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THE ROLE OF MELATONIN IN SIRT1 AND p-AMPK REGULATION IN HT-29 CELL LINE

Role melatoninu v regulaci SIRT1 a p-AMPK v buněčné linii HT-29

Diploma thesis

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V Hradci Králové Anastasiya Shkut

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Abstrakt

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Název diplomové práce: Role melatoninu v regulaci SIRT1 a p-AMPK v buněčné linii HT-29.

Sirtuin 1 (SIRT1) je na NAD^+ závislá deacetyláza vyskytující se v mnoha organismech včetně savců. Bylo zjištěno, že SIRT1 prodlužuje životnost kvasinek v podmínkách s nedostatkem živin. SIRT1 deacetyluje mnohé regulační proteiny a tím kontroluje metabolický stav buňky stejně jako AMP-aktivovaná kináza (AMPK), která je také energeticky regulační enzym závislý na úrovni NAD^+ v buňce. Oba enzymy hrají roli v kancerogenezi a mohou ovlivňovat autofágii, i když přesný mechanismus zůstává nejasný. V naší práci jsme se zaměřili na hormon melatonin, který má protizánětlivé a protirakovinné vlastnosti, abychom studovali jeho vliv na lidskou rakovinnou linii buněk tlustého střeva HT-29. Zda melatonin ovlivňuje SIRT1 a AMPK a jaký je mezi nimi hierarchický stav. Také jsme pozorovali možný vliv melatoninu na autofágii. Použili jsme techniku Western blotting (WB) a z našich výsledků se zdá, že melatonin má výrazný efekt na SIRT1, což může aktivovat AMPK a také autofágii. Nicméně některé výsledky neobsahovaly dostatečný počet experimentů, abychom vyvodili jasné závěry. Je potřeba více studií užívající různé metody ke stanovení, zda melatonin má významný vliv na SIRT1 a AMPK, jestli je tento fakt užitečný v některém směru pro léčbu rakoviny tlustého střeva, a jakou roli by mohla hrát autofágie v tomto procesu.

Abstract

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Title of diploma thesis: The role of melatonin in SIRT1 and p-AMPK regulation in HT-29 cell line.

Sirituin 1 (SIRT1) is NAD^+ dependent deacetylase present in wide range of organisms including mammals. It was found to extend life span in yeasts during calorie restriction (CR) conditions. SIRT1 deacetylates many regulator proteins and thus control metabolic status of cell as well as AMP-activated kinase (AMPK), which is also energy regulator enzyme depending on NAD^+ levels in cell. Both of them play roles in cancer and could influence autophagy, although the exact mechanism remains unclear. We focused our study on hormone melatonin, which has anti-inflammatory and anti-cancer effects, to study its influence on human colon cancer cell line HT-29. If it has impact on SIRT1 and AMPK and what is hierarchic relationship between SIRT1 and AMPK. Also we observed its possible influence on autophagy. We used Western blotting (WB) technique and from our results it seems that melatonin has significant effect on SIRT1, which might activate AMPK as well as autophagy. Nevertheless some of results did not have sufficient number of experiments to make clear statement. More studies using different methods would be necessary to declare if melatonin has significant influence on SIRT1 and AMPK, if this fact is useful for colon cancer therapy in any way, and what role could play autophagy in this process.

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1. LIST OF ABBREVIATIONS

AB – antibody

AM – goat anti-mouse antibody

AMPK – adenine monophosphate-activated protein kinase

APS - ammonium persulfate

AR – goat anti-rabbit antibody

ATG – autophagy related gene

CC – compound C

CMA – chaperone mediated autophagy

CR – calorie restriction

diH_2O - distil H_2O

DMSO - dimethylsulphoxide

EDTA – ethylenediamine-tetraacetic acid

EGTA – ethylene glycol-bis(β-aminoethylether)-N,N,N,´N´-tetraacetic acid

eNOS – endothelial nitric oxide synthase

FOXO 3 - forkhead box class O 3

HRP - horseradish peroxidase

IC50 – inhibition concentration for 50% of cells

LKB1 – liver kinase B1

LC3B – protein 1 light chain 3B

LC3B I – inactivated form of LC3B in cytoplasm

LC3B II – activated, conjugated form of LC3B bound to membrane of autophagosome

Mel – melatonin

mTOR – mammalian target of rapamycin

mTORC1 – mammalian target of rapamycin complex 1

NAD^+ – nicotine adenine dinucleotide

Nf-κB – nuclear factor κB

PAGE – polyacrylamide gel electrophoresis

p-AMPK – phospho-AMPK

PBS 1X – phosphate buffer saline 1X

PBST - phosphate buffered saline with Tween 20 (0.05%)

PC – prostate cancer

PGC-1α - PPAR-γ coatcivator-1α

PMSF – phenylmethylsulfonyl fluoride

PPAR-γ - peroxisome proliferator-activated receptor

Ref. – reference

ROS – reactive oxygen species

SDS – sodium dodecyl sulfate

SIR2 – silent information regulator 2

SIRT1 – sirtuin 1

SRB – sulforhodamine B

SUR – suramine

TBS 1X – tris buffered saline pH 7.4

TEMED – N,N,N´,N´-tetramethylethylenediamine

Triton X-100 - t-octylphenoxypolyethoxyethanol

Trizma base – 2-amino-2-(hydroxylmethyl)-1,3-propanediol

WB – Western blotting

2. INTRODUCTION

Crohn´s disease and ulcerative colitis belong to intestinal bowel diseases (IBD) characteristic by chronic uncontrolled inflammation in intestine. Studies suggest that patients with IBD are at higher risk of colorectal cancer development. *(Chiba et al. 2012, Okayasu 2012, Canavan et al. 2006)* Colorectal cancer is third most common cancer worldwide with 1000 000 new cases every year and 500 000 deaths per year. Only 60% of diagnosed cases can be controlled by standard therapy. *(León et al. 2012)*

Hormone melatonin is produced in pineal gland, retina, intestinal tract and skin. It is released from pineal gland dependently on light-dark changes, where dark stimulates the production and light inhibits it. It is believed to be main regulator in circadian clock machinery. Compared to the pineal gland, gastrointestinal tract produces hundred times more melatonin, which is released into blood according to food intake as post-prandial response. *(Hardeland and Pandi-Perumal 2005)*

Melatonin has positive impact on esophageal and gastric ulcers, pancreatitis, colitis, IBD, and some types of cancer. *(Motilva et al. 2011)* There is a link between light disturbances and higher evidence of some cancers. Night shift workers have more often breast, prostate, colorectal and endometrial cancers in epidemiological studies. *(Jung-Hynes and Ahmad 2009)* It has been shown, that melatonin level is increased in patients suffering from colorectal cancer. *(Motilva et al. 2011, Schernhammer et al. 2003)*

Melatonin has anti-inflammatory and anti-cancer effect and might influent process of inflammation and cancer via energy regulator enzymes SIRT1 and AMPK with possible influence on autophagy. Currently there are no available studies analyzing effect of melatonin on human colon cancer cells HT-29 in connection with SIRT1 and AMPK and their effect on autophagy. Increasing results of link between SIRT1 and AMPK are evident *(Zhang et al. 2010, Hu et al. 2011)* as well as connection of these two enzymes to autophagy. *(Chen and Debnath 2010)* However final conclusions differ within different scientific groups, types of tissues, animal models or even within same types of cancer. This all suggest a need for further research with careful definition of conditions to establish possible connections among melatonin, sirtuins and autophagy, and the relationship of these processes in inflammatory intestinal diseases and colorectal cancer.

3. THEORETICAL PART

3.1. SIRT1

Sirtuin 1 (SIRT1) is member of class III nicotine adenine dinucleotide (NAD^+)-dependent protein deacetylases occurring in lower and upper organisms and having beneficial health effects. It is a part of mammalian sirtuin family, which contains 7 memebers (SIRT1-SIRT7). The most studied sirtuin-SIRT1 is human homologue to yeast´s silent information regulator 2 (SIR2), which was first discovered to extend lifespan in yeasts during calorie restriction (CR). *(Tang 2011)* The evidence for SIRT1 being a longevity factor in mammals is controversial. It influences many regulator molecules and pathways and its role is more complicated in upper organisms than in lower organisms. *(Chung et al. 2010)*

SIRT1 is coded by genes that control other genes. The regulation mechanism of SIRT1 on all processes is due to its ability to deacetylate histone and non-histone proteins in cell nucleus and cytoplasm, e.g. nuclear factor (Nf)-κB, forkhead box class O (FOXO) 3, p53, peroxisome proliferator-activated receptor (PPAR)-γ, PPAR-γ coatcivator-1α (PGC-1α), endothelial nitric oxide synthase (eNOS) and others. (Fig. 1) The deacetylation reaction catalyzed by all sirtuins is dependent on availability of cellular NAD^+ and thus is effectively influenced by cell metabolic status. *(Morris 2013, Tang 2011, Gallí et al. 2011, Chung et al. 2010)*

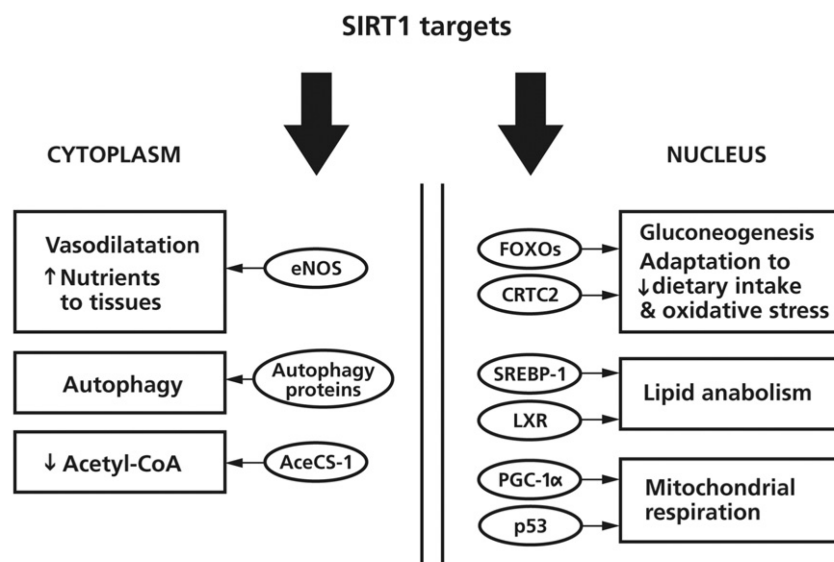


Fig. 1 <u>Targets of SIRT1 in cell cytoplasm and nucleus</u> SIRT1 deacetylates many regulator molecules in cell cytoplasm and nucleus and thus impact many key processes.

Taken from *Morris 2013*

SIRT1 regulates cell metabolism, stress resistance, apoptosis/proliferation, senesce/aging, inflammation-immune function, endothelial functions, and circadian rhythms. Thanks to its impact on the physiological and pathological processes SIRT1 plays role in many age-related diseases e.g. inflammation, arthritis, type II diabetes, obesity, cardiovascular diseases, osteoporosis, neurodegenerative diseases, aging, frailty and cancer. (Fig. 2) *(Morris 2013, Chung et al. 2010)*

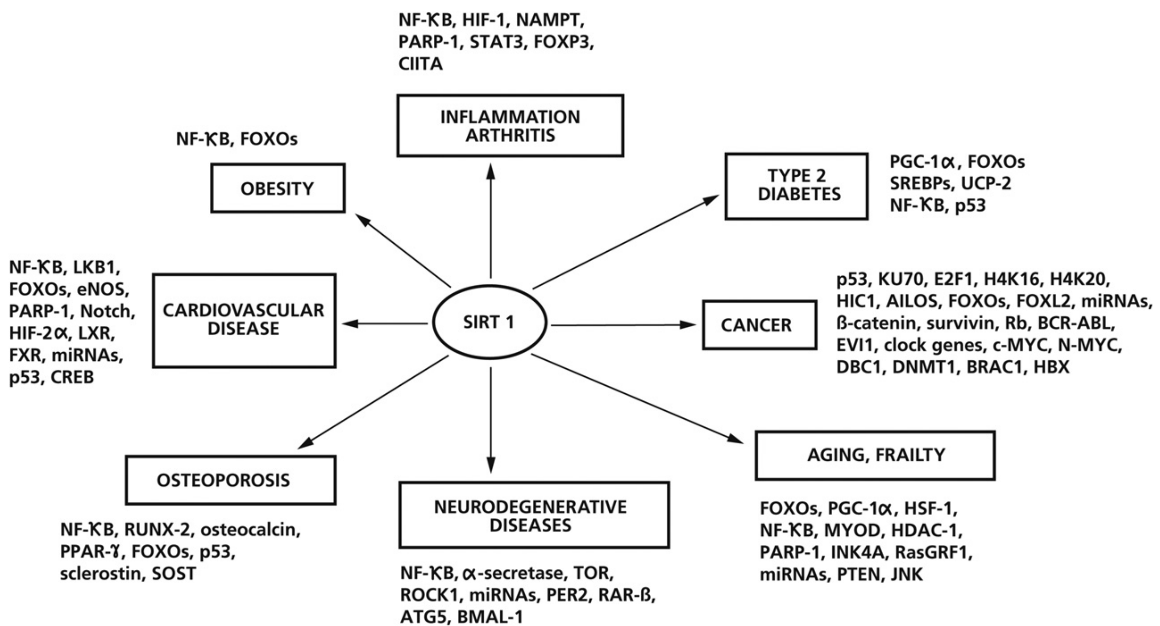


Fig. 2 Influence of SIRT1 on many regulator molecules SIRT1 has impact on age-related diseases such as inflammation arthritis, obesity, cardiovascular disease, osteoporosis, neurodegenerative diseases, aging, frailty and cancer through regulation proteins mentioned in figure.

