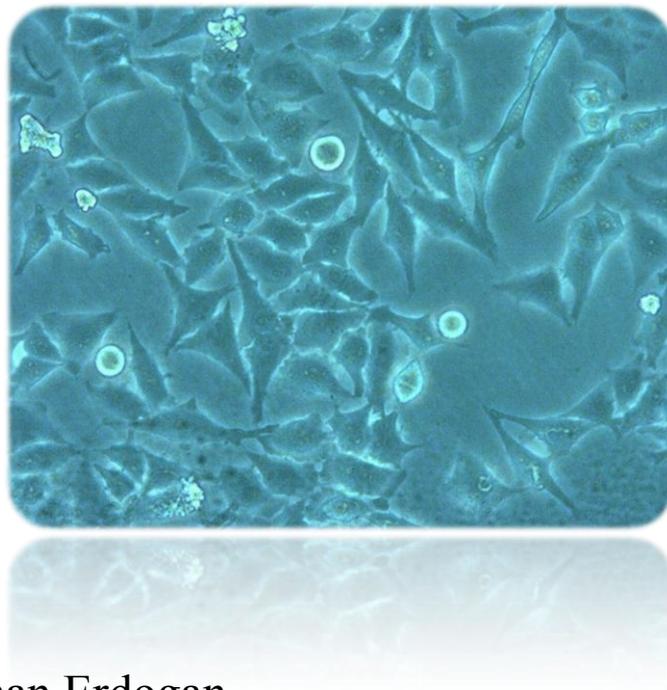# The Relation between Resveratrol, Sirt1, mTOR and Autophagy

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Abstract

Autophagy is an intracellular degradation system of proteins and organelles. It has several roles in the cell under different conditions. That is why; autophagy is highly regulated in the cell.

Sirt1 is member of mammalian sirtuins, which are the homologous of yeast silent information regulator 2 (sir2). It regulates several cellular process under different conditions, such as cancer, aging, metabolic regulation and cellular differentiation. It also functions in autophagy regulation by activating autophagy. Mammalian target of rapamycin (mTOR) is a main regulator protein of autophagy. It forms two complexes, which are called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Resveratrol is a polyphenol naturally found in several plants. It brings hope for treatment of several diseases. It is indicated as an activator of Sirt1. On the other hand, EX-527 is a novel inhibitor of Sirt1 which binds to the nicotinamide adenine dinucleotide (NAD^+) binding pocket of it. LC3A and LC3B are two novel autophagy markers, which are found cytosolic and autophagosomal membrane bound.

In this study, we propose to identify the relation between autophagy with mTOR and Sirt1. HeLa cells were exposed to 15 µM of resveratrol and 300 nM of EX-527, only 15 µM of resveratrol, only 300 nM of EX-527 or nothing. Protein concentrations of three experiment groups with four samples in each experiment were determined and western blotting analyses were done. The most LC3A/B accumulation was found in the cells, which were treated with both resveratrol and EX-527. Addition to this, we found that cells, which were treated with resveratrol, were found to have bigger cell size relative to the other experiment sets, indicating that resveratrol has an inhibitory effect on cell growth. On the other hand, cells treated only with EX-527 were found to have smaller cell size and least LC3 accumulation. These results were reproduced in three separate experiment groups. We concluded that resveratrol has an effect on cell growth either via activation of autophagy or by arresting the cell division cycle.

Keywords:

Acknowledgement

Table of Contents

1. Introduction

Autophagy

Autophagy is the catabolic process of the cytoplasmic components (proteins, organelles, RNAs etc.), which are degraded (Seglen and Bohley, 1992). There are three kinds of autophagy; macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Mizushima *et al*., 2008). In this work, macroautophagy is referred to as autophagy unless it is specified. Cytoplasmic components are degraded directly in lysosome (microautophagy and CMA) or in double membrane structure called autophagosome that is fused with lysosomes and endosomes (macroautophagy) (Mizushima, 2007; Mizushima *et al*., 2008; Tooze *et al*., 2010).

Autophagy has several roles in various conditions in the cell, such as starvation, aging, programmed cell death, and elimination of damaged organelles, tumor suppression and development (Mizushima, 2005). In parallel with various roles of autophagy, it is highly regulated *in vivo* (Mizushima, 2007; He and Klionsky, 2009). Under normal conditions, autophagy is mainly induced by target of rapamycin (TOR) (He and Klionsky, 2009), which functions as a major cellular nutrient sensor and key regulator of the balance autophagy and cellular growth (Jung *et al*., 2010).

Autophagy is generally triggered by starvation (Mizushima, 2007). In case of deprivation, TOR is inhibited, and autophagy is induced (Jung *et al*., 2010). In mammalian cells, mainly amino acid deprivation triggers autophagy in various cell types, except some distinct amino acids (Mizushima, 2007). Furthermore, it is reported that insufficient glucose causes autophagy induction in human embryonic kidney cells (Kim *et al*., 2011). On the other hand, autophagy is also regulated by the hormone signal pathways. Presence of insulin and insulin like growth factor (IGF) inhibits autophagic flux through TOR (He and Klionsky, 2009). It is suggested that insulin functions in autophagy regulation via TOR-dependent signaling pathway (Kanazawa *et al*., 2004). It is also reported that IGF-I counteracts the accumulation of autophagic vesicles and blocks the autophagic cell death of Purkinje neuron, which are cerebral granule neurons (Bains *et al*., 2009). Additionally, hypoxic stress, which is the situation of causing by low levels of oxygen at or below 1%, induces autophagy via AMP-activated protein kinase (AMPK), hypoxia-inducible factor-1 (HIF-1), E2F transcription factor or protein kinase Cδ (He and Klionsky, 2009). Autophagic flux is found to be induced under hypoxic conditions, and this leads to neuronal cell survival in mouse (Tanabe *et al*., 2011). Additionally, it is suggested that hypoxic conditions causes the induction of autophagy and

extended hypoxia causes autophagic cell death in several human breast cancer and glioma cell lines (Azad *et al.*, 2008). Formation of reactive oxygen species (ROS) is another kind of stress, which induces autophagy in cell through AMPK or autophagy-related gene 4 (Atg4). Autophagy is also reported as a cellular response after bacterial or viral infection. (He and Klionsky, 2009)

After induction of autophagy, autophagosome is formed. As the first step of autophagosome formation, cytoplasmic components are sequestered by phagophore, which is a unique membrane. Phagophore elongates and forms autophagosome. Afterwards LC3B binds to the autophagosomal membrane (Wu *et al.*, 2006). Then, autophagosome fuses with lysosome. At this level, degradation does not occur. Afterwards autophagosome fuses with lysosome and forms the final form of the autophagic vacuole, which is called autophagolysosome or autolysosome, before degradation process. As the final step, inner membrane of autophagosome and cytoplasmic constituents are degraded with special hydrolases. (Mizushima, 2007) (See Figure 1)
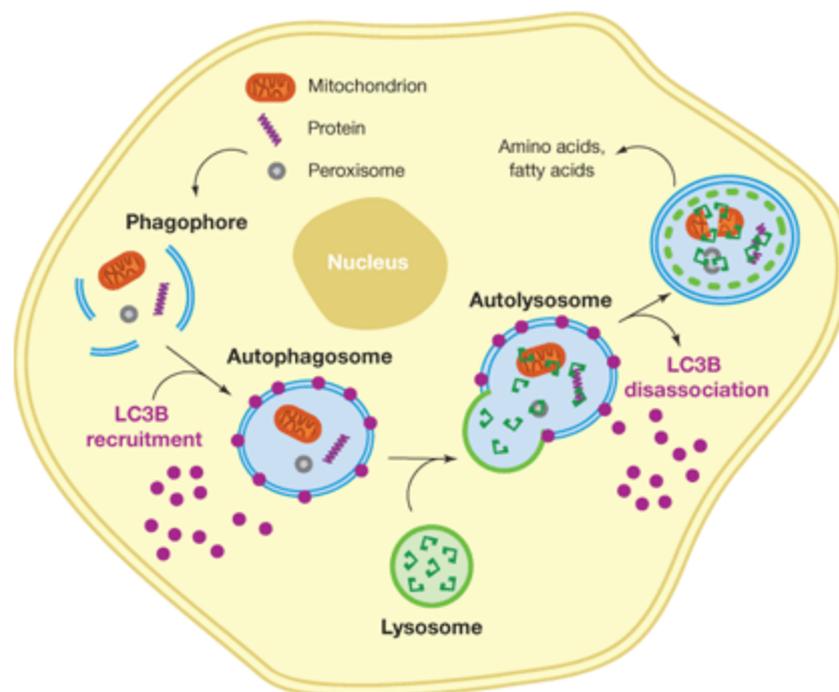
Figure 1. The autophagy process in eukaryotes. Purple circles indicate LC3B proteins. (Invitrogen, 2012)

The structures of the outer and inner autophagosomal membranes are highly different (Mizushima, 2007). Light chain 3 (LC3), which is the mammalian homolog of yeast Atg8, is a subunit of microtubule-associated proteins (MAPs), and it takes place on the inner membrane of autophagosome (Kabeya *et al.*, 2000; Mizushima, 2007). As the consequence of cleavage of carboxy termini of LC3, LC3-I is produced, and LC3-I is converted to LC3-II (Kabeya *et al.*, 2000;

Ohsumi and Mizushima, 2004). LC3A and LC3B are the human variants of LC3-I and LC3-II, respectively (Wu *et al.*, 2006). LC3-I is primarily cytosolic, whereas LC3-II is found as membrane bound (Kabeya *et al.*, 2000). The amount of LC3-II is suggested to be in a correlation with autophagosome formation (Kabeya *et al.*, 2000).

