer, is able to induce autophagy similarly to XM462, resveratrol and deuterated dihydroceramide (Fig. 8, panel C). In addition during ER stress, the translation inhibition of cyclin D1 causes cell cycle arrest in G1 phase, giving cells the opportunity to re-establish cellular homeostasis [146]. This evidence fits with our results on the cell cycle delay induced by dihydroceramide accumulation.

Several authors reported that the balance of saturated/unsaturated lipids affect biological membrane properties. Saturated, but not unsaturated, fatty acids accumulation within cells specifically slowed ER-to-Golgi protein trafficking, inducing protein overload inside the ER compartment, with consequent ER stress and UPR [240, 241]. Hence, the enrichment of ER membrane in dihydroceramide could decrease its protein permeability. Another demonstration of the effect of dihydro-sphingolipids enrichment in membranes was reported by Stiban et al. in mitochondria. They proposed that the increase of saturated/unsaturated sphingolipid ratio in the mitochondrial membrane uploaded with dihydroceramide, modify its biophysical properties, inhibiting the channels formation [10]. They demonstrated that ceramide, biosynthesized onto ER membranes, can move to mitochondria and that sufficient translocated amounts result in the permeabilization

of the outer mitochondrial membrane to proteins [242]. Moreover, Vieira et al. showed that dihydrosphingomyelin enriched membranes exhibit lower penetrability to viruses materials [243].

We speculate that the disruption of saturated/unsaturated sphingolipid ratio can affect the permeability of ER membrane and that dihydroceramide accumulation could reduce the ER-to-Golgi protein transport impairing vesicles formation and thus inducing UPR.

Taken all together these data design a possible picture of the different events following stimuli inducing dihydroceramide accumulation (Fig. 10). Different mild stimuli such as hypoxia, nutrient starvation, or different molecules such as resveratrol, XM462, jaspin B, would trigger dihydroceramide accumulation acting, directly or indirectly, on DEGS-1 activity. XM462 treatment leads to a rapid dihydroceramide accumulation, within 4 hours after treatment (Fig. 6), possibly at the ER (Fig. 9). This accumulation induces an unfolded protein response 6 hours after treatment, that in turn induces autophagy and reduces the proliferation rate as a survival strategy. When dihydroceramide amount inside ER starts to be cleared, due to its metabolism in complex sphingolipids, cell can proceed the cell cycle (Fig. 6) and autophagy is reduced to baseline level (48 hours after treatment, data not shown)



**Fig. 10 Events following the stimuli inducing dihydroceramide accumulation.** Different stimuli induce DEGS-1 inhibition and dhCER accumulation within ER. Cell respond to ER stress with the UPR activation that is followed by autophagy induction and cell cycle arrest.

We demonstrated that resveratrol is an inducer of dihydroceramide desaturase activity inhibition. Dihydroceramide accumulation, in response to resveratrol or HPR or jaspine B or to specific pharmacological enzyme inhibitor or dihydroceramide analogues treatment, mediate UPR activation, autophagy induction and cell cycle arrest and as a pattern of pro-survival response to stress. This may be the very first cell response to stress. According to cell specific setting and to the transient or prolonged nature of stress, this response may be overcome

by cell demise. Our gastric cell model is deficient for p53, which acts as a proliferation cycling gate keeper and it was reported as autophagic repressor [231, 244]. This is in agreement with a baseline autophagic activity in these cells and a high basal expression of LC3. Such deficiency may also cause the absence of ceramide induction that we and others previously obtained in response to resveratrol in a variety of cancer cells. We hypothesize that ceramide and apoptosis response is secondary to the cell cycle arrest, possibly via p53 induced cascade. In this view, the balance between dihydroceramides and ceramides can be important for cell to decide between survival or death. Finally, the growing evidences showing that dihydroceramides are signalling molecules advices to the need of taking with caution some of the roles previously attributed to ceramides, specially if the de novo pathway is involved.

## 5. CONCLUSIONS

Classically dihydroceramide was thought as an inactive precursor of the bioactive ceramide [101]. Only recently some groups proposed a biological activity of dihydroceramide [8, 9, 100, 104].

The results from my doctorate project first demonstrated that dihydroceramide accumulation is the first step of a well orchestrated cell survival strategy that involves: ER stress with the following induction of UPR, the modulation of cell cycle progression and the activation of a pro-survival autophagy.

We first demonstrated that resveratrol, a polyphenol known for its anti-tumoral and antioxidant properties, induces autophagy in the human gastric cancer cell line HGC-27, without affecting cell viability. Next we demonstrated that resveratrol-mediated autophagy is downstream of dihydroceramide, but not ceramide, signaling. We proved that resveratrol induces dihydroceramide accumulation through the inhibition of DEGS-1 activity, which results comparable with that observed treating cells with a DEGS-1 specific inhibitor. This is the first time in which resveratrol is described as a DEGS-1 inhibitor. Moreover, we demonstrated that dihydroceramide-mediated autophagy is independent from the type of the stimulus used for its accumulation (hypoxia, resveratrol, specific inhibitor of DEGS-1, deuterated dihydroceramide, fenretinide or jaspine B), or from the cell model in which it occurs.

We also described the dihydroceramide action on the modulation of cell cycle. Other groups reported that cell cycle is arrested in G0/G1 phase after DEGS-1 activity inhibition, and that cells are then committed to cell death. Here we demonstrated that dihydroceramide accumulation in HGC-27 cells induces a temporary cell cycle arrest in G0/G1 phase, that is overcome once dihydroceramide is metabolized and that it is associated with autophagy induction and cell survival.

Finally we demonstrated that dihydroceramide accumulation into the ER, obtained either via DEGS-1 inhibition or via a metabolic inactive dihydroceramide analogue, provokes ER stress and triggers unfolded protein response, as shown by the splicing of XBP-1.

Taken all together the data here reported described dihydroceramide as a molecule implicated in the triggering of a pro-survival response. Its accumulation induces ER stress and UPR, followed by the parallel delay of cell cycle progression and the activation of autophagy. Thus the dihydroceramide accumulation at the ER may result in a strategy to guarantee the cell-survival under stressor conditions.

Our data open a new perspective on the signaling role of sphingolipid metabolites which may be exploited in pharmacological approaches requiring cell proliferation and survival control.

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