Taken from *Morris 2013*

It has been known since 1930s that reduced calorie intake can extend life span in lower organisms (yeast, worms, flies, mammals). Recent studies also indicate that CR can decrease age-related diseases in mammals and improve biomarkers in humans. When SIRT1 was administrated to mice on a high-fat diet, CR-pathways were induced and their life span was extended. Meanwhile mice on normal diet did not show any evidences of longevity with SIRT1 over-expression nor with administration of SIRT1 activators. *(Hu et al. 2011, Kelly 2010)*

The role of SIRT1 seems to differ in different types of cancer. It has been shown, that SIRT1 promotes cell survivor by inhibiting apoptosis or cellular senesce in prostate

cancer (PC). Over-expression of SIRT1 was measured in PC and when SIRT1 was inhibited, cancer cell growth was inhibited as well. *(Jung-Hynes and Ahmad 2009, Jung-Hynes et al. 2011)* SIRT1 is considered as facilitator of tumorigenesis also in colon cancer, acute myeloid leukemia, and some skin cancers. *(Yamacuchi 2012)* In other types of cancer SIRT1 acts as tumor suppressor, e.g. ovarian cancer, glioblastoma, and bladder carcinoma. Conversely there are studies indicating role of SIRT1 in colon cancer also as suppressor. *(Motilva et al. 2011, Jung-Hynes et al. 2011)*

Suramin is polyanionic naphthylurea originally used for the treatment of trypanosomiasis and onchocerciasis. Foremost it is very potent SIRT1 inhibitor having also anti-proliferative and ant-viral properties. *(Villalba and Alcaín 2013)*

3.2. AMPK and p-AMPK

AMP-activated protein kinase is heterotrimetric Serine/Threonine (Ser/Thr) kinase composed of α, β and γ subunits with different isoforms (α1 and α2; β1 and β2; γ1, γ2 and γ3). AMPK belongs to family of highly conserved kinases that are regulated by nutritional and metabolic stresses. AMPK influents cellular energy and acts to maintain AMP/ATP and ADP/ATP ratios. It represses pathways that consume energy, and promote ATP-producing catabolic pathways by phosphorylation of downstream targets. Phospho-AMPK (p-AMPK) is active form of AMPK and is required for full enzymatic activity. *(Lin et al. 2010, Suchankova et al. 2009, Cantó and Auwerx 2010, Hardie 2011)*

AMPK activation stimulates metabolic responses in order to prevent metabolic and energetic crisis in situations with low ATP production (hypoxia, ischemia, low nutrient availability, glucose deprivation) or where ATP consumption is accelerated (muscle contraction). *(Cantó and Auwerx 2010, Hardie 2011)* Some of drugs and nutraceuticals activate AMPK as well, e.g. metformin, thiazolidinediones, resveratrol, or epigallocatechin gallate. AMPK is also modulated by numerous hormones and cytokines that regulate energy balance in the whole body, including leptin, adiponectin, ghrelin, cannabinoids, and even thyroid hormones, although detailed molecular mechanism remains unclear. *(Hardie 2011)*

Resveratrol is naturally occurring polyphenol in red wine and grapefruit juice having antioxidant and anti-inflammatory effects via modulating different signaling pathways. Resveratrol is also known for its activation of SIRT1 according to some studies directly *(Kelly 2010)* and/or indirectly. *(Chung et al. 2010)* Dependence of SIRT1 on NAD^+ as on cofactor links its activity to energetic state of cell and AMPK. *(Zhang et al. 2010)*

It has been noticed that resveratrol mimics calorie restriction (CR)-induced metabolic changes in response to AMPK activation. Resveratrol significantly increases the protein expression of SIRT1. Both AMPK and SIRT1 signaling pathways are energy sensing. According to this model, CR or resveratrol treatment increases NAD^+, which activates SIRT1 function that leads to activation of AMPK. *(Lin et al. 2010, Suchankova et al. 2009)*

Conversely *Baur (2010)* and *Hu et al. (2011)* point out in their reviews that resveratrol can increase AMPK phosphorylation directly, not through SIRT1. AMPK knock-out mice lost beneficial metabolic effects after administration of resveratrol, suggesting that resveratrol acts through AMPK activation. On the other side there are also results where hepatocytes and muscle cells were treated with SIRT1 inhibitors (nicotinamide and splitomicin) with further abrogation of resveratrol induced AMPK phosphorylation and activation.

Compound C (CC) is pharmacological inhibitor of AMPK that blocks actions of AMPK e.g. pro-apoptotic and anti-apoptotic actions as well as AMPK-induced autophagy. *(Vucicevic et al. 2011)*

3.3. Autophagy and LC3B II

Autophagy refers to process in cell, where cytoplasmic components are delivered to lysosome for bulk degradation. Three types of autophagy have been identified – macroauphagy, microautophagy and chaperone-mediated autophagy (CMA). Difference among the processes is in type of delivery to lysosomes. This study is focused on macroautophagy (herein referred to as autophagy). *(Tsuchihara et al. 2009, Morselli et al. 2009)*

Macroautophagy is characterized by formation of specialized vesicles called autophagosomes with double membrane structure (phagophore) to capture degradation products selectively and non-selectively. Autophagosomes fuse with lysosomes to form autolysosomes. (Fig. 3) Lysosomes contain acidic hydrolases, which degrade the content in autolysosomes. (*Swampillai et al. 2012, Choi et al. 2013)*

Autophagy has several phases: initiation, vesicle elongation, maturation, docking and fusion, and final vesicle breakdown and degradation of content. (Fig. 3)

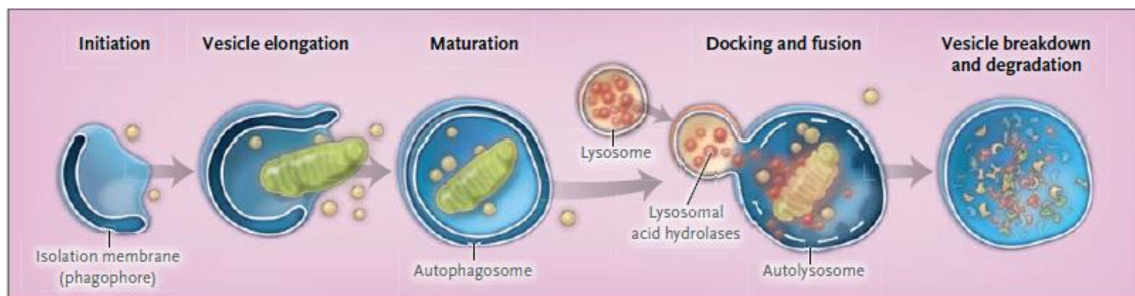


Fig. 3 <u>The process of autophagy</u> First phase of autophagy is initiation with forming isolation membrane (phagophore), then vesicle elongation, cargo sequestration and maturation, and last phase autophagosome-lysosome fusion. In the last phase content is degraded by lysosomal acid hydrolases and then released for metabolic degradation.

Taken from *Choi et al. (2013)*

Defective autophagy has been involved in the pathogenesis of diverse diseases, such as myopathy, neuronal degeneration, microbial infection, inflammatory bowel disease, aging and cancer. *(Chen & Karantza-Wadsworth 2009)*

Autophagy is present at basal level in all cells to maintain cell homeostasis. It plays important role in stress conditions e.g. deprivation or hypoxia and acts as a survival mechanism. It protects cells providing an alternative energy source, new materials for cell, and eliminating dysfunctional, excessive or aged proteins and organelles (e.g. mitochondria, endoplasmatic reticulum). (*Choi et al. 2013, Swampillai et al. 2012, Tsuchihara et al. 2009, Hoare et al. 2011)*

Autophagy plays a dual role in tumorigenesis: the promotion of cell death as tumor suppressor and the prevention of cell death as an oncogenic mechanism. *(Motilva et al. 2011)*

Hoare et al. (2011) and *Tsuchihara et al. (2009)* point out in their reviews cytoprotective role of autophagy in cells. They assume that loss of autophagy induces DNA damage and chromosomal instability, followed by increased tumor susceptibility. (Fig. 4A) The exact mechanisms for how autophagy maintains genome stability remain unclear. One possibility is that cells lacking autophagy are unable to reduce damaged mitochondria and peroxisomes, so they are potential sources of reactive oxygen species (ROS) that induce genotoxic stress.

When autophagy-deficient cells with inhibited apoptosis were exposed to starvation, necrotic cell death was observed in these cells. Furthermore as shows *Hoare et al. (2011)*, significant inflammation was induced in the tumor tissues in which autophagy-deficient cells with lack of apoptosis were transplanted. Necrosis is often associated with macrophage infiltration in vivo, and tumor-associated macrophages enhance tumor progression. It is an interesting hypothesis that the inflammation induced by a lack of autophagy may be correlated with tumor progression in vivo. (Fig. 4B)

Current evidence supports that autophagy suppresses tumorigenesis, particularly during the early stages of tumor initiation. However, in established tumors, autophagy can work as a survival pathway in response to stresses and during chemotherapy. (Fig. 4C) Moreover in some cases it may even lead to metastasis. *Chen and Debnath (2010)*

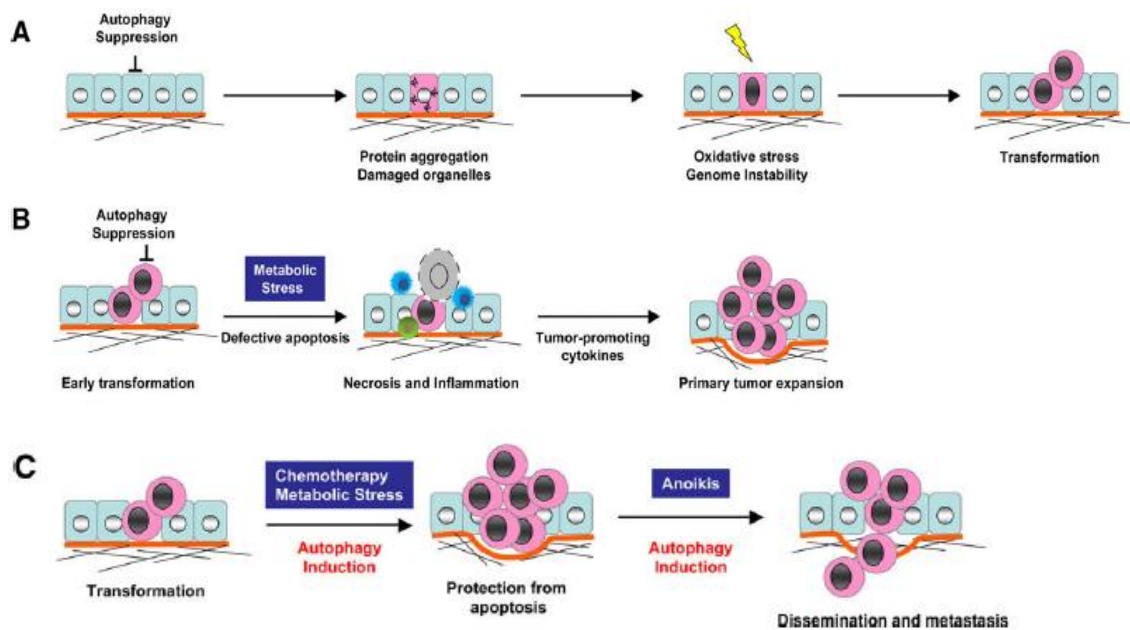


Fig. 4 **<u>Different states of cells in connection to autophagy</u>** A. Suppression of autophagy induces accumulation of damaged organelles such as mitochondria and aging proteins, it probably leads to

oxidative stress and genome instability and may cause malignant transformation. B. In cells with defective autophagy and with lack of apoptosis there was observed necrosis leading to inflammation under metabolic stress. C. Cancer cells with autophagy ability probably use their autophagy as survival mechanism under metabolic stress or during chemotherapy, which may lead even to metastasis

Taken from *Chen and Debnath (2010)*

The most extensively studied protein executing autophagy is microtubule-associated protein 1 light chain 3 (LC3). The LC3 has three mammalian isoforms (A, B and C), but only B isoform correlates with increased levels of autophagic vesicles and therefore is recommended to be measured. *(Barth et al. 2010)* The LC3 precursor (pro-LC3) is coded by Atg4 protease, resulting in the formation of LC3B I, which is in inactivated form present in cytoplasm. On initiation of autophagy cytoplasmic LC3B I is activated and conjugated to an amino group of phosphatidylethanol-amine generating LC3B II, which is bound to inner membrane of autophagosome. The existence of autophagosome-incorporated LC3B-II can be detected by Western blotting. However the removal of LC3B-II from membrane is required for fusion of autophagosome and lysosome in last phase of autophagy. (*Swampillai et al. 2012, Tsuchihara et al. 2009)*

AMPK can activate autophagy by inactivation of mTORC1, which is complex inhibiting autophagy in conditions of nutritional and energetic sufficiency. During metabolic stress, the reduction in ATP levels results in an elevated AMP/ATP ratio and activates the energy-sensing liver kinase B1 (LKB1), which phosphorylates and activates AMPK. p-AMPK inactivates mTORC1 and that induces autophagy. *(Chen and Debnath 2010, Levy and Thorburn 2011)*

There are few evidences that SIRT1 can activate initiation of autophagy as well. *(Choi et al. 2013)* Recent study of SIRT1 in Alzheimer´s and prion disease by *Jeong et al. (2013)* demonstrated that SIRT1 protects neurons in neurodegenerative disorders by activating autophagy, and that regulates mitochondrial homeostasis.