Target of rapamycin (Tor)

In the 1990s, genetic has identified two rapamycin target genes. These two genes were titled as *Target of rapamycin 1* and *2* (*TOR1* and *TOR2*). TOR1 and TOR2 play important roles in cell progression as responding different signals, mainly nutrients. Moreover, the conservations indicate that Tor genes are vital cell growth controller. (Yang and Guan, 2007) The mammalian Target of rapamycin also called mTOR, which is a catalytic subunit of the complex, is a serine/threonine protein kinase with molecular weight of 289 kDa. (Tsang *et al.*, 2007)

TOR proteins generate two TOR-complexes (See Figure 2). The TOR complex 1 (TORC1) contains KOG1 and lethal with SEC13 protein 8 (LST8), while the TOR complex 2 (TORC2) contains adheres-voraciously-to-target-of-rapamycin-2 protein 1/2/3 (AVO1/2/3) and LST8. It was found that rapamycin inhibits only TORC1, while TORC2 is rapamycin insensitive. In the mTORC1, regulatory associated protein of mTOR (Raptor), which is the mammalian homolog of KOG1, and mammalian LST8 (mLST8), which is also called G protein beta subunit-like (GβL), bind to mTOR. Interactions between these proteins result in mTORC1 that is sensitive to rapamycin inhibition. (Yang and Guan, 2007)

The mTOR signaling pathway plays a key role in many human diseases, for example Huntington's disease, or cancer. These diseases could be involved in immunosuppressive activity that causes cancer and cardiovascular diseases. (Tsang *et al.*, 2007) Moreover, mutations in the genes that encode components of the mTOR signaling network have been found in many human cancers, such as lymphomas, melanomas, carcinomas of the lung, bladder, kidney, ovary, stomach and prostate (Guertin and Sabatini, 2005). For instance, genetic anomalies those cause the overexpression of Protein Kinase B (Akt) or Ribosomal Protein S6 kinase 1 (p70S6K/S6K) lead to breast cancer (Tsang *et al.*, 2007).

Mammalian Target of Rapamycin Complex 1 (mTORC1)

The mTORC1 (See Figure 2) contains two subunits, Raptor and mLST8 (GβL). In the mTOR pathway, it shows that when Raptor is connected with mTORC1 by the stable interaction of Raptor and mTORC1, then mTORC1 strongly phosphorylates S6K and 4E-BP1 (4E-binding protein 1) (Hara *et al*., 2002). In the absence of Raptor in the mTORC1, phosphorylation levels of S6K and 4E-BP1 declined (Kim *et al*., 2002; Kim *et al*., 2003). In other words, Raptor has a strong influence on mTORC1 activity. Additionally, knockdown of Raptor causes alterations in cell morphology by disturbing the interaction between mTOR and its substrates (Ge *et al*., 2011).

mLST8 is another mTORC1 subunit. It contains seven WD40 repeats; therefore it interacts with the kinase domain of mTOR. mTOR kinase activity is thought to be regulated by mLST8 because it binds to mTOR kinase domain. (Yang and Guan, 2007) Nevertheless, mLST8 knockdown did not lead to a decrease in S6K and 4E-BP1 phosphorylation, which are the two substrates of mTORC1 complex, indicating that mLST8 is not essential for mTORC1 signaling during early embryonic developmental stages in mouse embryonic fibroblasts (MEFs) (Guertin *et al*., 2006). In addition to this, *mLST8*-null cells showed similar phosphorylation level of S6K and absence of *mLST8* did not disrupt the Raptor-mTOR interaction (Guertin *et al*., 2006). On the other hand, it was revealed that the nutrient- and rapamycin-sensitive interaction between mTOR and Raptor is positively regulated in complexes that contain GβL in HEK-293T cells (Kim *et al*., 2003).

S6K is characterized as one of the downstream targets of mTOR-signaling pathway that can directly phosphorylate mTOR (Holz and Blenis, 2005). 4E-BP1 is another downstream target of mTOR, which needs to be phosphorylated by mTOR to run a translation. Phosphorylation of S6K and 4E-BP1 result in the release of p4E-BP1, pS6K. Phosphorylated 4E-BP1 releases eukaryotic initiation factor 4E (eIF4E). Afterwards eIF4E and pS6K initiate protein translation. (See Figure 2) Additionally, protein translation is regulated by mTORC1 through S6K and 4E-BP1. Because mTORC1 signaling pathway is sensitive to nutrient, growth factors, insulin and stress factors, the phosphorylation of S6K and 4E-BP1 is therefore regulated by these factors. (Yang and Guan, 2007)

Mammalian Target of Rapamycin Complex 2 (mTORC2)

mTORC2 contains four components (See Figure 2) as rapamycin-insensitive companion of mTOR (Rictor), mTOR, stress-activated protein kinase-interacting protein-1 (Sin1) and mLST8 have been identified in mTORC2. Rictor, which is the characterizing member of the complex, is the homolog

of AVO3 in yeast. Thus, mTORC2 is also named as the Rictor complex. Rictor is a large protein with a molecular weight about 190 kDa, and has several domains that are thought to be important for substrate binding and assembly of mTORC2 (Yang and Guan, 2007).

Sin1, which has a conserved region in the middle of the sequence but with a limited amino acid homology among the species, is another subunit of mTORC2. The interaction between Sin1 and Rictor seems to be relatively stable. Because of this interaction, it was suggested that these two proteins create a structural foundation of mTORC2 by stabilizing each other. (Yang and Guan, 2007) It was demonstrated that, knockdown of either Rictor or Sin1 in HEK293 and HeLa cells resulted in a reduction of the protein levels of the other components of mTORC2 (Yang *et al*., 2006). It is also suggested that mTORC2 assembly and activity depend on the interaction between Rictor and Sin1 (Yang *et al*., 2006).

mLST8 is also a subunit of mTOR2 and it is involved in mTORC1 and mTORC2 activity (Yang and Guan, 2007). *mLST8* knockout found lethal in mice and found to result in only impaired mTORC2 activity, as the phosphorylation level of Akt on S473 declined, phosphorylation level of S6K and 4E-BP1, which are the substrates of mTORC1, stayed stable (Guertin *et al*., 2006). These results indicate that mLST8 is a functionally important for mTORC2 and perhaps not essential for mTORC1 activity (Yang and Guan, 2007).

Upstream factors of mTOR

mTOR signaling is regulated by different factors, including growth factors, stress, energy and nutrients. In the case of hormone-induced activation of mTOR, Phosphatidylinositol 3-kinase (PI3K) is activated. Active PIK3 activates 3-phosphoinositide dependent protein kinase-1 (PDK1) by catalyzing the conversion of Phosphatidylinositol 4,5-bisphosphate (PIP2) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Phosphatase and tensin homolog (PTEN), which is a tumor suppressor, down regulates the conversion of PIP2 to PIP3. Afterwards PDK1 phosphorylates T308 residue of Akt and activates it. Then Akt phosphorylates TSC2 and inactivates TSC1/2 heterodimer, which inhibits mTOR signaling. (Tsang *et al*., 2007) (See Figure 2)

On the other hand, when the cell is under energy-stress conditions or starvation, the AMPK is activated by AMP binding. Afterwards TSC2 is phosphorylated and TSC1/2 complex inactivates the Ras homolog enriched in brain (Rheb) protein, which is a small GTPase, by accelerating the GTPase activity and convert it GDP-bound (inactive) form. (Yang and Guan, 2007) (See Figure 2)

Downstream factors of mTOR

Studies revealed that downstream targets of mTOR are S6K, 4E-BP1 (Hay and Sonenberg, 2004) and Atg13 (Hosokawa *et al*., 2009). Energy depletion by low glucose treatment affects mTOR activity, which leads to the reduction of phosphorylation level of S6K. Under latent conditions, 4E-BP1 and S6K are bound to eIF3. Under active conditions, mTOR phosphorylates of S6K and 4E-BP1. (Hay and Sonenberg, 2004) The phosphorylation of S6K activates it and the active S6K promotes protein translation. 4E-BP1 is inhibited by phosphorylation and it releases the translation initiator eIF4 for the protein translation process. In addition, active mTOR enhances cell growth by promoting protein translation and increasing cell mass. (Hay and Sonenberg, 2004; Yang and Guan, 2007).

mTOR also regulates translation through S6K. When mTOR phosphorylates S6K, kinase activity of S6K increases. This leads to phosphorylation of 40S ribosomal S6 protein. Besides, S6K regulates translation of some specific mRNAs, which are called 5′ terminal oligopyrimidine tract (TOP) mRNAs. Components of the translation machinery, containing elongation factors, ribosomal proteins and poly(A)-binding protein, are encoded by these mRNAs. (Hay and Sonenberg, 2004) Additionally, overexpression of S6K in the absence of rapamycin was found to result in increased cell size in mammalian cells, suggesting that mTOR/S6K signaling pathway is important for cell growth (Fingar *et al*., 2002).

Atg13 is another downstream factor of mTOR. When Atg13 is knocked-out by siRNA, autophagic flux was found to relatively decrease. In addition to this, under starvation, Atg13 was found to be dephosphorylated. (Hosokawa *et al*., 2009) This suggests that mTOR down regulates activity of Atg13.
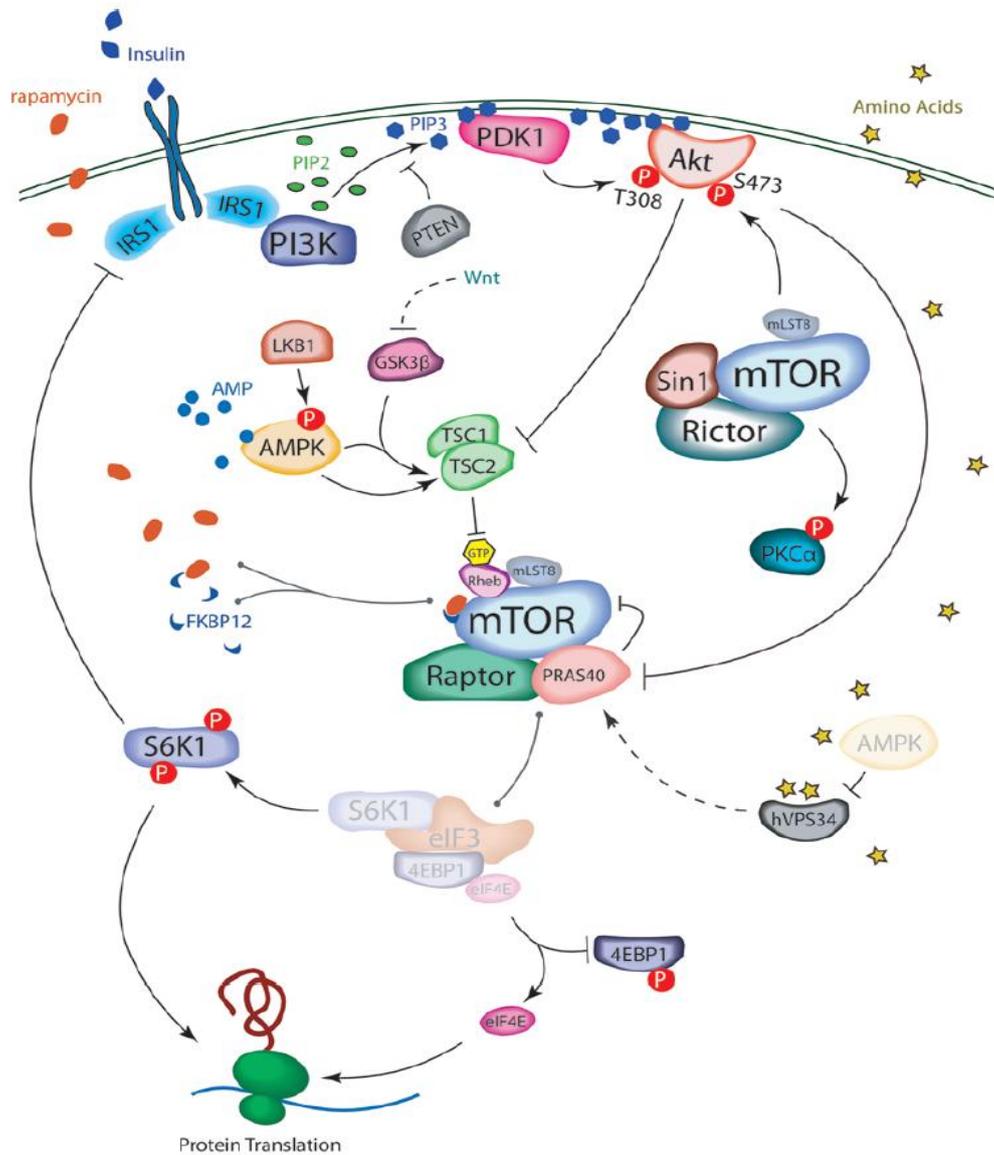
Figure 2. The mTOR signaling network in mammalian cells. mTORC1, the rapamycin-sensitive complex, consists of mTOR, Raptor, mLST8, and PRAS40. TSC1/2-Rheb is the major upstream regulator of mTORC1. Through the TSC1/2-Rheb axis, mTORC1 integrates cellular energy levels, growth factors, and Wnt signals to regulate protein translation by phosphorylating S6K and 4E-BP1. Phosphorylated S6K (active) inhibits IRS1 function and thus attenuates insulin/PI3K signaling. hVPS34 has been reported to sense nutrient availabilities for mTORC1. The mTORC2 subunits include mTOR, Rictor, Sin1, and mLST8. mTORC2 controls cell structure and survival by regulating PKCα and Akt. The upstream regulation of mTORC2 remains unknown. Arrows represent activation, bars represent inhibition, and dots represent binding (Yang and Guan, 2007)

