Charles University

1st Faculty of Medicine

Summary of Dissertation Thesis

Pharmacological perspectives and clinical benefits of SIRT1 and AMPK activators and inhibitors in inflammatory and oxidative stress in the liver

Perspektivy farmakologického a klinického přínosu aktivátorů a inhibitorů sirtuinu 1 a AMPK při zánětlivém a oxidativním poškození v jaterní tkáni

Mgr. Lea Njeka Wojnarová

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Chairperson of the council: Prof. MUDr. Ondřej Slanař, Ph.D.

Training institution: Institute of Pharmacology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Albertov 4, Praha 2, 128 00

Supervisor: MUDr. Nikolina Kutinová Canová, Ph.D.

Consultant: Prof. Dr. Hassan Farghali, DrSc

Abstract

Introduction: Liver diseases represent a significant cause of morbidity and mortality worldwide. Previous experimental studies have shown that polyphenolic compound, resveratrol, as a less specific activator of sirtuin 1 (SIRT1) and AMP-activated protein kinase (AMPK) can effectively attenuate acute liver injury. Although SIRT1 and AMPK have been widely studied for many years, further evidence for a mutual SIRT1/AMPK signaling mechanism and how it is modulated by drugs of small molecules had not been fully clarified at start of our experimental work.

Goal: The main objective of the presented research was to investigate the relationship of SIRT1 and AMPK in process of hepatotoxicity/hepatoprotection in *in vivo* and *in vitro* animal model of acute drug-induced liver injury.

Methods: Male Wistar rats were used for both *in vivo* and *in vitro* studies. Hepatotoxicity was induced by a single dose of D-Galactosamine (GalN)/lipopolysaccharide (LPS) or acetaminophen (APAP). Some rats and cultured hepatocytes were treated by resveratrol, synthetic selective activator or inhibitor of SIRT1 and AMPK. Biochemical markers of liver injury (aminotransaminases, total bilirubin), oxidative stress (nitrites) and lipid peroxidation (conjugated dienes, TBARS) were measured in the plasma, medium or liver homogenate. Liver histology, hepatocyte viability, SIRT1 and AMPK activity and protein expression were also assessed.

Results: Our findings demonstrate that the harmful effects of D-GalN/LPS and APAP were associated with decreased activity and/or protein expression of SIRT1 and AMPK alongside enhanced oxidative stress in hepatocytes which can be significantly attenuated by the administration of the SIRT1 activator. In addition, our results from *in vitro* experiments originally suggest that hepatoprotective effects of SIRT1 against APAP toxicity could be at least partially independent of AMPK activity.

Conclusion: The differentiated modulation of SIRT1 and AMPK activity, especially by their specific synthetic activators, could provide an interesting and novel therapeutic option for hepatocyte injury in the future.

Keywords: acetaminophen; adenosine monophosphate protein kinase (AMPK); AICAR; CAY10591; Compound C; D-Galactosamine (GalN)/lipopolysaccharide (LPS); enzyme activation; EX-527; hepatocyte protection; hepatotoxicity; sirtuin 1 (SIRT1).

Abstrakt

Úvod: Choroby jater se staly jednou z hlavních příčin morbidity a mortality u lidí po celém světě. Předchozí studie s přírodní polyfenolickou sloučeninou resveratrolem, jakožto nespecifickým aktivátorem sirtuinu 1 (SIRT1, silent information regulator T) a AMP-aktivované proteinové kinázy (AMPK), prokázaly jeho hepatoprotektivní působení při akutním poškození jater. Ačkoli SIRT1 a AMPK jsou široce studovány již řadu let, další důkazy o vzájemném propojení jejich signálních drah a o tom, jak jsou ovlivněny syntetickými látkami modulujícími jejich aktivitu o malé molekule, nebyly v době zahájení naší experimentální práce předloženy.

Cíle: Hlavním cílem naší studie bylo objasnění úlohy SIRT1 a AMPK v procesu hepatoprotekce na zvířecím modelu chemického poškození jaterních buněk *in vivo* a *in vitro.*

Metody: Akutní hepatotoxicita byla navozena jednorázovým podáním D-galaktosaminu (GalN)/lipopolysacharidu (LPS) nebo paracetamolu (APAP) *in vivo* u potkanů kmene Wistar nebo *in vitro* na buněčných kulturách primárních hepatocytů. Současně byl aplikován resveratrol nebo další látky modulující aktivitu sirtuinu 1 nebo AMPK. Biochemické markery hepatocelulárního poškození (aminotransaminázy, celkový bilirubin), oxidačního stresu (dusitany) a lipidové peroxidace (konjugované dieny, TBARS) byly měřeny v plazmě, kultivačním médiu nebo v jaterním homogenátu. Dále byla vyhodnocena histologie jater, životnost hepatocytů a aktivita a exprese proteinů SIRT1 a AMPK.