The study of autophagy is a rapidly changing field with potential for improving the treatment and cure of malignant diseases. There are still many factors that need to be investigated, most importantly, whether autophagy should be inhibited or stimulated to improve clinical outcomes in patients or whether some people should have autophagy induced while others should have it inhibited. Autophagy is a complex-multistep process that can be influenced by a number of cellular pathways as well as altered by

the tumor microenvironment and there may also be differences by blocking autophagy at the start of the process compared to halfway or at the end of it. It is clear from current research that it may also not be possible to have a unified treatment strategy for autophagy as there is conflicting evidence not only among tumor types, but also within the same tumor type when evaluating different treatment modalities. New and reliable methods for monitoring and measuring autophagy in patients will also be important in understanding the results of these clinical trials, as the changes in autophagy seen in vitro and in xenograft models may not directly translate into real clinical situations. *(Levy & Thorburn 2011)*

3.4. The role of melatonin

Melatonin is N-acetyl-5-methoxy-triptamine and was first isolated in 1958 from bovine pineal gland by Lerner et al. It is synthesized from tryptophan and its presence was observed in all major organisms from bacteria to unicellular eukaryotes, macroalgae, plants, fungi, invertebrate animals, and vertebrates. In mammals melatonin functions as signal for darkness and its blood levels are much higher during night (> 200 pg/mL) than during day (10 pg/mL). Additionally melatonin also shows antioxidant, oncostatic, anti-aging, and immonumodulatory effects. *(Carillo-Vico et al. 2005, Hardeland and Pandi-Perumal 2005)*

Melatonin has multiple functions: biological rhythm synchronization, sleep induction, vasoregulation or immunomodulation. Most of these actions are mediated via two G-protein membrane receptors MT1 and MT2, and melatonin nuclear receptor RORα. Other physiological functions such as antioxidant effect and free radical scavenger properties are not mediated by receptors. *(León et al. 2012)*

Melatonin has amphiphilic molecule that can enter any body fluid, cell or compartment. Its antioxidant properties are based on several different effects – direct radical scavenging, up-regulation of antioxidant enzymes (glutathione peroxidase, glutathione reductase), down-regulation of pro-oxidant enzymes (5- and 12-lipoxygenases, NO synthases), and improvement of mitochondrial metabolism. *(Hardeland and Pandi-Perumal 2005)*

It is also present in gastrointestinal tract from stomach to colon in 10 - 100 times higher concentration than in plasma. Melatonin is probably synthesized in enterochromaffin cells of mucosal lining to protect mucosa from erosion and ulcers and possibly to influence movement of gastrointestinal contents through digestive system. *(Reiter et al. 2003)*

Anisimov et al. (1997) showed in his research that melatonin after administration to rats with colon carcinogenesis (induced by DMH) in 21 mg/kg dose per day after 6 months decreased number of tumors in proximal parts of colon.

González-Puga et al. (2005) reported that melatonin significantly inhibited HT-29 cell proliferation and increased cancer cell death acting synergically with cholecystokinin-A antagonists (mainly devazepide). *García-Navarro et al. (2007)* suggested in their study that appeared anti-proliferative effect of melatonin on HT-29 cells was caused by nuclear receptors.

Melatonin could be activator of SIRT1. In senescence-accelerated mice SIRT1 was significantly lower than in control group. After adding 10 mg/kg of melatonin to drinking water during 9 months, SIRT1 increased significantly. *(Kelly 2010)* Additionally sirtuins play role also in circadian rhythm by deacetylating proteins crucial in clock machinery. Melatonin also increased autophagy in few experimental systems such as senescence-accelerated prone mice 8 or ischemia-injured neurons. *(Motilva 2011)*

However, little is known regarding the involvement of SIRT1 pathway on the effect of melatonin in HT-29 cells and its possible connection to autophagy.

4. AIMS OF RESEARCH

1) Determine inhibitory concentration 50 (IC50) of melatonin on HT-29 cell line to define appropriate doses of melatonin without toxic effect on cells using sulforhodamine B colorimetric assay (SRB).

2) Study the influence of different concentrations of melatonin on SIRT1 and AMPK including their hierarchical relationship using suramin – inhibitor of SIRT1 and compound C (CC) – inhibitor of AMPK. Application of Western blotting (WB) technique.

3) Study the influence of melatonin on autophagy measuring LC3B II through Western blotting technique.

5. EXPERIMENTAL PART

5.1. Chemicals, materials and their origin

5.1.1. Cell cultivation, freezing and unfreezing of cells

Human Caucasian colon adenocarcinoma (HT-29) cell line (Ref.: 91072201) from ATCC (USA), medium Mc Coy´s with glutamine (Ref.: E15-022), fetal bovine serum (Ref.: A15 - 101), penicillin + streptomycin (Ref.: P11-010) all from PAA (USA), sterile trypsin 1X (diluted from trypsin 10X-EDTA (Ref.: L-11-003) from Sigma-Aldrich (USA)), sterile ice-cold phosphate buffer saline (PBS) 1X (diluted from PBS 10X: 10.7 g $NaH_2PO_4 \cdot 2H_2O$ (Ref.: 121677) + 88 g NaCl (Ref.: 131659) from Pancreac Quimica S.A.U (Spain) + 3.9 g Na_2HPO_4 (Ref.: S-5136) from Sigma-Aldrich (USA) + distil H_2O (diH_2O) added to 1 L), dimethylsuphoxide (DMSO) (Ref.: 14195) from Pancreac Quimica S.A.U. (Spain), Trypan Blue solution 0.4% (Ref.: T-8154) from Sigma-Aldrich (USA), serological pipettes of 10 and 5 mL from Fisherbrand (USA), beaker with lye from Gelesan S.L. (Spain), flask 25 cm^2 or 75 cm^2 from Nunclon, Sigma-Aldrich (USA), 50 mL tube from Fisherbrand (USA), hemocytometer (Neubauer chamber) from Fisherbrand (USA), thermostatic bath from Orved (Italy), vertical laminar airflow cabinet BIO 48 from Faster (Italy).

5.1.2. Sulforhodamine B colorimetric assay for cytotoxicity screening

Materials mentioned above for cell cultivation, sulforhodamine B (SRB) sodium salt (Ref.: S9012-25G), trichloroacetic acid (Ref.: T9159-500G) from Sigma-Aldrich (USA), Tris buffer 10 mM (prepared from Trizma base from Sigma-Aldrich (Ref.: T6066) + diH_2O), easy flask with filter tampon from Nunc, Sigma-Aldrich (USA), micropipettes multichannel (100µl) from Eppendorf (Germany), sterile tips and sterile 1.5 mL tubes from Eppendorf (Germany), micropipettes (2 - 20 µl and 50 - 200 µl) from Pipetman (USA), sterile culture plates of 96 wells with cover from Nunc Sigma-Aldrich (USA), 96-well plates for dilution from Sigma-Aldrich (USA).

5.1.3. Sample preparation for Bradford protein assay and Western blotting

All materials mentioned above for cell cultivation. Moreover melatonin (Ref.: M5250-1G) from Sigma-Aldrich (USA), DMSO (Ref.: 14195) from Panreac Quimica S.A.U. (Spain), suramin (Ref.: BML – KI285) from Enzo Life Science (USA),

compound C (Ref.: Cat 171260) from Calbiochem, Merck Millipore (Germany), vortex from Labnet International (Spain), ultrasonic bath from Fungilab (UK), centrifuge (Ref.: 5415R) from Eppendorf (Germany).

Buffer of lyses: 8 mM $MgCl_2 \cdot 6\ H_2O$ from Panreac Quimica S.A.U. (Ref.: 141396), 50 mM pH 7.5 Tris HCl (Ref.: T5941-1kg), 5 mM ethylene glycol-bis(β-aminoethylether)-N,N,N,´N´-tetraacetic acid (EGTA) (Ref.: E-8145), 0.5 mM ethylenediamine-tetraacetic acid (EDTA) (Ref.: ED2SS), 0.01 mg/mL leupeptin (Ref.: L0649), 0.01 mg/mL pepstatin in DMSO (Ref.: P4265), 0.01 mg/mL aprotinin (Ref.: A1153), 1 mM phenylmethylsulfonyl fluoride (PMSF) in MetOH (Ref.: P7626), all from Sigma-Aldrich (USA), 250 mM NaCl (Ref.: 131659.1214) from Panreac Quimica S.A.U. (Spain), diH_2O.

1% Triton X-100 (Ref.: 121K009G), 1% inhibitors of phosphorylases cocktail 2 (Ref.: P0044-1mL) and 1% cocktail 3 (Ref.: P5726-1mL) from Sigma-Aldrich (USA), 500 µl and 1.5 mL tubes from Eppendorf (Germany), pipettes and tips from Pipetman (USA), scramble stick, 6-well plates (Ref.: 130184) from Thermo Scientific (USA), ice.

5.1.4. Bradfrod protein assay

Lyophilized bovine plasma gamma-globulin (Ref.: 500-000) and dye reagent concentrate – phosfic acid + methanol (Ref.: 500-006) from Bio-Rad Laboratories S.A. (Germany), diH_2O, 500 µl tubes from Eppendorf (Germany), pipettes and tips from Pipetman (USA), 96-well plate from Sigma-Aldrich (USA), microplate reader Asys HiTech UVM 340 from Biochrom (UK), MikroWin Program Version 4.36.

5.1.5. Western blotting

β-mercaptoethanol (Ref.: M7154) from Sigma-Aldrich (USA).

Tris 1M pH 8.8: 12.1 g trizma base (Ref.: T6066) from Sigma-Aldrich (USA), 100 mL diH_2O adjusting pH with NaOH on 8.8

Tris 1M pH 6.8: 12.1 g trizma base (Ref.: T6066) from Sigma-Aldrich (USA), 100 mL diH_2O adjusting pH with HCl on 6.8

Gel electrophoresis

Laemmli 1X: 1 mL glycerol (Ref.: 141339) from Panreac Quimica S.A.U., 2 mL sodium dodecyl sulfate 10% (SDS) (Ref.: L3771), 2 mg bromophenol blue (Ref.: B8026), 1.25 mL Tris 1M pH 6.8 from Sigma-Aldrich (USA), add diH_2O to 10 mL.

Precision plus protein standards (Ref.: 161-0373), **mini PROTEAN Tetra Cell system (Ref.: 165-8003)** (Fig. 5) includes short plates, plates of 1.5 mm, 15-well combs, casting stand with 2 casting frames, power supply with constant voltage, all from Bio-Rad Laboratories (Spain).

Fig. 5 **Mini PROTEAN Tetra Cell system** It contained short plates, plates of 1.5 mm, 15-well combs, casting stand with 2 casting frames, power supply with constant voltage.

Taken from *Bulletin 2895 Rev B. Bio-Rad Laboratories*

SDS-PAGE 10% running gel: 3.71 mL diH_2O, 3.75 mL Tris 1M pH 8.8, 3.3 mL acrylamide (Ref.: A3699), 50 µl SDS 20% (Ref.: L3771-100g), 100 µl ammonium persulfate 10% (APS) (Ref.: A3678-25g), 6.6 µl tetramethylethylenediamine (TEMED) (Ref.: T9281), all from Sigma-Aldrich (USA).
SDS-PAGE 15% running gel: 1.1 mL diH_2O, 3.75 mL Tris 1M pH 8.8, 5 mL acrylamide (Ref.: A3699), 50 µl SDS 20% (Ref.: L3771-100g), 100 µl APS 10% (Ref.: A3678-25g), 6.6 µl TEMED (Ref.: T9281) all from Sigma-Aldrich (USA).
SDS-PAGE 10% stacking gel: 2.3 mL diH_2O, 420 µl Tris 1M pH 6.8, 560 µl acrylamide (Ref.: A3699), 16.6 µl SDS 20% (Ref.: L3771-100g), 33.3 µl APS 10% (Ref.: A3678-25g), 3.3 µl TEMED from Sigma-Aldrich (USA). All calculations were made for 1 gel of 15 wells with thickness of 1.5 mm.

Electrophoresis buffer 10X: 30.2 g trizma base (Ref.: T6066), 188 g glycine (Ref.: G8898-1kg), 50 mL SDS 20% (Ref.: L3771-100g), all from Sigma-Aldrich (USA), add diH_2O to 1 L.

Tank blotting

Transfer buffer: 100 mL prepared electrophoresis buffer 10X, 250mL ethanol 96% (Ref.: 1.00983.1000) from Merck KGaA (Germany), add diH_2O to 1 L.
Precut nitrocellulose membrane 0.2 µm pore size 7 x 8.5 cm (Ref.: 162-0112) from Bio-Rad Laboratories (Germany), precut filter paper 7.5 x 10 cm (Ref.: 10427818) from Whatman (Germany), spatula from Bio-Rad Laboratories (Germany).

Blotting system Mini Trans-Blot cell from Bio-Rad Laboratories (Spain). (Fig. 6)

Fig. 6 **Blotting system Mini Trans-Blot cell** It contained buffer tank and lid, electrode assembly, cooling unit, gel holder cassette and foam pads.

Taken from *Bulletin 2895 Rev B. Bio-Rad Laboratories*

Ponceau S solution: 0.2 g Ponceau S (Ref.: P3504), 30 mL 10% trichloroacetic acid (Ref.: T8657) from Sigma-Aldrich (USA), add to 100 mL distil H_2O.