Sirtuin1 (Sirt1)

Histone deacetylases (HDACs) modify histone and non-histone proteins by removing the acetyl elements from ε-amino groups of the lysine residues, and take role in different regulatory pathways that both suppress and enhance gene transcription (Gregoretti *et al*., 2004; Glozak *et al*., 2005). HDCAs are divided into four definite classes based on bioinformatic analyses (Gregoretti *et al*., 2004). Class III HDACs, also called sirtuins, are the members of silent information regulator 2 (sir2) family of proteins (Carafa *et al.*, 2012) that are nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases (Imai, *et al*., 2000; Landry, *et al*., 2000) and ADP-ribosyl transferases (Tanny *et al*., 1999).

Mammalian sirtuins, which are homologous of yeast Sir2, are Sirt1, 2, 3, 4, 5, 6 and 7 (Zhang *et al*., 2009), and vary broadly in localization, activity, and role in the cell (Bosch-Presegué and Vaquero, 2011). Sirt1, 6 and 7 are mainly localized in nucleus; Sirt2 and 3 can be found in nucleus in limited levels, however, they are mainly cytoplasmic and nuclear, respectively; Sirt4 and 5 are rigorously mitochondrial (Haigis and Guarente, 2006; Vaquero *et al*., 2007; Michishita *et al*., 2005). Besides, localization is important for regulation of their function and they have different roles in the response to different conditions at both cellular and organism level that have been linked to different human diseases such as cancer, diabetes and neurodegenerative diseases (Bosch-Presegué and Vaquero, 2011; Zhang *et al*., 2011). One of sirtuins functions is regulating the genome stability, thereby the carcinogenesis. Main function of Sirt1, 2, 3, 6 and 7 is to regulate the chromatin stability, and except Sirt7, they take part in chromatin regulation via deacetylase activity on histone and non-histone proteins (Bosch-Presegué and Vaquero, 2011). Diverse and important roles of Sirt1, which is the most studied among the mammalian sirtuins (Yi and Luo, 2010), in DNA damage and repairing process are indicated (Wang *et al*., 2008a). Additionally, it is indicated that Sirt1 is involved in cellular response to stress by forming facultative heterochromatin, which is crucial for the cell (Bosch-Presegué *et al*., 2011; Vaquero *et al*., 2004). It was asserted that Sirt1 deacetylates forkhead transcriptional factor FoxO3a, which is a member of Forkhead box-containing protein family (Burgering and Kops, 2002), and represses its transcriptional activity (Motta *et al*., 2004). In addition to these, it is demonstrated that sirtuins have different roles in cancer formation and progression. First of all, expression of sirtuins is altered in many types of cancers (Saunders and Verdin, 2007). For example, it is reported that Sirt1 is overexpressed in a number of cancers such as gastric cardiac carcinoma, prostate cancer (Huffman, *et al*., 2007; Feng *et al*., 2011), whereas it is

down regulated in BRCA1-associated breast cancer (Wang *et al*., 2008b). Besides, it is suggested that Sirt1 role in premalignant cells as a tumor suppressor by inhibiting c-Myc oncogene, which is active in most of tumors (Yuan *et al*., 2009; Menssen *et al*., 2012). Oppositely, it is also known that Sirt1 has the activity of directly silencing particular tumor suppressor genes, suggesting that Sirt1 overexpression leads to promote cancer development (Bosch-Presegué and Vaquero, 2011). Moreover, Sirt1 regulates p53 tumor suppressor protein; thereby it regulates apoptosis in different ways (Yi and Luo, 2010). Taken together, Sirt1 has important roles in cancer development and progression.

Furthermore, Sirt1 is linked to metabolic regulation. It is reported that Sirt1 regulates hormonal regulation such as IGF and thyroid-stimulating hormone in mammals (Akieda-Asai *et al*., 2010; Lemieux *et al*., 2005). Besides the hormonal regulatory role of Sirt1, improved insulin sensitivity and glucose uptake are indicated in the case of Sirt1 overexpression under insulin-resistant conditions (Sun *et al*., 2007). The positive regulatory role of Sirt1 in insulin secretion process in pancreatic β-cells is indicated (Bordone *et al*. 2006). In addition to these findings, it is revealed that Sirt1 is under expressed in endothelial progenitor cells in the presence of high glucose (Balestrieri *et al*. 2008). In another study, Sirt1 was found to lessen the lipid accumulation in human HepG2 hepatocytes through stimulating AMPK (Hou *et al*., 2008). Altogether, these findings suggest that Sirt1 is a metabolic regulator in cell.

Sirt1 is also indicated to have connection with aging-related conditions, such as diabetes, cancer and neurodegeneration (Anastasiou and Krek, 2006). For example, it is indicated that calorie restriction decreases the risk factor of age-related cancer, diabetes and cardiovascular diseases (Lane *et al*. 1999; Hursting *et al*., 2003). Calorie restriction is a dietary therapy that was found to extend the lifespan of different organisms that were tested (Haigis and Guarente, 2006). It is suggested that Sirt1 has a complex role in response to calorie restriction in neurodegenerative diseases (Chen *et al*., 2008). A recent study also suggested that Sirt1 is a key interagent in the hypothalamus that regulates the metabolism and aging process in mammals under low-nutrient conditions (Satoh *et al*., 2010). Another process that is affected by aging is mitochondrial biogenesis (López-Lluch *et al*., 2008). It is reported that acetylation level of the transcriptional cofactor PGC-1α which controls the mitochondrial formation in liver and muscle (Wu *et al*., 1999), is regulated positively by Sirt1 (Rodgers *et al*., 2005). One group asserted that mitochondrial number was increased in mice were provided with the high calorie diet supplemented with resveratrol, which is suggested to increase

the deacetylase activity of Sirt1 (Baur *et al*., 2006) All in all, Sirt1 is indicated as a key mediator that roles in aging-related conditions.

Besides, it is indicated that Sirt1 has a relationship with cellular development and differentiation. It is reported that Sirt1 play a role in development and activation of differentiation in germ cells in both male and female mice (McBurney *et al.*, 2003). Also, it is found that inhibition of Sirt1 suppresses the keratinocyte differentiation, and expression level of Sirt1 affects the replication capacity of human keratinocyte cells, suggesting that the Sirt1 is an important interplay with the keratinocyte differentiation pathway and regulates skin aging potently (Blander *et al*., 2009). Moreover, Sirt1 is found function as a repressor of target genes that regulate white adipocyte differentiation and fat storage (Picard *et al*. 2004).

EX-527, the potent inhibitor of Sirt1

EX-527 (See Figure 3) (the maximal half inhibitory concentration [IC_{50}], 98 nM), which was identified by high throughput screening, is an indole-based, potent and selective inhibitor of Sirt1 enzymatic activity (Napper *et al*., 2005; Solomon *et al*., 2006). This compound binds to the NAD^+ pocket of the Sirt1 protein (Nayagam, *et al*., 2006). It is reported that inhibition of Sirt1 with EX-527 caused an increase p53 acetylation in several cell lines; however, this inhibition did not result in any problems with cell growth or proliferation (Solomon *et al*., 2006). Additionally, inhibition of Sirt1 leads to down regulation of peroxisome proliferator-activator receptor gamma (PPARγ), which is a key element of fat metabolism and adipocyte differentiation, and thus leads to fat accumulation (Nayagam, *et al*., 2006).
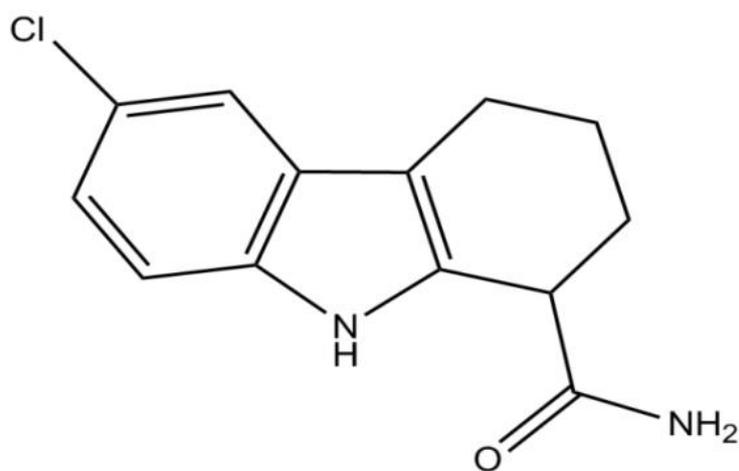

Figure 3. EX-527 (6-chloro-2, 3, 4, 9-tetrahydro-1*H*-carbazole-1-carboxamide)

Resveratrol

trans-Resveratrol (*trans*-3,4,5-Trihydroxystilbene) (See Figure 4) is a polyphenol, which is a natural product of several plant species, including nuts, grapes, and berries (Juan *et al*., 2012). UV exposure of *trans* isomer of resveratrol facilitates the production of *cis* isomer (Yu *et al*., 2012) (See Figure 4). *trans*-Resveratrol was found to extend the lifespan in *Caernohabtidis elegans* and *Nothobranchius furzeri* (Bass *et al*., 2007; Valenzano *et al*., 2006). Additionally, a variety of roles of resveratrol in different conditions in the cell was indicated, such as neurodegeneration, cancer and inflammation (Vang *et al*., 2011; Yu *et al*., 2012).
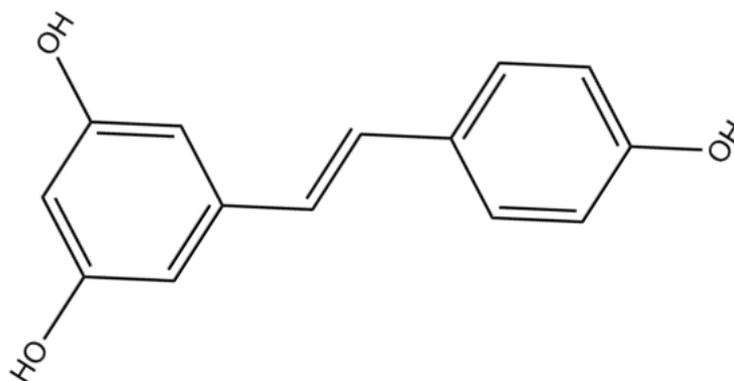
Figure 4. *trans*-Resveratrol (3,4,5-Trihydroxystilbene).

The relation between, mTOR, Sirt1, resveratrol, EX-527 and autophagy

TOR kinase is known to be the main regulators of autophagy signaling in the cell (Neufeld, 2010). In mammalian cells, mTOR signaling regulates autophagy (Jung *et al*., 2010), and Sirt1 regulates mTOR signaling (Ghosh *et al*., 2010). It is found that Sirt1-diminished HeLa, a human cancer cell line, cells showed higher phosphorylation of mTOR, and Sirt1 knockout caused an increase in mTOR activation in mouse embryonic fibroblasts (MEFs) (Ghosh *et al*., 2010). Inhibition of mTOR causes autophagy induction in the cell (Jung *et al*., 2010). On the other hand, it is suggested that Sirt1 inhibits mTOR signaling via TSC2 (Ghosh *et al*., 2010). It was also reported that Sirt1 induces autophagy by acetylating several components in the autophagy process (Lee, *et al*., 2008). Sirt1 deficiency was found to decrease LC3-I, LC3-II and autophagy-related gene 4 homolog C (Atg4c) levels in prostate cancer cells (Powell *et al*., 2011). Resveratrol was indicated as an activator and an inhibitor of autophagy through different pathways (Armour *et al*., 2009; Ghosh *et al*., 2010). It is suggested that autophagy is induced via AMPK activation by resveratrol is accompanied by the rise

of LC3-II level in SH-SY5Y, a human dopaminergic neuron cell line, cells (Wu *et al*., 2011). Resveratrol is also a specific Sirt1 activator (Borra *et al*., 2005). It is indicated that effect of resveratrol on the accumulation of LC3-II is arrested by suppression of Sirt1 (Wu *et al*., 2011). In addition to this, inhibitory effect of resveratrol on mTOR via Sirt1 was observed in MEFs (Ghosh *et al*., 2010). Additionally, inhibition of S6K by resveratrol disrupts autophagy in different mammalian cell lines in a Sirt1-independent manner (Armour *et al*., 2009). EX-527 is the potent Sirt1 inhibitor, which binds to the NAD^+ binding pocket of the protein (Solomon *et al*., 2006), and thereby inhibiting the deacetylase activity of Sirt1.

One of the goals of this project was to identify the effects of resveratrol, as an enhancer of Sirt1 and as a suppressor of autophagy via S6K, and inhibition of Sirt1 by EX-527 on the autophagy process. It was also an aim to understand more about the relations of these components in regards to autophagy. This was achieved by treating the cells with both resveratrol and EX-527, the inhibitor, only resveratrol, only inhibitor, or nothing. It was expected that treatment with both EX-527 and resveratrol would result in Sirt1 inhibition, which leads to autophagy induction, thereby less LC3A/B accumulation in the cell (See Figure 5a). On the other hand, treatment with resveratrol was expected to enhance Sirt1 activity, which results in autophagy induction (See Figure 5b), and treatment with EX-527 was expected to result in suppression the activity of Sirt1, which results in inhibition in autophagy process (See Figure 5c). Last experiment set was prepared as the control, in which the autophagy should continue at basal level (See Figure 5d).
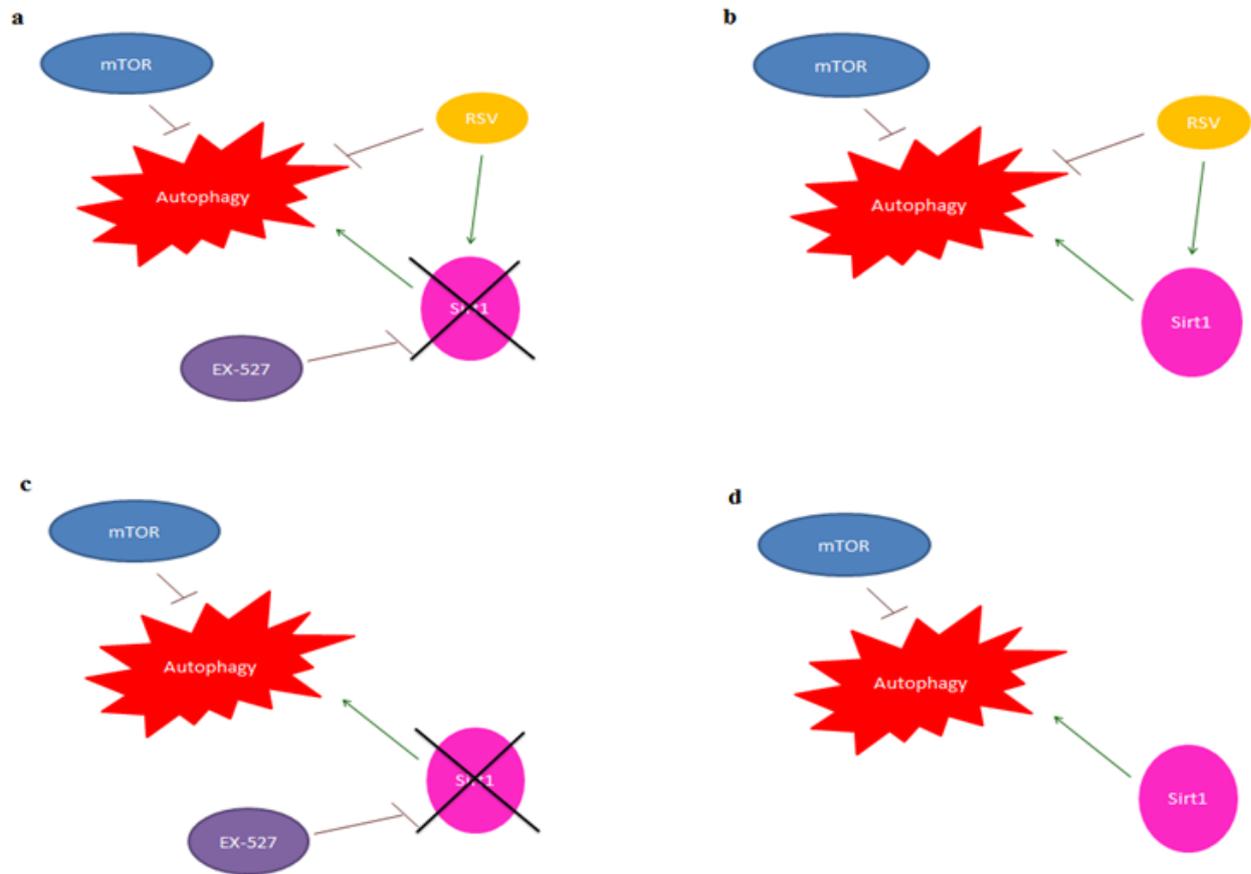
Figure 5. The setup of each experiment set and the effects of the components of autophagy. (a) Resveratrol (RSV) and EX-527 treatment results in Sirt1, which is an autophagy activator, inhibition. Resveratrol (RSV) also down regulates autophagy. (b) Resveratrol (RSV) treatment results in activation of Sirt1 and inhibition of autophagy. (c) EX-527 results in Sirt1 inhibition, which leads to inhibition of autophagy. (d) This experiment set was prepared as the control group without adding anything, which was expected to show autophagy at basal level.

2. Materials and Methods

Cell culture and treatments

HeLa cells, which are human adenocarcinoma of the cervix cells and the first human cancer cell line (Lucey *et al*., 2009), were thawed as it is described in Appendix 1. Cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM)-Glutamax™-I (GIBCO) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG) and 100 units/ml penicillin/streptomycin (Sigma-Aldrich), and cultured at 37 °C under humidified 5% CO_2 atmosphere. Resveratrol (Sigma-Aldrich) and EX-527 (Sigma-Aldrich) stocks were dissolved in dimethyl sulfoxide (DMSO) and 600 mM of resveratrol and 1 mM of EX-527 stocks were prepared. Cell dissociation solution was prepared with PBS (LONZA), 2% EDTA and 2.5% trypsin. HeLa cells were trypsinized as it is described in Appendix 2 to start new cell culture or to start the experiment.

Four petri dishes were prepared for the experiment sets, which is outlined in Table 1. Each petri dish was prepared as containing 250000, 500000 and 1000000 cells, which were counted by using a hemocytometer 16 quadrat as it is described in Appendix 3. Cells were treated with 15 µM of resveratrol 300 nM of EX-527 (1), 15 µM of resveratrol and DMSO (2), 300 nM of EX-527 and DMSO (3) or DMSO (Control) (4). Cell images were obtained before and after treatment. A Leica DMIRB microscope and Leica DC300F digital camera system with 10X and 20X magnifications were used for imaging the cells.