Immunodetection

Blocking and incubation solution: For non-phosphorylated proteins: 5 g non-fat milk powder Sveltesse from Nestlé (Switzerland), 100 mL prepared solution B.
For phosphorylated protein: 5 g phospho blocker^{TM} blocking reagent (Ref.: AKR-103) from Cell Biolabs (USA), 100 mL prepared **PBST:** 50 µl Tween 20 (Ref.: P1379) from Sigma-Aldrich (USA) + 100 mL PBS 1X.

Washing solutions:

Solution B: 5 mL Nonidet P-40 (Ref.: 74385) from Sigma-Aldrich (USA), 1 L PBS 1X.

Solution C: 50.83 mL NaCl 5M (Ref.: 131659.1214) from Panreac Quimica S.A.U. (Spain), 1 L prepared solution B.

PBS 1X: 100 mL PBS 10X, 900 mL diH_2O.

Primary Antibodies: human SIRT1 (Ref.: 2593), p-AMPKα (Ref.: 2535), LC3B (Ref.: 3868) from Cell Signaling (USA), β-actin (Ref.: A5316) from Sigma-Aldrich (USA).

Secondary antibodies: HRP-conjugated goat anti-rabbit antibody (AR) (Ref.: 31460) from Thermo Scientific (Spain), HRP-conjugated goat anti-mouse antibody (AM) (Ref.: P0447) from DakoCytomation (Denmark).

Chemiluminescence imaging and analytic softwares: SuPerSignal West Pico Chemiluminescent Substrate kit (Ref.: 34087), SuPerSignal West Femto Chemiluminiscent Substrate kit (Ref.: 34096) both from Thermo Scientific (USA), Fujifilm imaging system LAS 3000 mini from R&D Systems (USA), ImageJ 1.47 g Wayne Rasband National Institutes of Health (USA), GraphPad Prism 6 (USA) for statistical analysis.

5.2. Cell cultivation, freezing and unfreezing of cells

5.2.1. Cell cultivation

Before start working with HT-29 cells (Fig. 7) it was necessary to put Mc´Coy medium, previously filled with 10% fetal bovine serum and 1% antibiotics, into thermostatic bath for 20 min to reach 37°C and also to turn on UV in airflow cabinet for at least 30 min to prevent contamination. All materials used for working in cabinet were previously sterilized and splattered with 70% ethanol as well as cabinet itself to maintain sterility. Cells were observed in microscope and when confluence in flask reached 80 - 90%, they were sub-cultivated into new flask.

First the old medium in flask was thrown into beaker with lye and then flask was washed with 3 mL sterile ice-cold PBS 1X. After there was added 3 mL of trypsin/EDTA solution and it was incubated for 7 min until all cells were floating. 3 mL of medium was added to neutralize trypsin and suspension was very well resuspended and put into 50 mL tube. New middle flask was used and filled with 10 mL of new medium and with 2 - 3 mL of cell suspension. Flasks stayed in incubator under 37°C and 5% CO_2. The medium was changed every 3 or 4 days. As soon as cells reached 80% of confluence, they were sub-cultivated again, maximum until step number 22.

5.2.2. Freezing of cells

Firstly medium was warmed on 37°C and airflow cabinet was sterilized as mentioned above. After steps of washing, trypsinization and neutralization cells were counted in Neubauer chamber. 20 µl of trypan blue and 20 µl of cell suspension were well mixed in 500 µl tube and 10 µl of this blue mixture were inserted in Neubauer chamber to count the cells. Neubauer chamber has 4 quadrants. Cells were counted in each of them to calculate final average number, which was multiplied by 10 000 (dilution factor of chamber) and 2 (dilution factor) to get final concentration of cells in 1 mL. For one vial $2 \cdot 10^6$ cells should have been frozen. Known concentration of cells in suspension was diluted to get 900 µl of it to insert into vial together with 100 µl of DMSO. Everything was mixed properly and vial was rapidly wrapped in paper to cushion changes of temperature and placed in freezer at -80°C to be stored for weeks or after in liquid nitrogen for years.

5.2.3. Unfreezing of cells

To reach sterile conditions all steps described in cell cultivation were done before start working with cells.

Previously warmed medium on 37°C was added to 10 mL tube in sterile conditions of airflow cabinet. Also frozen vial was located in thermostatic bath for further use. Content from unfrozen vial was added into the tube with medium to dilute 10% of containing DMSO on 0.1%. Next the tube was centrifuged on 1200 rpm for 3 min and supernatant was replaced with 5 mL of new medium and resuspended very well with biomass. This suspension was placed in small flask and observed in microscope to see if cells were vital. Continuously cell line was cultivated.

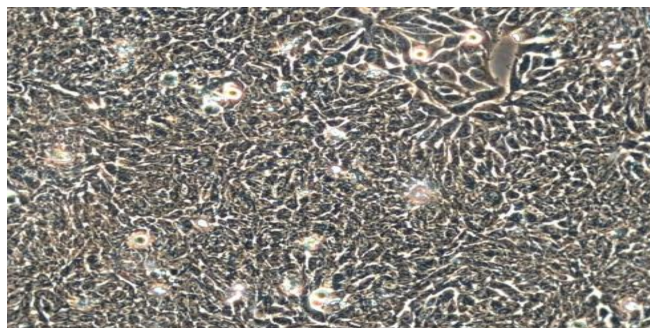


Fig. 7 <u>HT-29 cells</u>

Taken from *Health Protection Agency, Images of cell line HT-29*

5.3. Methods

5.3.1. Sulforhodamine B colorimetric assay for cytotoxicity screening

It is colorimetric assay using anionic dye sulforhodamine B (SRB) for screening and measuring of cell proliferation as a total protein synthesis. SRB bounds to proteins elecrostatically and after fixation and solubilization it is measured photometrically. Results correlate with total protein synthesis and therefore with cell proliferation, IC50 is stated. *(Keepers et al. 1991)*

Day 1 – Inoculation of cells into 96-well plate

Before start working with cells it was always necessary to sterilize airflow cabinet with UV for at least 20 min and splash all materials with 70% ethanol. Also it was essential to observe cells in microscope every time before using them. Then the old medium in flask was thrown into beaker with lye and flask was washed with ice-cold PBS 1X and trypsinized. Well resuspended cell suspension was neutralized with medium and placed in tube in the same way as mentioned above. After counting cells in Neubauer chamber it was necessary to dilute them on concentration 55 555.55 cells /mL to finally have 5000 cells in each of 96 wells of plate. Every well had final volume 100 µl – 90 µl cell suspension + 10 µl melatonin sample.

For one 96-well plate was necessary volume of 10 mL of cell suspension. According to real counted concentration of cells in flask and final needed concentration 55 555.55 cells /mL necessary volumes of medium and cell suspension were calculated and prepared. The volumes were homogenized in tube apart and then inserted 90 µl of suspension with multichannel pipette into every well to have 5000 cells in every well apart from first and last lines, these were blank. Prepared plate was placed in incubator for 24 hours.

Day 2 – Addition of melatonin samples

Before start the sterilization process was the same as mentioned in Day 1. Then 6 small tubes were prepared and labeled with final concentrations: 8 mM, 4 mM, 2 mM, 1 mM, 0.5 mM, 0.2 mM. According to previous calculations was prepared 3M solution of melatonin in DMSO. Then in every tube there was pipetted 389.34 µl of medium and in first tube 10.66 µl of melatonin 3 M accordingly to previous calculations. Using binary dilution of first tube we got the row of final concentrations. From every single tube 10 µl was inserted to 96-well plate two times. Two columns were controls without

melatonin. The same process was repeated with DMSO with concentrations: 2%, 1%, 0.5%, 0.25%, 0.125%, 0.0625%. One plate was incubated for 24 hours, the other one for 48 hours.

Day 3 or 4 – Fixation, staining, solubilization and reading in spectrophotometer

These steps could have been done in non-sterile conditions outside of airflow cabinet. After 24 and 48 hours both plates were observed in microscope, then 50 µl of trichloroacetic acid was added to every well using multichannel pipette and plate was placed into fridge for 1 hour to fixate it. Afterwards plate was washed 5 times with distil H_2O.

100 µl 1% sulforhodamine B was added into every well of plate and then it was placed into dark for 30 min in room temperature. Afterwards plate was washed 5 times with 1% acetic acid.

100 µl of Tris 10 mM was added at the end and the plate was read in spectrophotometer $\lambda = 492$ nm.

IC50 of value was calculated using Microsoft Excel.

5.3.2. Sample preparation for Bradford protein assay and Western blotting

Inoculation of plate

The process of sterilization before working with cells in airflow cabinet was the same as mentioned above in cell cultivation. Also first steps of handling with cells (washing. trypsinizing and counting) were the same. Cells were cultivated in 6-well plate. For Western blotting it was necessary to have 10^6 cells in every well. One sample consisted from cells of two wells. After counting in Neubauer chamber and diluting cell suspension with medium, the final concentration of cells had to be 10^6 cells /mL. First was inserted 1 mL of medium into plate and then 1 mL of cell suspension with final volume 2 mL per well. Very important was to shake the plate every time cell suspension was added to homogenize cells in all area of well. The plate was incubated for 24 hours in incubator, where the cells adherent to bottom with final confluence 80 - 90% per well.

Treatment

Sterilization steps before start working in airflow cabinet were the same as mentioned above. After 24 hours old medium was thrown into beaker with lye, the plate was washed with ice-cold PBS 1X and 2 mL of new medium was added into every well. Previously prepared 2 M melatonin was stored under - 40°C in tube and used for all experiments. It precipitated in Mc Coy´s medium when it was added directly to incubation plate in appropriate concentrations. However, if melatonin was diluted in medium in tube separately (1:75), and afterwards inserted into plate, it did not precipitate there. Adequately calculated volumes of mixture melatonin + medium from tube were inserted into the plate to treat cells with appropriate concentrations of melatonin (3 mM, 2 mM, 1.5 mM, 1 mM, 0.75 mM, 0.5 mM, 0.25 mM). There were also control wells without any treatment and control wells with 0.2% or 0.6% DMSO, highest concentrations appearing in samples with melatonin.

Suramin (SIRT1 inhibitor) and compound C (AMPK inhibitor) were pretreated 30 min before treatment with melatonin. Plates were incubated in incubator for 24 hours.

Supernatant preparation

Sterile conditions were not necessary for supernatant preparation, but it was important to keep everything on ice while working with cells. After 24 hours medium was thrown away and plate was washed with ice-cold PBS 1X. 500 μl of ice-cold PBS 1X were added in every well, cells were scrubbed from two wells with same treatment and inserted into 1.5 mL tube. Then all tubes with samples were centrifuged in pre-cold centrifuge (4°C) on 1200 rpm for 2 min. The supernatant – PBS 1X was thrown away and centrifuged cells on the bottom of tube were resuspended with 50 μl buffer of lyses. Buffer of lyses was prepared and stored in fridge at 4°C. Right before using there was added 1% of triton X-100, 1% of inhibitors of phosphorylases cocktail 2 and 1% of cocktail 3. Well resuspended and vortexed mixture was put into ultrasonic bath filled with ice to sonicate for 15 min. When the mixture was homogenized it was centrifuged again and final supernatant was inserted into small 500 μl tubes and stored under -40 °C until next use.

5.3.3. Bradford protein assay

This colorimetric technique was used to determine concentration of proteins (mg/mL) in biological sample. It utilized dilution series of a known protein (γ-globulin)

to create standard curve from which the concentration of the sample for Western blotting was delivered.

First of all it was essential to dilute dyeing reagent 1:4 with diH_2O. Then frozen samples were placed into ice to get liquefied. After shaking on vortex, 5 µl of sample was used for dilution 1:20 with diH_2O, the rest of sample was frozen again to be used later in Western blotting. Plate of 96-wells was used in this method. First 6 wells in two columns were marked for standards to create calibration curve. All others well were used for samples, always as a duplicate. γ-globulin was used as protein standard. According to Tab. 1 first were pipetted standards, then 5 µl of diluted samples as duplicate. Using multichannel pipette there was pipetted 240 - 250 µl of prepared dye reagent. After 15 min the plate was read in spectrophotometer, λ = 595 nm.

Tab. 1 *Standard curve*

Standard	**γ-globulin (mg/mL)**	**H_2O (µl)**	**γ-globulin (µl)**	**Final volume**	**Dye Reagent (µl)**
P0	2.982	0	15	15	240
P1	1.988	0	10	10	245
P2	0.994	0	5	5	250
P3	0.596	2	3	5	250
P4	0.198	4	1	5	250
Blank	0	5	0	5	250

Results were generated in computer using MikroWin program, version 4.36. Different detecting proteins in Western blotting need different concentrations for ideal band visualization. These amounts of proteins were used in this study: SIRT1 50 µg, p-AMPK 75 µg, LC3II 40 µg.

Computer generated concentrations for every single sample and due to formula also generated amounts of sample and Laemmli used in Western blotting.

X = concentration of diluted sample $\quad R = X/5 * 20 \quad R = 7.032$ mg/mL

R = real concentration of sample

Every well of gel in WB was loaded with Laemmli + sample to get final volume 30 µl. For example for measuring SIRT1 it was needed to load 50 µg of proteins.