Table 1. The outline of the four petri dishes. Cells were exposed for 24 hours.

Treatment	# 1	# 2	# 3	# 4
Resveratrol (15 µM)	+	+	-	-
EX-527 (300 nM)	+	-	+	-
Control	-	+	+	+

Cells were exposed for 24 hours and trypsinized for protein collection.

Protein collection and concentration determination

Proteins were collected from the cells after 24 hours exposure as it is described in Appendix 4. Complete protease inhibitor (Roche) was used as the protease inhibitor in protein collection process.

0.8 mg/ml of BSA stock solution was diluted to 0.5 mg/ml, 0.375 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.063 mg/ml, and a standard curve was obtained for protein concentration determination. Protein concentrations of each experiment set were determined. Bio-Rad protein assay reagent A, reagent B and reagent S were used for the standard curve in Appendix 8 and the protein concentration determination by using a Heλios γ spectrophotometer, as it is described in Appendix 5.

Western Blotting

Western blot analyses were done for our 3 experiment groups. Buffers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were prepared as it is described in Appendix 6 and 7. Bio-Rad PAGEgel Dual Run and Blot Unit were used for SDS-PAGE and western blotting.

12-well RunBlue™ Precast SDS gels (4%-12%) (Expedeon), LDS sample buffer (Expedeon) and 1X LDS running buffer (Expedeon) were used in SDS-PAGE. Bio-Rad PAGEgel dual run and blot unit was used as SDS-PAGE and western blot apparatus. Page Ruler™ Protein Ladder (Fermentas) and Page Ruler™ PreStained Ladder (Fermentas) were used as the size-indicator. 8.92 μg of protein samples were denatured at 95 ^{o}C for 5 minutes and loaded into the wells of the SDS gels. Electrophoresis was set to 60 V. After 20 minutes, voltage was increased to 100 V. Two hours later SDS-PAGE was ended.

Proteins on SDS gels were transferred to western membranes, and the membranes were exposed with primary and secondary antibodies as they are described in Appendix 6. LC3A/B antibody (Cell Signaling Technology) and P0217 (DAKO) were used as primary and secondary antibodies, respectively. BioSpectrum® AC Imaging System (UVP) was used to obtain western blot membrane images. SuperSignal® West Femto Maximum Sensitivty Substrate (Thermo Scientific) was used to elicit the bands.

3. Result

Resveratrol treatment results in increased cell size in HeLa cells

In order to examine whether treatment with resveratrol and EX-527 together or separately affects cell morphology, cells were exposed to 15 μM of resveratrol and 300 nM of EX-527, 15 μM of resveratrol, 300 nM of EX-527 or without anything for 24 hours and images were obtained with 20X and 10X magnifications.

In first experiment, $5x10^5$ cells were plated in each petri dish (See Figure 6). It was observed that cells that were exposed with only resveratrol showed bigger sizes relative to the other three experiment sets (See Figure 6b). On the other hand, treatment with EX-527 led to smallest cell size (See Figure 6c). Treatment with resveratrol and EX-527 resulted in homogenous cell culture and average cell size was found less than resveratrol-treated cells (See Figure 6a). Cell sizes of each experiment set in the first experiment are indicated in Table 2.

Figure 6. Fluorescent microscopy images of HeLa cells exposed to (a) 15 μM of resveratrol and 300 nM of EX-527, (b) 15 μM of resveratrol, (c) 300 nM of EX-527 or (d) without anything for 24 hours. Cells were plated at the concentration of 5×10^4 cells/ml in a petri dish. Images were obtained by using a Leica DMIRB microscope with 20X magnification.

Table 2. The size and relative cell sizes of the cells in the first experiment.

Treatment	Real size (μm^2)	Relative cell size
RSV+EX-527	166.33	0.97
RSV	173.39	1.01
EX-527	162.05	0.95
Control	170.32	1.00

HeLa cells were exposed to 15 µM of resveratrol and 300 nM of EX-527, 15 µM of resveratrol, 300 nM of EX-527 or without anything (Control) for 24 hours. Cell sizes were measured by using ImageJ 1.45s software.

In second experiment, cells were plated at the concentration of 2.5 x 10^5 cells and incubated one extra day to obtain the same number of cells with other experiments, so a comparison with the other experiments is possible. This situation resulted in cells, which were overgrown in petri dishes (See Figure 7). Interestingly, effect of resveratrol on cell size was reproduced in this experiment as well (See Figure 7b). However, sizes of the cells that were treated with EX-527 (See Figure 7c) or without anything (See Figure 7d) were found similar, probably because of the density of the cells. Moreover, cells that were exposed with resveratrol and EX-527 (See Figure 7a) were observed having smaller cell size relative to the cells that were treated with resveratrol. Sizes of the cells in the second experiment are given in Table 3.

Table 3. The size and relative cell sizes of the cells in the second experiment.

Treatment	Real size (μm^2)	Relative cell size
RSV+EX-527	123.52	1.27
RSV	124.15	1.27
EX-527	79.42	0.81
Control	97.24	1.00

HeLa cells were exposed to 15 µM of resveratrol and 300 nM of EX-527, 15 µM of resveratrol, 300 nM of EX-527 or without anything (Control) for 24 hours. Cell sizes were measured by using ImageJ 1.45s software.
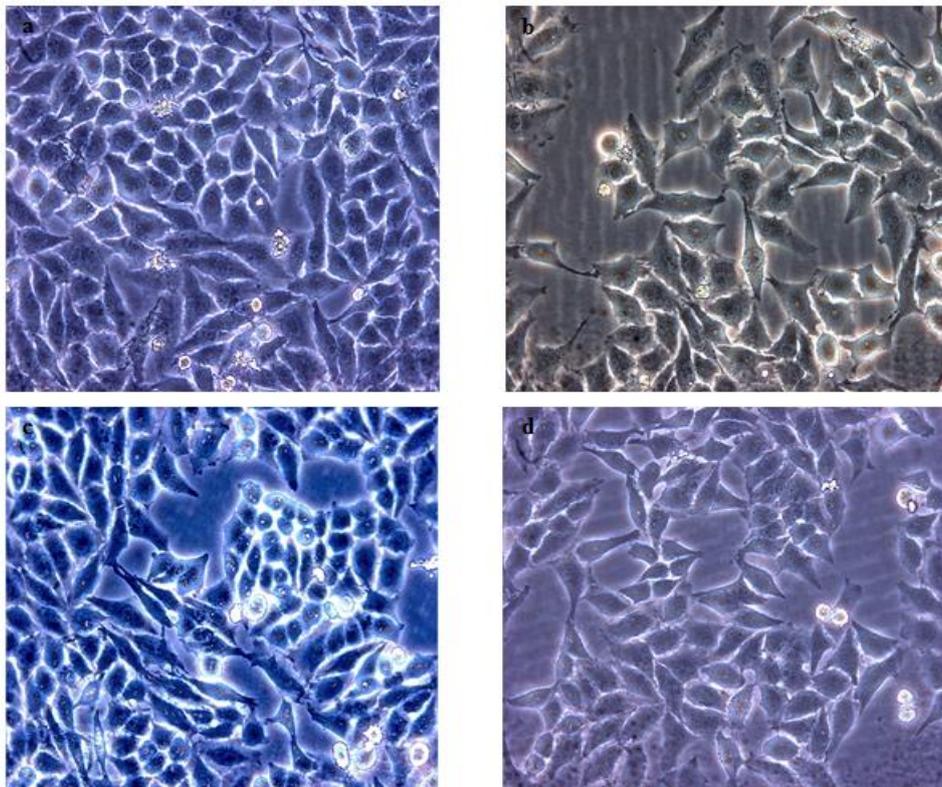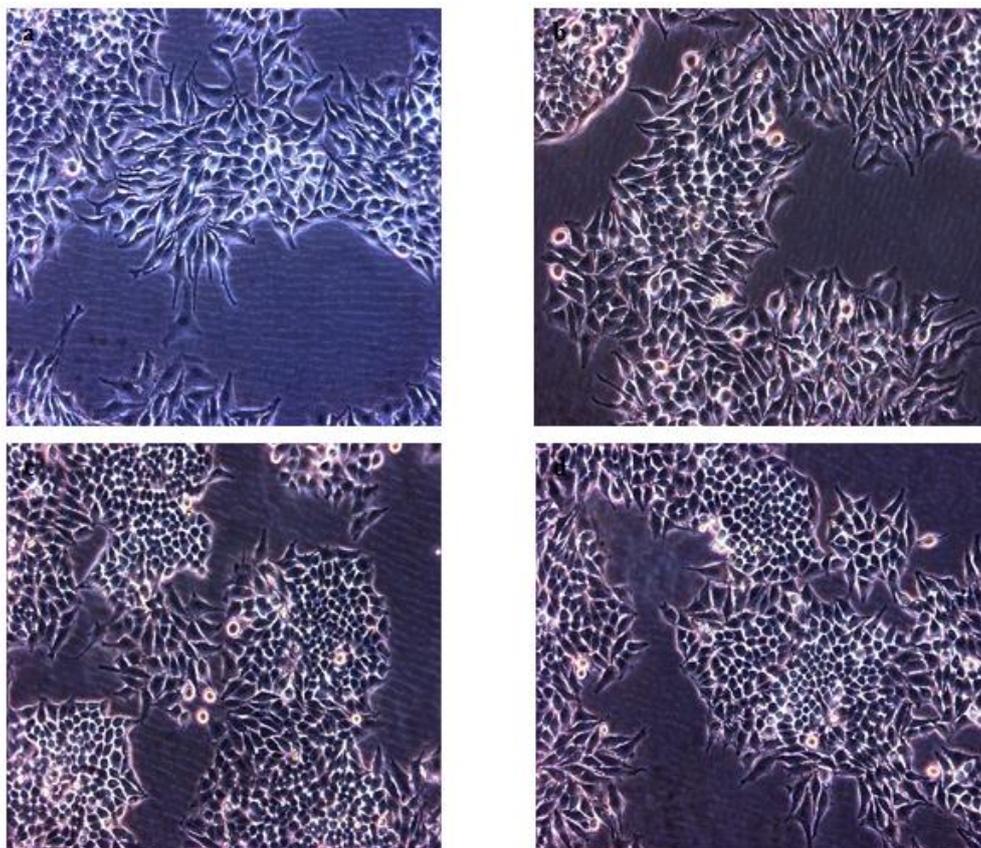
Figure 7. Fluorescent microscopy images of HeLa cells exposed to (a) 15 µM of resveratrol and 300 nM of EX-527, (b) 15 µM of resveratrol, (c) 300 nM of EX-527 or (d) without anything for 24 hours. Cells were plated at the concentration of 2.5 x 10^4 cells/ml in a petri dish. Images were obtained by using a Leica DMIRB microscope with 10X magnification.

In third experiment, $1x10^6$ cells were plated in each petri dish (See Figure 8). As it is parallel with the other experiments, cell size of resveratrol-treated cells had bigger cell size than the others (See Figure 8a). Moreover, treatment with EX-527 caused a reduction in sizes of HeLa cells, which were found to be the smallest in all of the experiment set (See Figure 8c, 6 and 7). On the other hand, resveratrol and EX-527 treatment resulted in cells that had bigger sizes than EX-527 or the ones without anything added, as it was observed in the first and the second experiment (See Figure 8b, 6 and 7). Sizes of the cells in the third experiment are shown in Table 4.
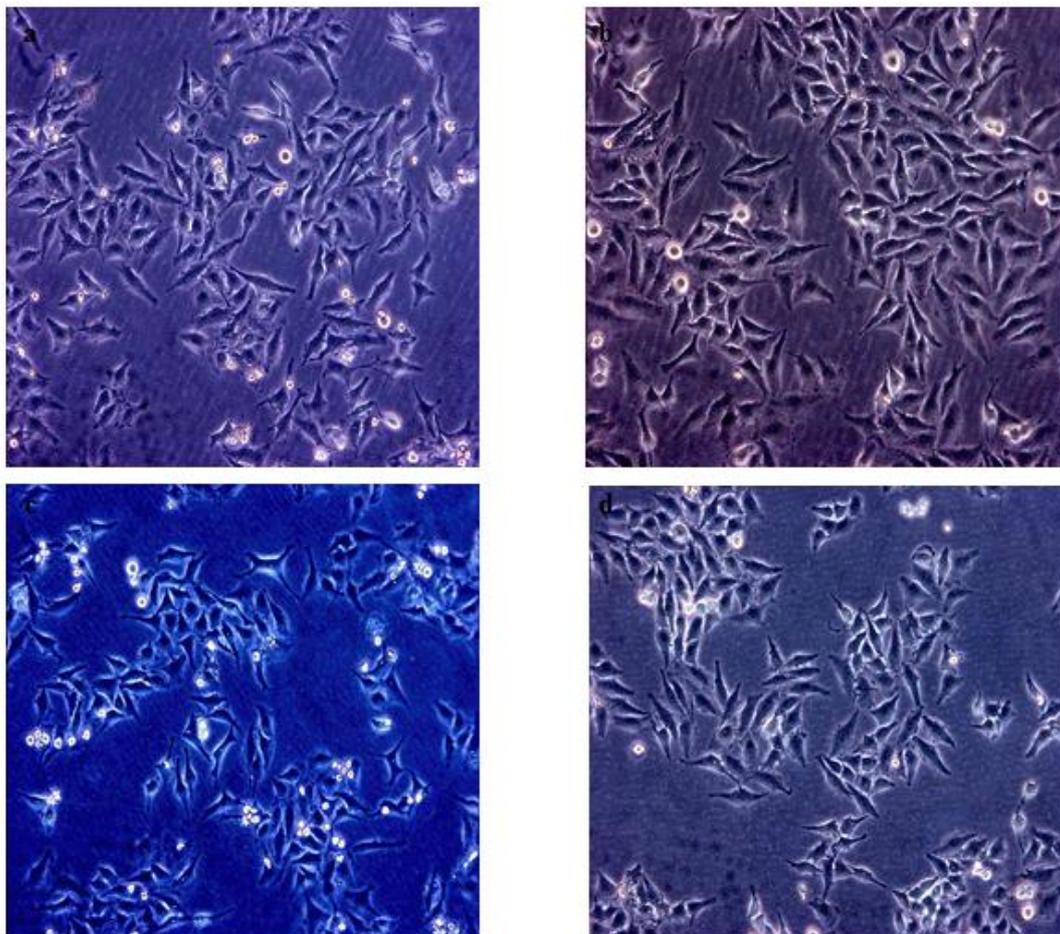
Figure 8. Fluorescent microscopy images of HeLa cells exposed to (a) 15 µM of resveratrol and 300 nM of EX-527, (b) 15 µM of resveratrol, (c) 300 nM of EX-527 or (d) without anything for 24 hours. Cells were plated at the concentration of 2.5 x 10^4 cells/ml in a petri dish. Images were obtained by using a Leica DMIRB microscope with 10X magnification.

Table 4. The size and relative cell sizes of the cells in the second experiment.

Treatment	Real size (μm^2)	Relative cell size
RSV+EX-527	127.36	1.31
RSV	131.99	1.35
EX-527	88.98	0.91
Control	97.15	1.00

HeLa cells were exposed to 15 µM of resveratrol and 300 nM of EX-527, 15 µM of resveratrol, 300 nM of EX-527 or without anything (Control) for 24 hours. Cell sizes were measured by using ImageJ 1.45s software.

Altogether, resveratrol treatment led to having the biggest cell size in all three experiment groups, suggesting that resveratrol has an inhibitory effect on cell growth. It is also noteworthy that the biggest cell sizes of all three experiment groups were observed in the first experiment, in which the cells that were treated with resveratrol. This suggests that effect of resveratrol on cell growth might be affected by the cell density. EX-527-treated cells were found slightly smaller than the cells without anything added in first and third experiments, and were found having similar cell size with and without anything added in second experiment, indicating that EX-527 might have an inhibitory effect on cellular development in HeLa cells. On the other hand, results of exposure of the cells with resveratrol and EX-527 showed that EX-527 may counteract the effect of resveratrol on cellular growth.

Resveratrol and EX-527 has no significant effect on protein synthesis in the cell

After cells were exposed for 24 hours with resveratrol and EX-527, resveratrol, EX-527 or without anything, cells were trypsinized for protein collection. In order to determine the protein concentration of the samples, the equation from the standard curve (Appendix 8) was used.

Table 5. The protein concentrations which were obtained from three separate experiment groups.

Experiment No	Protein Concentration (µg/µl)			
	Resveratrol+ EX-527	Resveratrol	EX-527	Control
1	1.426	1.580	1.770	1.675
2	0.803	1.209	0.889	1.494
3	0.693	0.558	0.726	0.834

HeLa cells were exposed to15 µM of resveratrol and 300 nM of EX-527, 15 µM of resveratrol, 300 nM of EX-527 or without anything (Control) for 24 hours and proteins of the cells were collected as described in *Material and Method part*.

All in all, we did not observe any correlation between protein synthesis and treatment in all experiments (See Table 5).

Sirt1 inhibition suppresses autophagy induction

Resveratrol is indicated as a suppressor of autophagy by inhibiting mammalian S6K (Armour *et al*., 2009), and is also suggested as an activator of autophagy by activating AMPK (Wu *et al*., 2011) and Sirt1 (Ghosh *et al*., 2010). Therefore, we examined if EX-527, which is the novel Sirt1 inhibitor, and resveratrol treatments have any effect on autophagy accumulation in HeLa cells. LC3-I is converted to LC3-II in the case of autophagy induction, and LC3-II binds to the membrane of autophagosome (Kabeya *et al*., 2000). Because autophagy can be measured by the amount of LC3-II (Mizushima and Yoshimori, 2007), we made western blot analyses to determine the amount of LC3A and LC3B, which are the human homologs of LC3-I and LC3-II, respectively.

Our results showed that inhibition of Sirt1 by EX-527 led to the decrease in LC3A/B accumulation in all three experiments, as it was expected. Beside this, in first and third experiment the least amount of LC3A and LC3B were monitored, indicating that Sirt1 is important for autophagy induction in HeLa cells (See Figure 9a and 9c). Interestingly, in first experiment, resveratrol and EX-527 treatment initiated autophagy more than control (DMSO-treated) cells (See Figure 9a). This was unexpected because resveratrol is known to mostly inhibit autophagy and Sirt1 inhibition by EX-527 should have blocked LC3A/B increase in the cell. Additionally, resveratrol treatment for 24 hours led to an increase in protein levels of LC3A/B in first experiment (See Figure 9a). Nevertheless, these results could not be reproduced in second and third experiments and protein levels of LC3A/B in resveratrol and EX-527 or resveratrol-treated cells were found lesser than DMSO-treated cells (See Figure 9b and 9c). Furthermore, in second and third experiments, resveratrol was observed to down regulate LC3A/B accumulation, thereby, autophagy (See Figure 9b and 9c). However, in second experiment, LC3A/B levels were found to be similar in resveratrol or EX-527 treated cells.
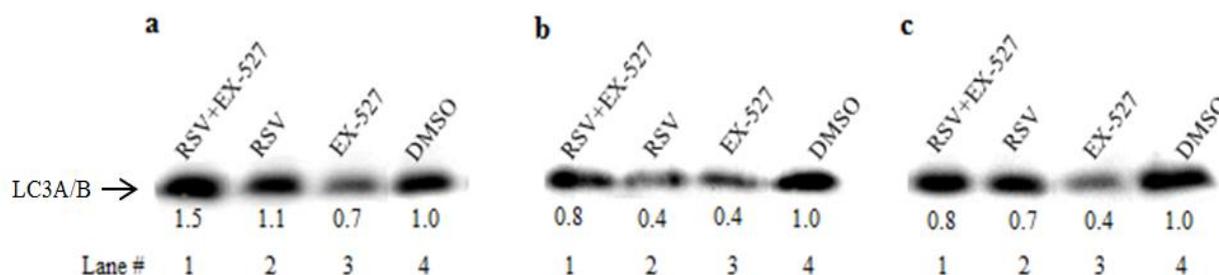
Figure 9. Western blot results of HeLa cells. (a) In first experiment, 500000 cells, (b) in second experiment, 250000 cells, and (c) in third experiment 1000000 cells were plated. Cells were treated with (Lane #1) 15 µM of resveratrol (RSV) and 300 nM of EX-527, (Lane #2) 15 µM of resveratrol (RSV), (Lane #3) 300 nM of EX-527 or (Lane #4) control (DMSO) for 24 hours. Values of intensity were calculated for each experiment individually by using VisionWorks®LS Image Acquisition and Analysis Software.

4. Errors and Error Corrections

It is important to count precise number of the cells. It would be the best if cell counting was done by only one person through the project in order to avoid error variation. Because of the personal variations, cells might be plated in different amounts.