S = µl of sample for well

$S = 50/R \qquad S = 50/7.032$

$S = 7.11\ \mu l$

$L = 30 - S \qquad L = 30 - 7.11$

L = μl of Laemmli for well

$L = 22.89\ \mu l$

Prepared samples for WB had to be heated on 100°C for 10 min to denaturalize proteins. Because Laemmli was evaporating out of prepared sample, all calculated volumes of samples and Laemmli were multiplied 1.2 X.

5.3.4. Western blotting

Western blotting was method using gel-electrophoresis for protein separation in electric field according to weight to transfer them on nitrocellulose membrane and to analyze them.

Gel-preparation

Polyacrylamide gels were prepared by free radical polymerization of acrylamide. Crystal plates were first degreased with ethanol and placed in casting stand. 15% running gel was used for LC3B II analyses and 10% running gel was used for p-AMPK and SIRT1 analyses, 10% stacking gel was used for both running gels. Preparation of gels was according to Tab. 2 and Tab. 3. The mixtures without tetramethylethylenediamine (TEMED) were prepared separately in different 50 mL tubes. Polymerization was initiated by ammonium persulfate (APS) with TEMED acting as a catalyst. That is why TEMED was added as last compound and it was essential to homogenize mixture very well and fast, so polymerization was the same in all area of plate. Also it was crucial to insert the mixture very quickly between two plates in casting stand to avoid polymerization in tube. The mixture was inserted approximately 2 cm above upper edge. Immediately 3 mL of 1-butanol was inserted on top of running gel mixture to avoid air bubbles, to support polymerization and to secure straight surface line. Usually it took around 15 - 20 min for polymerization. Then 1-butanol was washed with distil H_2O and surface between plates was dried well with filter paper. Afterwards TEMED was inserted into stacking gel mixture in other tube and immediately inserted on top of the running gel. To create sample wells in the gel, a comb was directly placed into the top of the gel prior to polymerization.

Calculations were made for 10 mL ≈ 1 gel of 15 wells, 1.5 mm thick. (Tab. 2 and Tab. 3)

Tab. 2 *Preparation of running gel*

Running gel		
	10%	15%
H_2O	2.76 mL	1.1 mL
Tris 1M pH 8.8	3.75 mL	3.75 mL
Acrylamide	3.3 mL	4 mL
SDS 20%	50 µl	50 µl
APS 10%	100 µl	100 µl
TEMED	6.6 µl	6.6 µl

Tab. 3 *Preparation of stacking gel*

Stacking gel	
	10%
H_2O	2.3 mL
Tris 1M pH 6.8	420 µl
Acrylamide	560 µl
SDS 20%	16.6 µl
APS 10%	33.3 µl
TEMED	3.3 µl

Sample preparation, sample loading and electrophoresis

Calculated amounts of Laemmli and sample supernatant were pipetted into 500 µl tubes and heated on 100°C for 10 min.
Meanwhile gels were placing in electrophoresis chamber between two electrodes in tank. Tank was filled with electrophoresis buffer first in space between glasses, then in rest of tank until special mark.

After careful elimination of comb, protein standard (10 µl) was loaded in first well, then 30 µl of samples were loaded in rest of wells. Tank was closed with lid and connected to power supply. Approximately first hour electrophoresis ran on 70 V until blue sample line entered the running gel and then it ran on 120 V around one more hour.

Wet transfer, dyeing of membrane with ponceau S, incubation with primary AB

2 filter papers (7.5 x 10 cm) and one nitrocellulose membrane (7 x 8.5 cm) were cut for protein transfer of one gel.

To realize transfer first was necessary to make "sandwich" of gel and membrane in correct sequence. On black side of gel holder cassette there was put sponge, filter paper, gel, membrane, second filter paper and second sponge, all well soaked in transfer buffer. Gel should have been stick very well to membrane. To remove air bubbles 5 mL tip was used as a blot roller. Sandwich was placed into blotting module with black side of cassette facing the black side of blotting module. Freshly prepared transfer buffer was added into the tank until the buffer level reached the fill line and whole cassette

with gel membrane sandwich was under the buffer. The tank was connected to power supply for 90 min, 100 V. Colors of cables had to match with those of inputs.

Transfer process was exothermic and it was essential to keep it in cool using cooling unit inside the tank and ice around the tank. Upon completion of run, cassettes were removed and sandwich disassembled.

Dyeing of membrane was done with ponceau S after wet transfer as a control if all proteins transferred well. Then it was washed with PBS 1X.

SIRT1 and p-AMPK were measured in membrane transferred from 10% gel and LC3B II in membrane from 15% gel. The membranes were cut according to marks from protein standards, so all proteins could have been incubated and analyzed at the same time.

To reduce background signals membranes were blocked with non-fat milk in case of non-phosphorylated proteins (β-actin, SIRT1 and LC3B II) and in case of phosphorylated proteins (p-AMPK) with special blocker. Blockage ratio was 5 g of non-fat milk in 100 g of Solution B and 5 g of special blocker powder in 100 g of PBST. Membranes were located in 50 mL tubes and blocked with 20 mL of required milk for 90 min. They were gentle agitating on special rolling to ensure that membranes will be sodden in all their area all the time.

Then membranes were incubated with primary antibodies for 60 min in room temperature and later in fridge at 4°C over-night. One membrane was always incubated with one antibody. Primary and secondary antibody dilution buffer was the same as for blockage of phosphorylated or non-phosphorylated proteins. Buffers were freshly prepared just before use. Dilutions used for different antibodies were according to Tab. 4.

Tab. 4 *Dilutions of antibodies*

Primary antibody	Dilution	Secondary antibody	Dilution
SIRT1	1:1000	AR	1:1000
p-AMPK	1:1000	AR	1:2500
LC3B II	1:1666	AR	1:1666
β-actin	1:1000	AM	1:000

AR – goat anti-rabbit antibody, AM – goat anti-mouse antibody

Washes, incubation with secondary AB and immunodetection

Next day the membranes were washed with special washing buffers for 15 min to eliminate excessive primary antibody. First with C buffer, then with B buffer and then with PBS 1X.

Then the memabranes for detection of SIRT1, p-AMPK and LC3B II were incubated with secondary HRP-conjugated goat anti-rabbit antibody (AR) using same dilution buffers as for primary antibodies. Membrane for detection of β-actin was incubated with HRP-conjugated goat anti-mouse antibody (AM). Dilution used for secondary antibodies was according to Tab. 4. Membranes were incubated with secondary antibodies for 90 min and then washed again in the same manner as mentioned for primary antibodies.

The membranes were detected through chemiluminescent reaction using Fujifilm imaging system LAS 3000 mini and special Kit (Pico Chemiluminiscent substrate) for SIRT1, p-AMPK and LC3B II antibodies and another Kit (Femto Chemiluminiscent substrate) for β-actin.

The images were analyzed with ImgaeJ program to quantify protein bands, and GraphPad Prism 6 program was used for statistical analysis.

5.4. Statistical analysis

Data were collected from three or more independent experiments and the results were expressed as mean ± SD. Some data were collected from one experiment without SD, some from two with mean ± SD. One way ANOVA followed by multiple-comparison Tukey´s test was used for statistical analysis in GraphPad Prism 6 program. Asterisks indicate that the values were significantly different from control. ($P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***)

6. RESULTS

6.1. Sulforhodamine B colorimetric assay for cytotoxicity screening

To define concentration of melatonin that decreased cell viability on 50% (IC50), melatonin was incubated with cells in these concentrations: 8 mM, 4 mM, 2 mM, 1 mM, 0.5 mM, 0.25 mM for 24 hours and 48 hours. Melatonin was diluted in DMSO. Concentration of DMSO did not exceed 2% (Fig. 8)

After 24 hours results did not show any changes in cell viability (Fig. 8 Left). DMSO did not have any influence on cell viability (Fig. 8 Right)

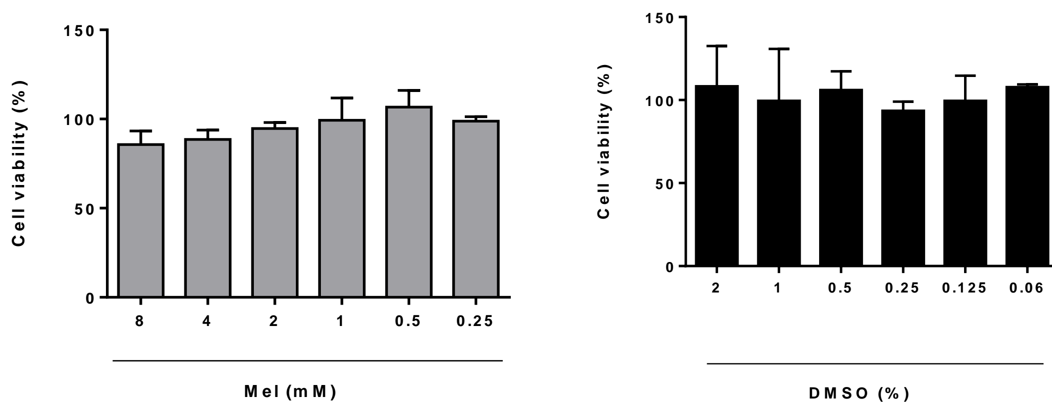


Fig. 8 <u>Influence of different concentrations of melatonin on viability of HT-29</u> (Left) HT-29 cell viability has not changed significantly in any concentration of melatonin after 24 hours of treatment. The bars are marked as follows: 8 - 0.25 mM melatonin. (Right) 2 - 0.06% of DMSO

Cell viability decreased significantly in 8 mM and 4 mM melatonin on less than 50% after 48 hours. IC50 was calculated using Microsoft Excel as 3.6 mM melatonin (Fig. 9 Left). DMSO did not have any influence on cell viability (Fig. 9 Right)

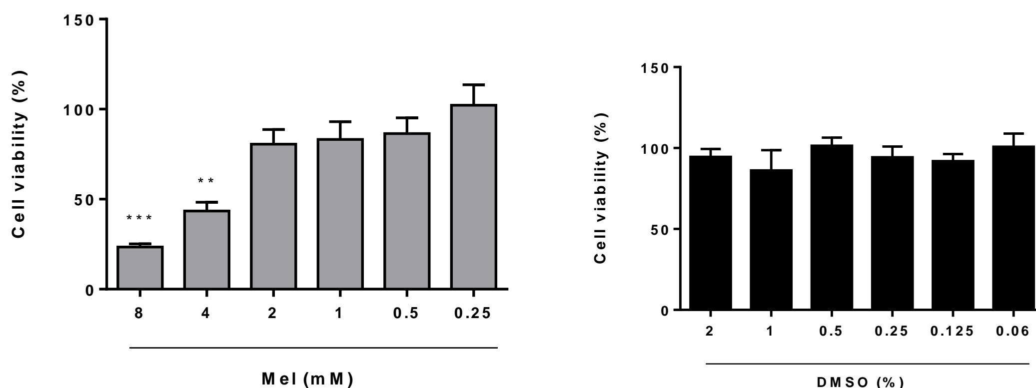


Fig. 9 **Influence of different concentrations of melatonin on viability of HT-29** (Left) HT-29 cell viability after 48 hours changed significantly in concentrations 8 mM and 4 mM. IC50 was determined as 3.6 mM. (Right) 2 - 0.06% DMSO

6.2. Western blotting

6.2.1. SIRT1 dependence on melatonin

Effect of melatonin on SIRT1 was measured after 24 hours of incubation in melatonin concentrations 0.25 mM, 0.5 mM, 1 mM and 2 mM. DMSO did not exceed 0.2% and did not have any significant effect on cells. (Fig. 10) SIRT1 increased with increasing concentration of melatonin up to 2 mM. Number of experiments (n) was averaged from n ≥ 3.

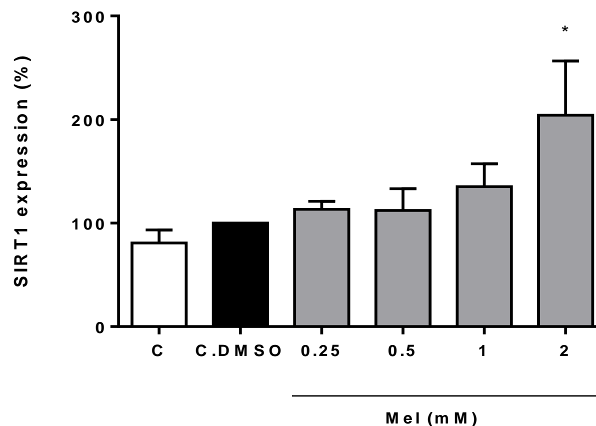


Fig. 10 **Dependence of SIRT1 on concentration of melatonin** The bars are marked as follows: C-control cells without any treatment, C.DMSO-control cells with 0.2% DMSO, 0.25 - 2 mM melatonin treatment. SIRT1 had significant increase in Mel 2 mM, n ≥ 3.

SIRT1 had significant increase in Mel 2 mM concentration. (Fig. 10, 11)

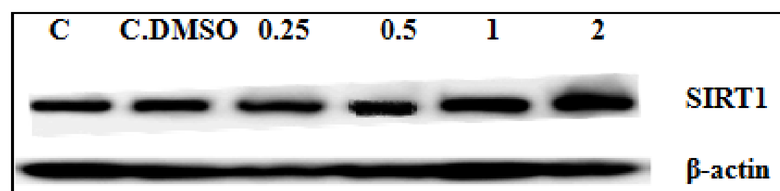


Fig. 11 **Dependence of SIRT1 on concentration of melatonin** The bars are marked as follows: C-control cells without any treatment, C.DMSO-control cells with 0.2% DMSO, 0.25 - 2 mM melatonin treatment. SIRT1 had significant increase in Mel 2 mM, n ≥ 3.

Effect of melatonin on SIRT1 was also measured in higher concentrations of melatonin 0.75 mM, 1.5 mM and 3 mM. DMSO did not exceed 0.6%. (Fig. 12) Number of experiments (n) was averaged from n ≥ 3.

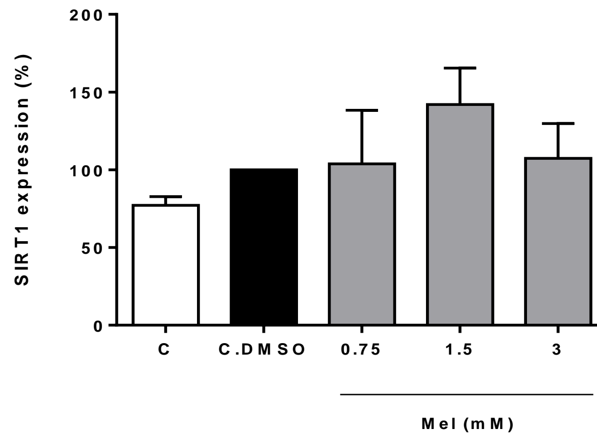


Fig. 12 **Dependence of SIRT1 on concentration of melatonin** The bars are marked as follows: C-control cells without any treatment, C.DMSO-control cells with 0.6% DMSO, 0.75 - 3 mM melatonin treatment. SIRT1 had no significant change, n ≥ 3.

SIRT1 had no significant change in these concentrations. (Fig. 12, 13)

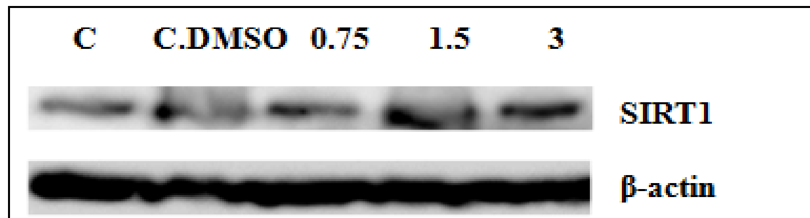


Fig. 13 **Dependence of SIRT1 on concentration of melatonin** The bars are marked as follows: C-control cells without any treatment, C.DMSO-control cells with 0.6% DMSO, 0.75 - 3 mM melatonin treatment. SIRT1 had no significant change, n ≥ 3.

Final tendency of SIRT1 dependence on melatonin concentration is seen in Fig. 14. It is increasing until 2 mM and decreasing in 3 mM. Graphical expression was made from all experiments done with SIRT1. C.DMSO bar was used as mean from different experiments with 0.2% and 0.6% of DMSO with insignificant SD.

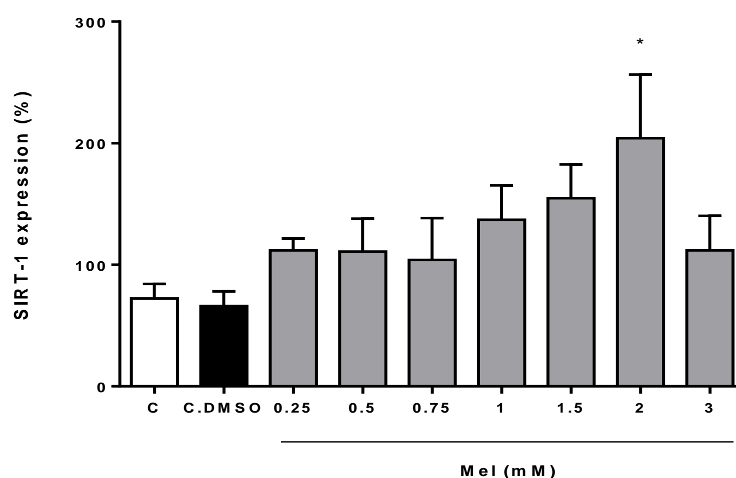


Fig. 14 **Dependence of SIRT1 on concentration of melatonin** The bars are marked as follows: C-control cells without any treatment, C.DMSO-control cells with 0.2% and 0.6% DMSO, 0.25 - 3 mM melatonin treatment. SIRT1 was significantly increased in 2 mM, n ≥ 3.

6.2.2. p-AMPK dependence on melatonin

Effect of melatonin on AMPK was measured as effect on its active phosphorylated form p-AMPK after 24 hours in 0.25 mM, 0.5 mM, 1 mM, 2 mM concentrations of melatonin. Maximum concentration of DMSO was 0.2%. Number of experiments (n) was averaged from n ≥ 3. (Fig. 15)

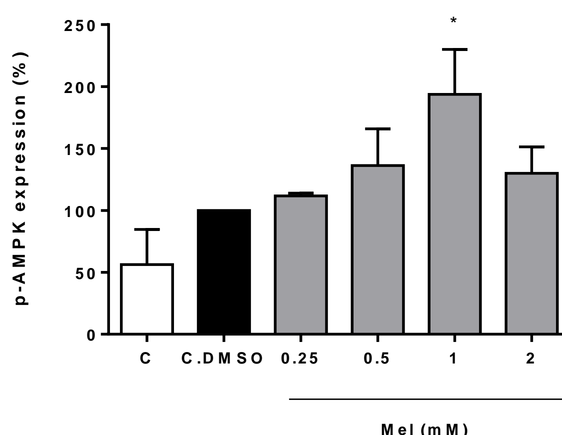


Fig. 15 **Dependence of p-AMPK on concentration of melatonin** The bars are marked as follows: C-control cells without any treatment, C.DMSO-control cells with 0.2% DMSO, 0.25 - 2 mM melatonin treatment. p-AMPK had significant increase in 1 mM, n ≥ 3.

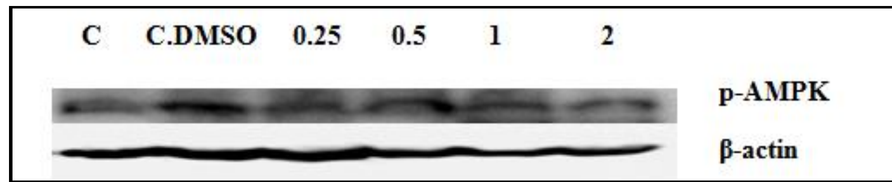


Fig. 16 **Dependence of p-AMPK on concentration of melatonin** The bars are marked as follows: C-control cells without any treatment, C.DMSO-control cells with 0.2% DMSO, 0.25 - 2 mM melatonin treatment. p-AMPK had significant increase in 1 mM, n ≥ 3.

p-AMPK was also measured in 0.75 mM, 1.5 mM, and 3 mM concentrations of melatonin without any significant effect. Maximum concentration of DMSO was 0.6 %. Number of experiments (n) was averaged from n ≥ 1 (Fig. 17, 18).

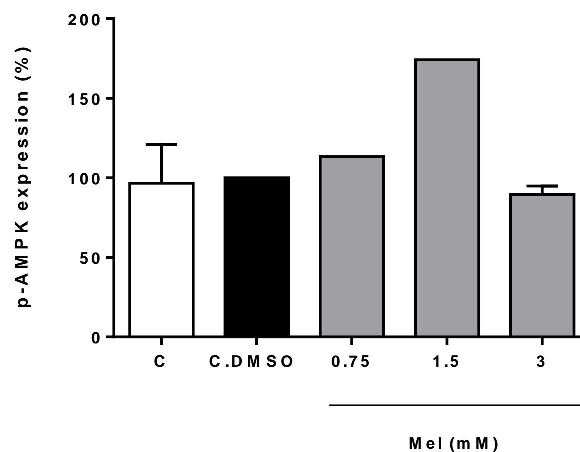


Fig. 17 **Dependence of p-AMPK on concentration of melatonin** The bars are marked as follows: C-control cells without any treatment, C.DMSO - control cells with 0.6% DMSO, 0.75 - 3 mM melatonin treatment. p-AMPK had no significant increase, n ≥ 1.

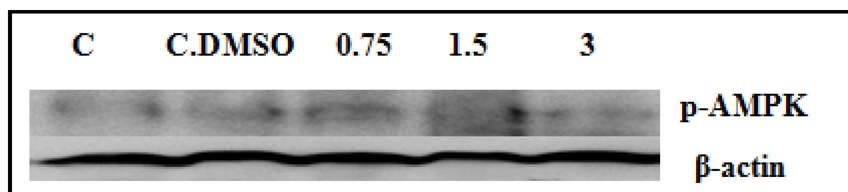


Fig. 18 **Dependence of p-AMPK on concentration of melatonin** The bars are marked as follows: C-control cells without any treatment, C.DMSO-control cells with 0.6% DMSO, 0.75 - 3 mM melatonin treatment, n ≥ 2.

Fig. 19 shows tendency of p-AMPK dependence on concentration of melatonin from all experiments done with p-AMPK. C.DMSO bar was used as mean from different experiments with 0.2% and 0.6% of DMSO with insignificant SD.

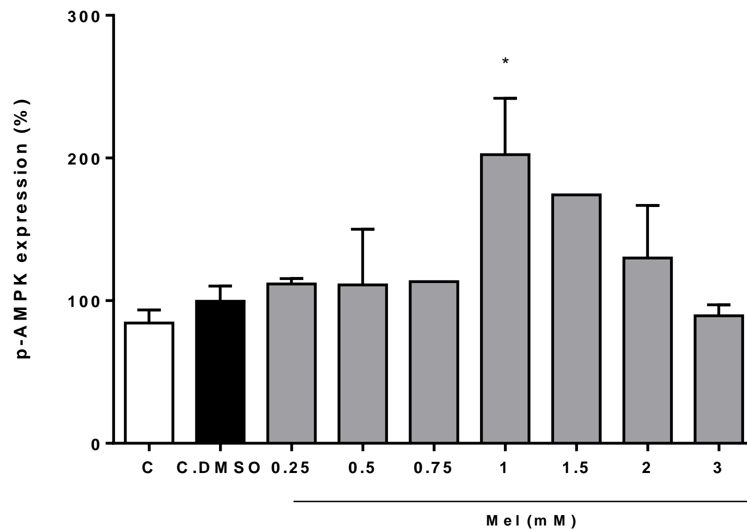


Fig. 19 Dependence of p-AMPK on concentration of melatonin The bars are marked as follows: C-control cells without any treatment, C.DMSO-control cells with 0.2% and 0.6% DMSO, 0.25 - 3 mM melatonin treatment. p-AMPK had significant increase in 1 mM. n ≥ 1 in 0.75 mM, 1.5 mM and 3 mM; n ≥ 3 in 0.25 mM, 0.5 mM, 1 mM and 2 mM.

6.2.3. Inhibitors of SIRT1 and AMPK

To define hierarchical relationship between SIRT1 and p-AMPK, suramin (SUR) was used as inhibitor of SIRT1 and compound C (CC) as inhibitor of AMPK. The SIRT1 production was observed with both inhibitors as well as p-AMPK. SUR was used in concentration of 2.5 μM *(Sturges et al. 2012)* Cells were pretreated with SUR 30 min before treatment with melatonin. We selected doses of melatonin 1 mM and 2 mM. Number of experiments (n) was averaged from n = 2. (Fig. 20, 21)

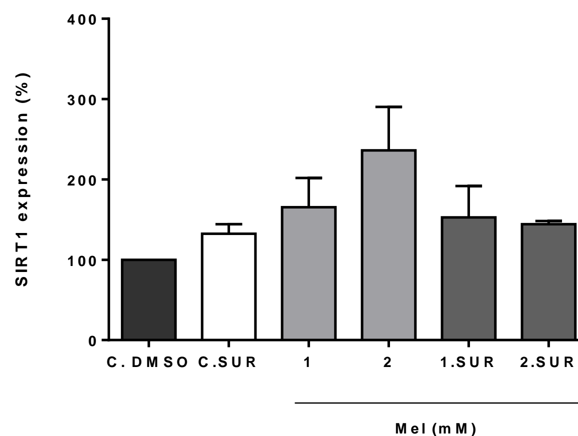


Fig. 20 **Dependence of SIRT1 on SUR comparing to Mel** The bars are marked as follows: C.DMSO-control cells with 0.2% DMSO, C.SUR–cells with SUR 2.5 µM, 1 - 2 mM melatonin treatment, 1.SUR–1 mM melatonin + pretreated cells with SUR 2.5 µM, 2.SUR–2 mM melatonin + pretreated cells with 2.5 µM. No significant results were observed, n = 2

SIRT1 increased in Mel 1 mM and 2 mM, although not significantly. SUR 2.5 µM in combination with Mel 1 mM and Mel 2 mM inhibited SIRT1 production. (Fig. 20, 21)

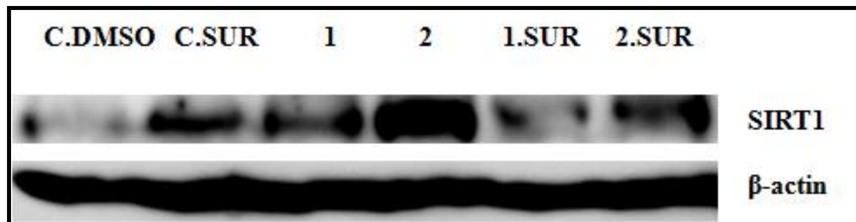


Fig. 21 **Dependence of SIRT1 on SUR comparing to Mel** The bars are marked as follows: C.DMSO-control cells with 0.2% DMSO, C.SUR–cells with SUR 2.5 µM, 1 - 2 mM melatonin treatment, 1.SUR–1 mM melatonin + pretreated cells with SUR 2.5 µM, 2.SUR–2 mM melatonin + pretreated cells with SUR 2.5 µM. No significant results were observed, n = 2.