Another important error in the project was the calculation errors of the number of the cells that were plated. In the first experiment 500000 cells, in the second experiment 250000 cells, in the third experiment 1000000 cells were plated. This also affected the results of each experiment. Cells in the second experiment were grown one extra day in order to possibly do comparison of the cell morphology. Unfortunately, one extra day resulted in the increase of the cell density in the petri dish (See Figure 7). As the consequence of this situation, sizes of cells in this experiment group were found smaller than the first experiment.

Cell scraping process is important, because one have to harvest all the cells in the petri dish. In order to obtain reliable data from the experiments, cells should be harvested properly. One of the reasons of the variations of the protein concentration between three experiment groups might be the failure in the cell harvesting process.

Pipetting technique is also important in these experiments. Calibrations of the pipettes should be checked before the experiments and errors in of the pipettes while measuring the volume should be less than 4.9%. In our experiments this might cause an error. In example, we might measure 18 µl instead of 20 µl.

A reason to why the amount of proteins was found fewer on the blotting membrane of second experiment might be due to the transferring process. In the western blotting process of the second experiment, we set the power supply to 100 volts as the limiting factor. This might not be enough to transfer all the proteins. In the transfer process of first and third experiments, we used 180 miliamperes as the limiting factor, so we could obtain better result. Failure of the protein transfer would lead to fewer protein of interest, thereby a decrease in the intensity of the visualized bands. Another possible reason for the failure in the transfer process might be the heating of the buffer. Buffer was tried to be held as cold as possible by placing an ice pack in the blotting apparatus.

5. Discussion

In this project, we examined the role of resveratrol in the presence and absence of the potent Sirt1 inhibitor, EX-527, with regards to autophagy. Resveratrol is suggested to regulate mTOR signaling through both Sirt1-dependent and Sirt1-independent pathways (Ghosh *et al*., 2010), thereby autophagy.

We started our experiments by treating the cells with resveratrol and EX-527, resveratrol, EX-527 or without anything. After 24 hours exposure, we obtained pictures of each experiment set in all three experiments. We investigated that resveratrol treatment resulted in bigger cell size than other experiment sets in all three experiment groups. It was found that resveratrol extended the lifespan in *C. elegans*, and *N. furzeri* (Bass *et al*., 2007; Valenzano *et al*., 2006). It is also suggested that resveratrol interrupts cell division in several human cell lines in a dose-dependent manner, which changes from a cell line to another (Ragione *et al*., 1998; Joe *et al*., 2002). These properties of resveratrol on cell cycle and lifespan might be a reason for bigger cell sizes of resveratrol-treated HeLa cells relative to the other experiment sets. On the other hand, Sirt1 overexpression in vascular smooth muscle cells was found to prolong the replicative lifespan if it is accompanied by NAD^+ salvage pathway amplification (Ho *et al*., 2009). Resveratrol is known as a specific activator of Sirt1 *in vivo* (Borra *et al*., 2005). An increase of the activation in Sirt1 by resveratrol in the cell may mimic the activation by NAD^+ salvage pathway amplification. On the other hand, inhibition of Sirt1 by EX-527, which is the novel Sirt1 inhibitor, resulted in the smallest sizes of cells in all three experiment groups. These findings are parallel with the role of Sirt1 in lifespan extension in the cell. Moreover, treatment with both resveratrol and EX-527 resulted in the smaller cell size than resveratrol-treated cells. Resveratrol and EX-527 treatment was expected to result in inhibition of

Sirt1 because of the presence of EX-527; however, although Sirt1 is inactive, resveratrol may interact with other cell cycle components and led to arrest cell division. As an addition to these, although the number of cells or the cell density in the petri dish was different, resveratrol treatment, which results in bigger cell size relative to the other experiment sets, were reproduced in all experiments. This result suggests that effect of resveratrol on cell development is not dependent to cell density in the environment. Moreover, Sirt1 is suggested to increase longevity via autophagy (Salminen and Kaarniranta, 2009). Enhancing the activity of Sirt1 by resveratrol and autophagy activity may lead to prolonged lifespan.

After 24 hours exposure, we collected the proteins of all three individual experiment groups and did the western blot analyses in order to examine the effect of treatment with resveratrol and EX-527, resveratrol, EX-527 or without anything. To investigate the level of autophagy accumulation in the cells, LC3A and LC3B proteins were blotted. LC3B is found as membrane bound and there is a correlation between the amount of LC3B and autophagosome formation (Kabeya et al., 2000). Sirt1 is suggested to regulate autophagy by deacetylating several Atg proteins, such as Atg5, Atg7 and Atg8, and Sirt1 knockout in MEFs were found to result in decreased level LC3 accumulation relative to wild-type MEFs (Lee et al., 2008). mTOR, which is the one of the important cellular nutrient sensors and autophagy regulators, is known to be down regulated by Sirt1 through tuberous sclerosis complex 2 (TSC2) (Ghosh et al., 2010), thereby it inhibits autophagy. Parallel with this role of Sirt1, its inhibition is expected to result in down regulation of autophagy. Consistent with this, our results showed that when Sirt1 was inhibited by EX-527, autophagy was suppressed in all experiments.

mTOR is known as a regulator of the autophagy machinery (Jung et al., 2010). It interacts with Raptor, GβL, DEPTOR and PRAS40 and generates the mTORC1, and when mTORC1 is active, autophagy is inhibited (Jung et al., 2010). Besides, Sirt1 activation by resveratrol was found to down regulate mTOR signaling in HeLa cells and MEFs (Ghosh et al., 2010). This interconnection between Sirt1 and mTOR signaling, which is shown in Figure 10, was demonstrated by our results. Furthermore, the autophagy machinery is stimulated by AMPK. Resveratrol was found to activate AMPK in Neuro2a cells (Dasgupta and Milbrandt, 2007) and HEK293 cells (Vingtdeux et al., 2010). Activation of AMPK by resveratrol treatment resulted in the inhibition of mTOR, which leads to autophagy accumulation in the cells (Vingtdeux et al., 2010). Consistent with that, resveratrol treatment resulted in LC3A/B accumulation more than control group in the first

experiment as it was expected. However, in the second experiment, less LC3A/B accumulation than control was found probably because of the protein concentration difference. Moreover, another possible reason for that may be the cell densities in the petri dishes. Although cell density has an inhibitory effect on cell division cycle, HeLa cells were found not to be inhibited by confluency and they went on growing exponentially (Castor, 1972). However, because of the density, cells were observed smaller in the second experiment than the first and the third experiments. Smaller size might result in less protein synthesis; therefore cells might need the autophagy accumulation for protein turnover. Possible reasons of these different results are given in *Errors and Error Corrections part.*

Interestingly, resveratrol and EX-527 treatment led to autophagy formation more than the resveratrol treatment. Theoretically, Sirt1 inhibition would lead to a decrease in autophagy formation. Besides, resveratrol is known to down regulate the autophagy process by inhibiting S6K via a Sirt1-independent pathway (Armour *et al*., 2009). Although AMPK activation by resveratrol would result in autophagy induction, this was expected to be balanced by Sirt1 and S6K inhibitions. One possible explanation for this might be that the difference between the amount of resveratrol concentration that interacts with Sirt1 in the presence of EX-527. Exposure with resveratrol and EX-527 began at the same time in the petri dishes. Some of the Sirt1 proteins might interact with resveratrol before binding with EX-527. It is suggested that interaction of Sirt1 with resveratrol results in a conformational change of Sirt1 (Borra *et al*., 2005). As a result of this conformational change inhibition of protein with EX-527 might be prevented. On the other hand, AMPK is suggested to increase transcriptional activity of peroxisome proliferator-activated receptor gamma (PPARδ; PPARβ/δ) (Narkar *et al*., 2008). It was also demonstrated that expression of PPARβ/δ led to an increase in the 5′-promoter activity of Sirt1 gene (Okazaki *et al*., 2010). Consistent with these findings, activation of AMPK by resveratrol leads to increase in Sirt1 gene expression, thereby increase in Sirt1 protein level. Activity of these newly-produced and active Sirt1 proteins may lead to increase in autophagy accumulation.

In case of deprivation, mTOR is inhibited and this inhibition triggers the autophagy process (Mizushima, 2007; Jung *et al*., 2010). Resveratrol treatment resulted in bigger cell size in all three experiments. In parallel with the increase of the cell size, nutrient level that the cell needs to continue the anabolic processes is expected to increase. In addition to this, the increase in AMP/ATP ratio results in increase in AMPK activation by binding with AMP (Yang and Guan,

2007), thereby mTOR activity is inhibited and autophagy is triggered (Tsang *et al*., 2007). This might be another explanation for the observation of the higher autophagy induction in resveratrol-treated cells than the other experiment groups.
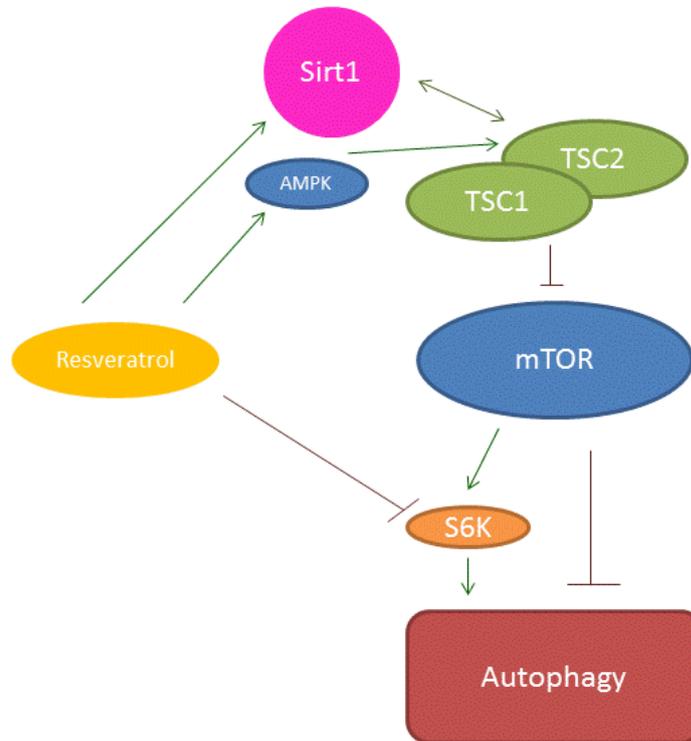
Figure 10. mTOR signaling network in relation to autophagy. Autophagy is down regulated by mTOR by itself. S6K, which activates autophagy, is the downstream target of mTOR. Sirt1 and AMPK are the upstream factors of mTOR and they down regulates mTOR activity through TSC1/TSC2 complexes. Resveratrol is the activator complex of Sirt1 and AMPK, whereas it inhibits S6K. Arrows represent activations and bars represent inhibitions.