We used 10 µM inhibitor of AMPK–compound C (CC) to see expression of SIRT1. *(Alves et al. 2010, Peyton et al. 2011)* Cells were pretreated with CC 30 min before treatment with melatonin. We selected again doses of melatonin 1 mM and 2 mM. Number of experiments (n) was averaged from n = 2. (Fig. 22, 23)

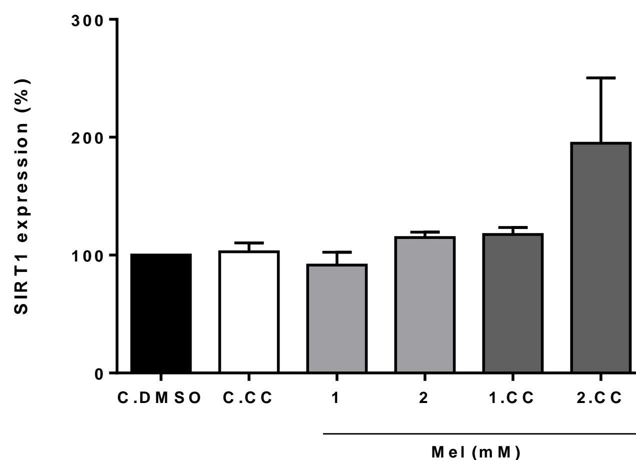


Fig. 22 **Dependence of SIRT1 on CC comparing to Mel** The bars are marked as follows: C.DMSO-control cells with 0.2% DMSO, C.CC-cells with CC 10 µM, 1 - 2 mM melatonin treatment, 1.CC–1 mM melatonin + pretreated cells with CC 10 µM, 2.CC–2 mM melatonin + pretreated cells with CC 10 µM. No significant results were observed, n = 2.

SIRT1 did not show any increase in Mel 1 mM and 2 mM, nor in Mel 1 mM pretreated with CC 10 µM. SIRT1 relatively increased in Mel 2 mM pretreated with CC. No results were observed as significant.

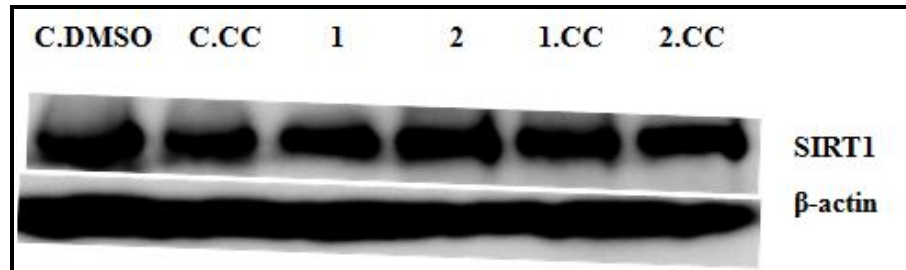


Fig. 23 **Dependence of SIRT1 on CC comparing to Mel** The bars are marked as follows: C.DMSO-control cells with 0.2% DMSO, C.CC–cells with CC 10 µM, 1 - 2 mM melatonin treatment, 1.CC–1 mM melatonin + pretreated cells with CC 10 µM, 2.CC–2 mM melatonin + pretreated cells with CC 10 µM. No significant results were observed, n = 2.

Some decrease of p-AMPK was observed with both inhibitors: SUR and CC, although it was difficult to measure phosphorylated protein. Cells were pretreated with inhibitors in the same way as for measurement of SIRT1. n = 1 (Fig. 24, 25)

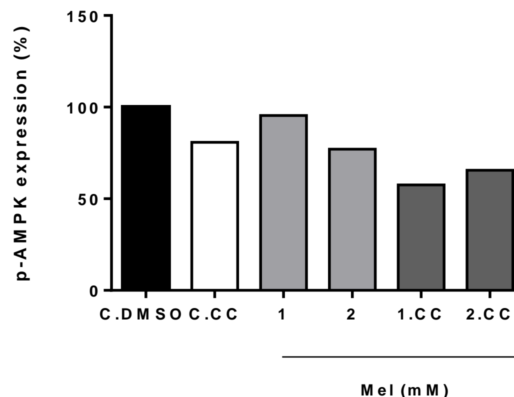


Fig. 24 **Dependence of p-AMPK on CC comparing to Mel** The bars are marked as follows: C.DMSO-control cells with 0.2% DMSO, C.CC–cells with CC 10 µM, 1 - 2 mM melatonin treatment, 1.CC–1 mM melatonin + pretreated cells with CC 10 µM, 2.CC–2 mM melatonin + pretreated cells with CC 10 µM. No significant results were observed, n = 1.

p-AMPK measured in 1.CC and 2.CC showed slight decrease comparing to Mel 1 mM and 2 mM (Fig. 24). C.DMSO was quiet high for unknown reasons. The increase of expression of p-AMPK after co-incubation with 1 mM and 2 mM melatonin was not high comparing to C.CC. and without changes comparing to C.DMSO. However in Fig.

25 the increase of expression is more noticeable. This might be caused by amount of b-actin.

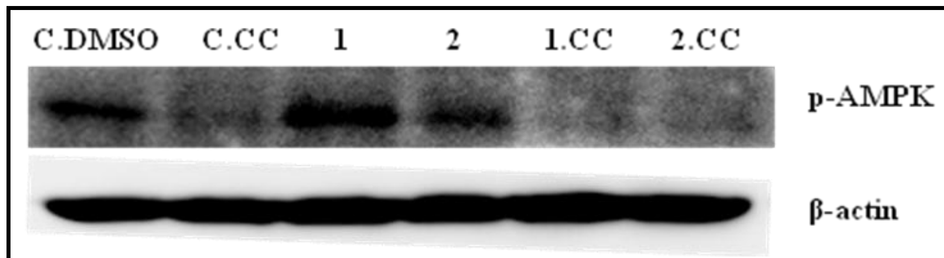


Fig. 25 **Dependence of p-AMPK on CC comparing to Mel** The bars are marked as follows: C.DMSO-control cells with 0.2% DMSO, C.CC–cells with CC 10 μM, 1 - 2 mM melatonin treatment, 1.CC–1 mM melatonin + pretreated cells with CC 10 μM, 2.CC–2 mM melatonin + pretreated cells with CC 10 μM. No significant results were observed, n = 1.

p-AMPK measured with SUR also did not show any significant results because of low number of measurable experiments. n = 1 (Fig. 26, 27)

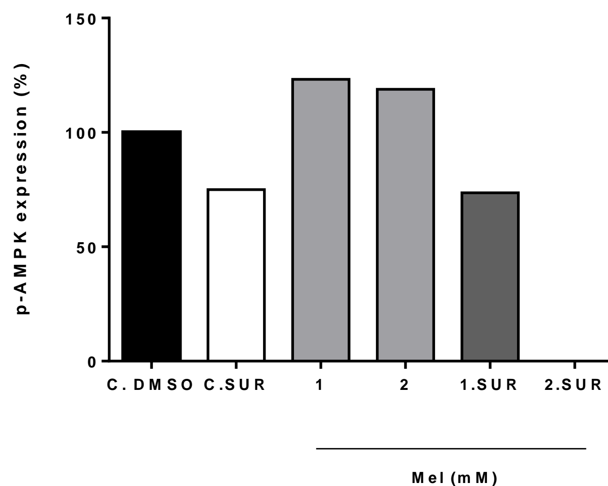


Fig. 26 **Dependence of p-AMPK on SUR comparing to Mel** The bars are marked as follows: C.DMSO-control cells with 0.2% DMSO, C.SUR– cells with SUR 2.5 μM, 1 - 2 mM melatonin treatment, 1.SUR–1 mM melatonin + pretreated cells with SUR 2.5 μM, 2.SUR–we did not get the result. No significant results were observed, n = 1.

p-AMPK showed slight increase in Mel 1 mM and 2 mM, and decrease in incubation with 1.SUR comparing to Mel 1 mM. We did not get any result with 2.SUR. Also C.DMSO was quiet high for unknown reasons, although it is not evident from Fig. 27.

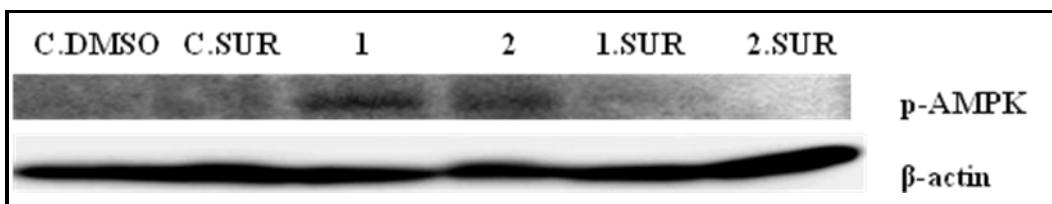


Fig. 27 **Dependence of p-AMPK on SUR comparing to Mel** The bars are marked as follows: C.DMSO-control cells with 0.2% DMSO, C.SUR–cells with SUR 2.5 µM, 1 - 2 mM melatonin treatment, 1.SUR–1 mM melatonin + pretreated cells with SUR 2.5 µM, 2.SUR–we did not get the result. No significant results were observed, n = 1.

6.2.4. Autophagy, LC3B II and its dependence on melatonin

Activation of autophagy is measured by increase of LC3B II, which is activated from cytoplasmic LC3B I and bound to inner membrane of new formed autophagosome. The influence of melatonin on autophagy was measured in these concentrations: 0.25 mM, 0.5 mM, 1 mM and 2 mM. DMSO concentration did not exceed 0.2%, number of experiments was n ≥ 3. (Fig. 28, 29)

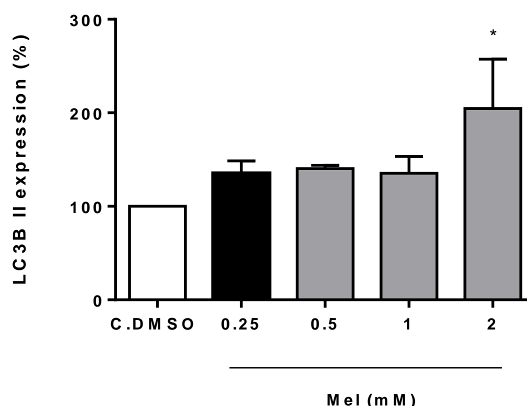


Fig. 28 **Dependence of LC3B II on Mel** The bars are marked as follows: C.DMSO-control cells with 0.2% DMSO, 0.25 - 2 mM melatonin treatment. LC3BII had significant increase in Mel 2 mM, n ≥ 3.

LC3B II increased with increasing concentration of Mel being significant in 2 mM. Together with LC3B II increased also LC3B I. This result suggests that melatonin activates autophagy in HT-29. (Fig. 28, 29)

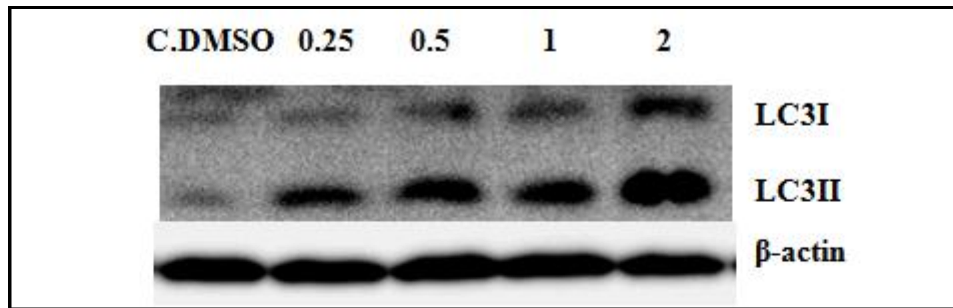


Fig. 29 **Dependence of LC3B II on Mel** The bars are marked as follows: C.DMSO-control cells with 0.2% DMSO, 0.25 - 2 mM melatonin treatment. LC3B II had significant increase in Mel 2 mM, $n \geq 3$.

7. DISCUSSION

The present results further support positive activity of melatonin on HT-29 cells through SIRT1 and AMPK. These results also present activation of autophagy.

Melatonin did not have cytotoxic effect on HT-29 in lower measured concentrations, although these concentrations were several hundred times higher than physiological level of melatonin in plasma $10^{-11} – 10^{-9}$ M. *(Papazisis et al 1998)* In the study of *Papazisis et al (1998)* there were showed similar results as ours testing effect of melatonin on cell lines HT-29, HeLA (human cervical cancer), OAW-42 (human ovarian cancer) using SRB. Their results showed that doses higher than 3 mM exhibited potent cytotoxic effect on cancer cells after 72 hours in general. We determinated IC50 of melatonin for HT-29 cells as 3.6 mM after 48 hours using SRB colorimetric assay.

According to results in SRB assay we selected the probe doses of melatonin as 0.25 mM, 0.5 mM, 1 mM, and 2 mM to avoid toxic effect on cells. We incubated cells with melatonin for 24 hours and further used them for Western blotting experiment measuring SIRT1, p-AMPK and LC3B II.

SIRT1 had significant increase in 2 mM concentration of melatonin. We tested higher doses of melatonin up to 3 mM in another experiment and interestingly SIRT1 decreased with higher dose 3 mM. (Fig. 15)

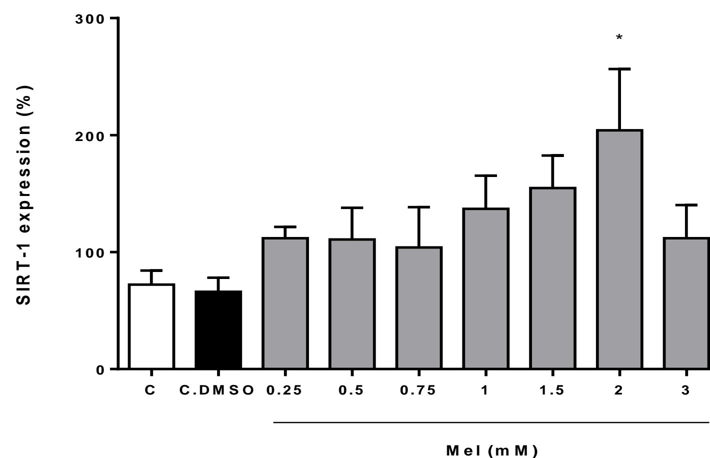


Fig. 15 <u>Dependence of SIRT1 on concentration of melatonin</u> C-control cells without any treatment, C.DMSO-control cells with 0.6% and 0.2% DMSO, 0.25 - 3 mM melatonin treatment. SIRT1 was significantly increased in 2 mM, n ≥ 3.

Interesting and similar results were measured in study of *Lim et al. (2012)*, where melatonin was tested in hydrogen peroxide stimulated human chondrocyte cells. SIRT1 was increasing with increasing level of H_2O_2 until 5 mM and then decreasing with 10 mM and 20 mM H_2O_2. (Fig. 30 left) When Mel was used, SIRT1 was at high levels and then decreased with higher levels of Mel – 10 ng and 100 ng. (Fig. 30 right) Interestingly these concentrations of Mel are much lower (ca. 10^{-9} M) than we used in our study (10^{-3} M). Probably there are certain ranges of Mel affecting the increase or decrease of SIRT1.

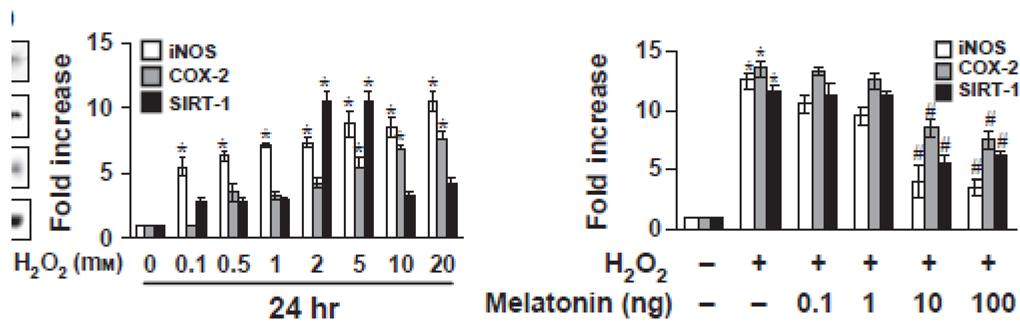


Fig. 30 Dependence of SIRT1 on H_2O_2 and melatonin (Left) 0 - 20 mM H_2O_2, SIRT1 was increasing until 5 mM and then decreasing. (Right) With addition of Mel SIRT1 increased until 1 ng and then decreased with higher levels of 10 and 100 ng Mel.

Taken from *Lim et al. (2012)*

As well AMPK dependence on melatonin was changing as we measured its active phosphorylated form p-AMPK. First we measured dependence of p-AMPK on melatonin in lower concentrations up to 2 mM. Level of p-AMPK was the highest and significant at Mel 1 mM. Then we measured p-AMPK in higher concentrations of Mel and it had decreasing tendency as well as SIRT1. However number of experiments was not sufficient because of difficulties with measuring phosphorylated protein. Final tendency seems to copy SIRT1 results with pick of p-AMPK in 1 mM Mel. (Fig. 19)

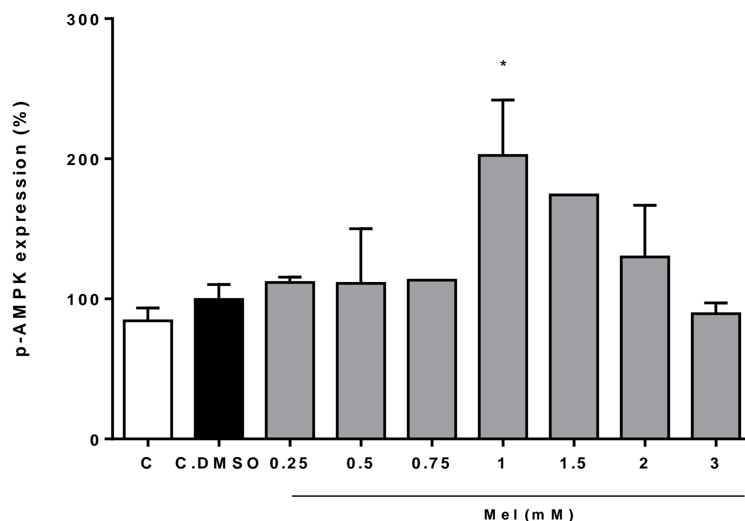


Fig. 19 Dependence of p-AMPK on concentration of melatonin C-control cells without any treatment, C.DMSO-control cells with 0.6% and 0.2% DMSO, 0.25 - 3 mM melatonin treatment. p-AMPK had significant increase in 1 mM. n ≥ 1 in 0.75 mM, 1.5 mM and 3 mM. n ≥ 3 in 0.25 mM, 0.5 mM, 1 mM and 2 mM.

To see whether SIRT1 activates AMPK or AMPK activates SIRT1 we used suramin as SIRT1 inhibitor and compound C as AMPK inhibitor. We picked up two doses of Mel 1 mM and 2 mM and pretreated cells with inhibitors. The results we got were not significant. Probable explanation could be low number of experiments (n = 2 and n = 1) because of time and measuring difficulties. But some tendency was seen. Tab. 5 and Tab. 6 indicate possible outcomes.

If SIRT1 activates AMPK (Tab. 5)

SIRT1 ⇨ AMPK

Tab. 5 *SIRT1 activates AMPK*

	SUR	*CC*
SIRT1	⇩	⇧
p-AMPK	⇩	⇩

If AMPK activates SIRT1 (Tab. 6)

AMPK ⇨ SIRT1

Tab. 6 *AMPK activates SIRT1*

	SUR	*CC*
SIRT1	⇩	⇩
p-AMPK	⇧	⇩

In our study results suggest that SIRT1 activates AMPK according to Tab. 5.

SIRT1 was inhibited by SUR (Fig. 20), but not by CC, at least not by 2.CC (Fig. 22). Nevertheless SIRT1 did not increase in Mel 1 mM and 2 mM in Fig. 22 for unknown reasons comparing to Fig. 15, neither did in 1.CC. Probably with more experiments we would see increase in 1 mM and 2 mM as it was seen in Fig. 15, which was averaged from n ≥ 3.

p-AMPK did not increase in Mel 1 mM, which was not expected, and decreased slightly in 2 mM comparing to C.DMSO in Fig. 24. p-AMPK did decrease in 1.CC and 2.CC. However C.DMSO was very high comparing to Fig. 19 for unknown reasons. Possible problem could have been in cells used for this experiment, or mistake in process of experiment, or there is another unknown factor important for the experiment. In any case more experiments would show better and more reliable result. Interestingly when p-AMPK was co-incubated with SUR – SIRT1 inhibitor (Fig. 26) in bar marked 1.SUR, p-AMPK was also inhibited. In 2.SUR unfortunately we did not get any result. C.DMSO was quiet high as well in this figure.

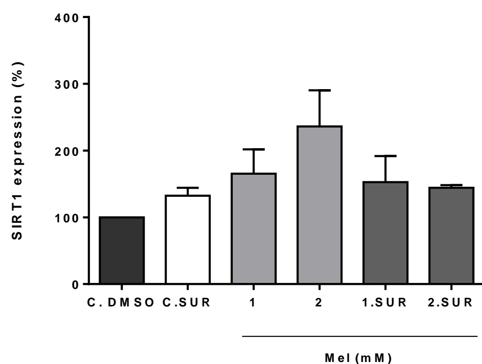


Fig. 20 **SIRT1 dependent on Mel and SUR**

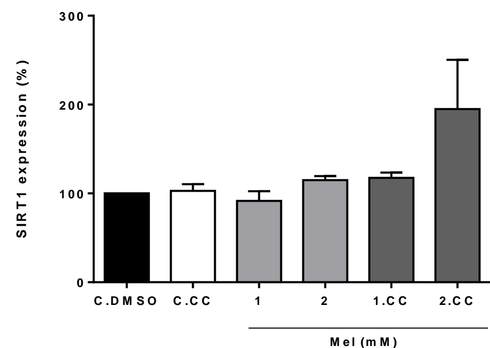


Fig. 22 **SIRT1 dependent on Mel and CC**

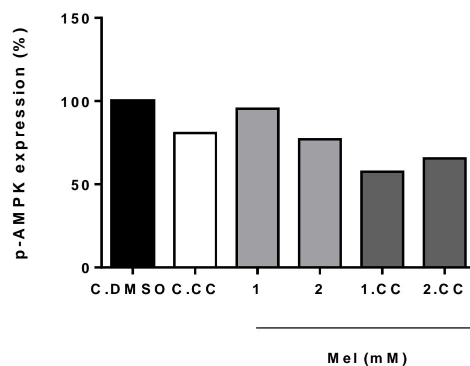


Fig. 24 **p-AMPK dependent on Mel and CC**

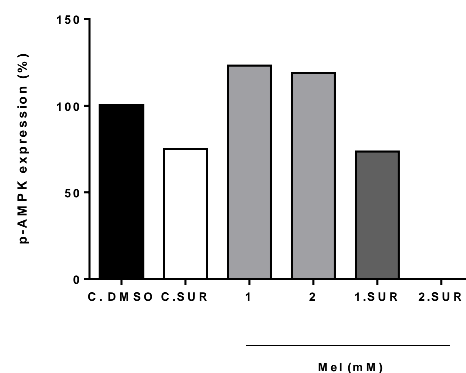
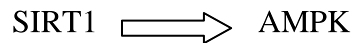


Fig. 26 **p-AMPK dependent on Mel and SUR**

These results suggest that SIRT1 activates AMPK, although they are not significant, and come from only one or two experiments.



Suchankova et al. (2009) presented in their study similar results when they were observing whether glucose-induced changes in AMPK in HepG2 cells could be mediated via SIRT1. SIRT1 activated and inhibited AMPK by using different SIRT1-activators and inhibitors.

It is difficult to interpret results connected to autophagy as there are a lot of different and contrary studies. In our case, autophagy was augmented by measuring increased LC3B II levels, which is protein significant for forming membrane of autophagosome and indicates activation of autophagy. LC3B II was augmeneted the most significantly in Mel 2 mM as well as SIRT1 suggesting possible role of SIRT1 in its activation. Similar results were observed in study of *Jeong et al. (2013).* They concluded that SIRT1 activates autophagy in neurons and so protects neuron cells in neurodegenerative disorders. However in our case literature differs, if the augmented atuophagy has positive or negative effect on colon cancer cells. We observed that in low doses of Mel atuphagy was at low levels increasing with increased doses of Mel.

Assuming SIRT1 has positive anti-cancer effect on HT-29 and one of its mechanisms is induced autophagy as anti-cancer mechanism, then autophagy induction by certain doses of melatonin could be used as adjuvantive anti-cancer treatment as was already reported with success in some clinical trials. *(Chen and Debnath 2010, Levy and Thorburn 2011, Swampillai et al. 2012)* However there is number of clinical cases *(Levy and Thorburn 2011, Swampillai et al. 2012)* where augmented autophagy plays tumor protective role against anti-cancer treatment. Specifically for HT-29 it differs within studies and there is lack of evidences on relationship between melatonin and autophagy.

More experiments using different methods and clinical trials would be necessary to determinate positive anti-cancer impact of melatonin on HT-29 cells, and if so, whether SIRT1 plays a key role through activating AMPK, and what role plays autophagy in this process.

8. CONCLUSIONS

This study demonstrates that melatonin has significant impact on HT-29 cells. We found out that melatonin activates SIRT1 as well as AMPK. Also there are evidences, that SIRT1 activates AMPK. However more experiments would be necessary to make final conclusions. SIRT1 might activate autophagy as we measured increased tendency of LC3B II dependently on concentration of SIRT1.

1) We determined IC50 of melatonin as 3.6 mM after 48 hours of incubation in HT-29 cells. After 24 hours melatonin did not show any significant changes in cell viability. According to results we stated main studying concentrations: 0.25 mM, 0.5 mM, 1 mM, and 2 mM, although we measured changes in HT-29 also in 0.75 mM, 1.5 mM, and 3 mM.

2) We measured that melatonin activates SIRT1 and it has increasing tendency with highest pick in 2 mM. However SIRT1 decreased in Mel 3 mM. AMPK also had increasing tendency with highest pick in Mel 1 mM. After that it was decreasing as well. When we measured their hierarchical relationship using SUR as inhibitor of SIRT1 and CC as inhibitor of AMPK, our results showed that SIRT1 activated AMPK.

3) LC3B II was measured as indicator of autophagy. Our results suggested its activation with highest pick in Mel 2 mM the same as SIRT1. According to some studies SIRT1 could activate autophagy.

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