Based on the findings, mTOR pathway should be regulated by several components (See Figure 10). As it was explained above, resveratrol treatment has different effects on autophagy, including activation of Sirt1 and AMPK, which leads to activation of autophagic flux, and inhibition of S6K, which results in inhibition of autophagy.

6. Conclusion

We concluded that Sirt1 inhibition with EX-527 suppresses autophagy induction. In addition to this, resveratrol treatment might slightly induce autophagy in HeLa cells, whereas resveratrol is also known to block the autophagy process via S6K. Furthermore, resveratrol and EX-527 treatment caused LC3A/B accumulation in the cell more than only resveratrol-treated cells, suggesting that autophagy might be induced via a Sirt1-independent pathway, probably through AMPK. Moreover, resveratrol has an inhibitory effect on cell growth either via activation of autophagy or by arresting the cell division cycle. Furthermore, the inhibition or activation of Sirt1 may cause an effect on the components that regulates cell cycle and cell growth.

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8. Appendix

Appendix 1

1. 18 ml of culture media (with 20% FBS) is added to a T75 culture flask. The flask is placed in the incubator.

2. Selected cryotube containing is taken from the cryo-container and kept on ice

3. The cryotube is defrosted by submerging it in 37 ^{o}C water. The defrosting should be complete within 1 minute. The cryotube is cleaned with 70% EtOH and the culture is added to preheated T75 flask with culture media.

4. The next day the culture is investigated under a microscope, and the culture media is changed with medium containing 10% FBS.

Appendix 2

1. Old culture media is sucked out with Pasteur pipette.

2. A suiting amount of PBS is applied and the cells are carefully washed (T25: 10 ml, T75: 20 ml, T150: 25 ml).

3. PBS is removed by suction.

4. A suiting amount of fresh trypsin-EDTA is applied and is dispersed over the cells (T25: 0.5 ml, T75: 1.5 ml, T150: 2.5 ml).

5. The culture flask/dish with trypsin-EDTA is put in the incubator for 3 minutes (after which the cells are inspected whether they are loosened by slapping the culture flask/dish. If the cells have not been successfully loosened, the flask/dish is incubated for additional 3 minutes. The cells must however not be exposed to trypsin for more than 8-10 minutes total).

6. Loosened cells are resuspended in culture media (T25: 4.5 ml, T75: 3.5 ml, T150: 2.5 ml – could add 5 ml culture media so total is 10 ml).

7. If the cells are to be used in further experiments the cells are transferred to a 15 ml falcon tube which can be placed on ice. (Count the cells – count in a hemocytometer 16 quadrat (Appendix 3)) add suiting volume to flask or well.

8. If further growth is wanted, the cells are split (often 1/5, 1/10 or 1/20) and culture media is added to flask (T25: 5-6 ml, T75: 17-20 ml, T150: 35-40 ml).

Appendix 3

1. A droplet (20 µl) of the cell solution is taken and added to the hemocytometer and a coverslip is put on top of it.

2. Sample is inspected under a microscope by using 10X objective. (Cell suspension is diluted in case of having a concentrated suspension.)

3. Number of the cells is counted.

4. One chamber of the hemocytometer is indicated and nine squares are counted (Each square is 1 mm^2)

5. Cell density in ml is calculated by multiplying cell count with 10000.

Appendix 4

For each petri dish

1. Cells are washed with 2 X 10 ml PBS.

2. 1 ml PBS is added, and cells are loosened with a rubber cell scraper and collected in an eppendorf tube.

3. 0.5 ml PBS is added to loosen remainder cells and cells are collected in the eppendorf tube.

4. Tubes are centrifuged at 15000 G for 15 seconds at 4 $^{\circ}$C.

5. Supernatant is removed.

6. Pellet is resuspended in 150 µl of protease inhibitor buffer.

7. Suspension is sonicated 3 X 15 seconds on ice.

8. Sonicated suspension is centrifuged at 4000 G for 30 minutes at 4 $^{\circ}$C.

9. The supernatant is divided into 3 tubes.

   1 for total protein determination – Store at -20 $^{\circ}$C

   2 for SDS-PAGE – Store at -80 $^{\circ}$C

Appendix 5

1. 100 µl of blind/ diluted standard/ diluted sample is added to a disposable cuvette.

2. 100 µl of reagent A (20 µl reagent S/ ml reagent A) is added to the disposable cuvette.

3. 800 µl of reagent B is added to the disposable cuvette.

4. Cuvette is mixed gently and kept dark for 15 minutes.

5. Absorbance values are measured at 750 nm.

Appendix 6

1. Membrane is established in ethanol for 10 minutes.

2. Membrane is established in blotting buffer for 20 minutes.

3. Pad and filter papers are established in blotting buffer for 20 minutes.

4. Blotting apparatus is filled with blotting buffer until it covers whole membrane.

5. Blotting is started with the current of 220 mA as the limit. After 1 hour, blotting process is ended.

6. Membrane is treated with TBS-T for 5 minutes and then with blocking buffer for 1 hour.

7. Membrane is washed with TBS-T for 3 X 5 minutes.

8. If it is necessary, membrane is divided.

9. Primary antibody (1:1000 (10 µl + 10 ml) diluted in 5% (w/v) BSA in 1X TBS-T) is applied to the membrane and membrane is overnighted.

10. Membrane is washed with TBS-T for 3 X 5 minutes.

11. Membrane is treated with secondary antibody (1:2000 diluted (20 µl + 40 ml) in 1X TBS-T for 1 hour.

12. Membrane is washed with TBS-T for 3 X 5 minutes.

13. Membrane is treated with a blot enhancer to elicit the bands.

14. Images of the membrane are obtained.

Appendix 7

5X TBS-T pH 7.6

6.05 g Tris (Trizma® Base) (Sigma-Aldrich)

20 g NaCl (Merck)

5 ml Tween 20 (Sigma-Aldrich)

Add 1L H_2O

Adjust pH with HCl (1 M)

Blotting Buffer

6.00 g Tris (Trizma® Base) (Sigma-Aldrich)

28.8 g Glycine (Sigma-Aldrich)

400 ml 96% Ethanol (Kemetyl)

2.0 g SDS

Add H_2O until 2L

Blocking Buffer

5.00 Skimmed Milk Powder (Merck)

100 ml 1X TBS-T

5% BSA TBS-T

2.5 g Bovine Serum Albumin (BSA) (Sigma-Aldrich)

50 ml 1X TBS-T

Appendix 8

Appendix Table 1. Turbidity measurements for standard curve.

BSA Concentration (µg/µl)	Measurement 1	Measurement 2	Measurement 3	Average	Average – Blind
0 (Blind)	0.025	0.024	0.019	0.023	0.00
0.5	0.838	0.806	0.799	0.814	0.792
0.375	0.639	0.591	0.587	0.606	0.583
0.25	0.333	0.308	0.316	0.319	0.296
0.125	0.169	0.182	0.183	0.178	0.155
0.0631	0.117	0.107	0.106	0.110	0.087

All measurements were done at 700nm by using. Average – Blind value was used to obtain the standard curve.
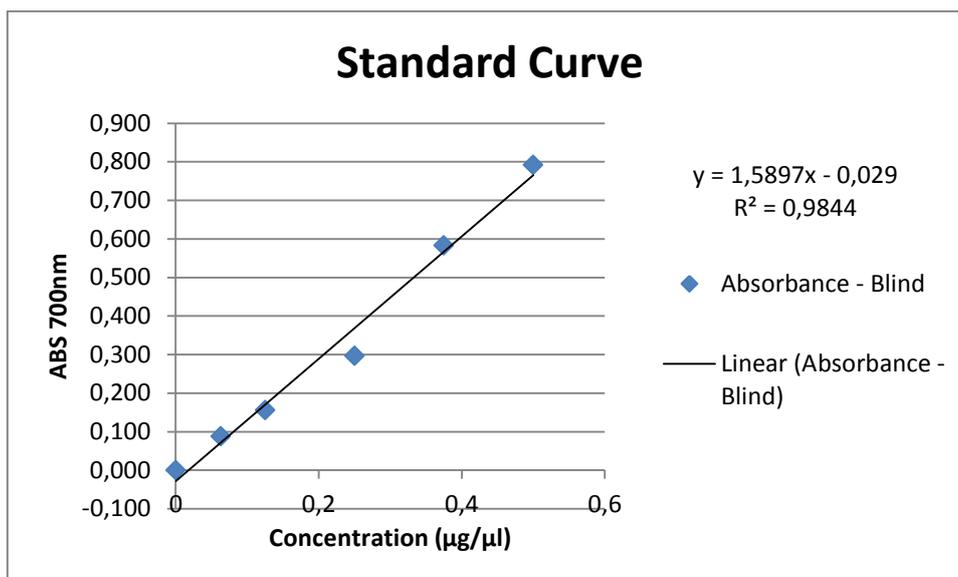
Appendix Figure 1. Standard curve for protein determination

Appendix Table 2. Raw data for protein concentrations of each experiment set.

	Absorbance at 700 nm			
Experiment No	**RSV+EX-527 (5X diluted)**	**RSV (5X diluted)**	**EX-527 (10X diluted)**	**Control (7X diluted)**
1	0.447	0.496	0.275	0.374
2	0.249	0.378	0.135	0.333
3	0.214	0.171	0.109	0.183

In order to calculate the protein concentration, average value of blind measurement from Appendix Table 1 was subtracted from the values in Appendix Table 2, and Appendix Table 3 was obtained. Values from Appendix Table 3 and equation from standard curve (Appendix Figure 1) was used to calculate protein concentrations of the samples. Protein concentration values that were obtained from previous step were multiplied with dilution factors in order to determine the protein concentration of each experiment set for all three experiment groups, and Appendix Table 4 was obtained.

Appendix Table 3. Absorbance values after subtraction of blind value.

	Absorbance at 700 nm			
Experiment No	RSV+EX-527 (5X diluted)	RSV (5X diluted)	EX-527 (10X diluted)	Control (7X diluted)
1	0.424	0.473	0.252	0.351
2	0.226	0.355	0.112	0.310
3	0.191	0.148	0.086	0.160

Appendix Table 4. Protein concentration of each experiment set

	Protein Concentration (μg/μl)			
Experiment No	RSV+EX-527	RSV	EX-527	Control
1	1.426	1.580	1.770	1.675
2	0.803	1.209	0.889	1.494
3	0.693	0.558	0.726	0.834