Výsledky: Naše výsledky naznačují, že škodlivý účinek D-GalN/LPS a APAP byl spojen se sníženou aktivitou a/nebo expresí SIRT1 a AMPK spolu se zvýšeným oxidačním stresem v hepatocytech, který může být významně zmírněn podáním selektivního aktivátoru SIRT1. Kromě toho naše výsledky z *in vitro* experimentů naznačují, že hepatoprotektivní účinky SIRT1 při toxicitě APAP by mohly být alespoň částečně nezávislé na aktivitě AMPK.

Shrnutí: Diferencovaná modulace aktivity SIRT1 a AMPK, zejména jejich specifickými syntetickými aktivátory, by mohla v budoucnu poskytnout zajímavou a novou terapeutickou možnost pro poškození hepatocytů.

Klíčová slova: AICAR; aktivace enzymu; AMP-aktivovaná proteinová kináza (AMPK); CAY10591; Compound C; D-galaktózamin (GalN)/lipopolysacharid (LPS); EX-527; hepatotoxicita; hepatoprotekce; paracetamol; sirtuin 1 (SIRT1).

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1. Introduction

Liver diseases represent a significant cause of morbidity and mortality and account for approximately 2 million deaths per year worldwide (Asrani *et* al., 2019). Many plants have emerged as a great source of pharmaceutical products. It has been reported in the publication of Bhaargavi *et* al., 2014 that about 160 phytoconstituents from 101 medicinal herbs have hepatoprotective action. Many plants have been used to mitigate diverse liver diseases, of which the favorite ones include for example silymarin from *Silybum marianum* and curcumin from *Curcuma longa* (Bhaargavi *et* al., 2014; Farghali et al. 2015). Today, the main problem with herbal medicines is that many plants are consumed as polyherbal formulations where multiple constituents work synergistically. The active component responsible for the pharmacological and therapeutical effects in most cases remains unknown. So today, the worldwide research of potent hepatoprotective drugs have led towards the screening of numerous plant products, their purification and characterization of various bioactive compounds, and searching for their probable mode of action (Dey *et* al., 2013).

Previous experimental studies on resveratrol (Farghali *et* al., 2009), silymarin (Farghali *et* al., 2000), curcumin (Černý *et* al., 2011) and quercetin (Lekić *et* al., 2013) at our institute have shown definite hepatoprotective properties with alteration in some intracellular signaling molecules which contributed to these effects. In addition, many other studies have suggested that polyphenol resveratrol (2,3,40-trihydroxystilbene) has anti-inflammatory, antioxidant, anti-aging, and anti-carcinogenic properties that might be pertinent to chronic diseases and/or longevity in humans.

Resveratrol, among others, has been described (Howitz *et* al., 2003) as an activator of silent information regulator T1 (SIRT1) that can also increase adenosine-5′-monophosphate-activated protein kinase (AMPK) phosphorylation and reduce the oxidative stress biomarkers in laboratory settings (Ruderman *et* al., 2010; Farghali *et* al., 2013; Farghali *et* al. 2015; Lan *et* al., 2017). However, there is still an open question of whether resveratrol can activate SIRT1 directly or indirectly through AMPK or act independently (Farghali *et* al., 2019).

The sirtuins are a family of evolutionarily conserved NAD^+-dependent histone/protein deacetylases that are expressed in mammalian cells and have been studied in many tissues, including liver, skeletal muscle, adipose tissue, pancreas (β-cells), brain, and endothelium. A common feature about the activity of sirtuins as fuel-sensing molecules is their dependence on

intracellular levels of nicotinamide adenine dinucleotide (NAD) in its oxidized (NAD^+) or reduced form (NADH). Seven human sirtuin isoforms (SIRT1–7) were identified. SIRT1 has been found to enhance insulin sensitivity and secretion, decrease oxidative stress and inflammatory activity, and help in glucose and lipid metabolism (Silva and Wahlested, 2010). AMPK is a fuel-sensing enzyme that is activated by a decrease in a cell's energy state as reflected by an increased AMP/ATP ratio and/or ADP/ATP ratio. AMPK plays a key role in many physiological processes as homeostasis of glucose/lipid, insulin signaling, body weight, food intake, and mitochondrial biogenesis and it is a big therapeutical player in many metabolic diseases such as diabetes or obesity, or tumorigenesis (Liang *et* al., 2007; Kim *et* al., 2016). There are some similarities between AMPK and SIRT1 since AMPK and SIRT1 have a regulatory impact on each other and share many common target molecules (Ruderman *et* al., 2010; Kutinová Canová *et* al., 2012; Farghali *et* al., 2013; Hubbard *et* al., 2014) (**Fig. 1**). Therefore, we focused our research work on these two molecules and their signaling pathways.

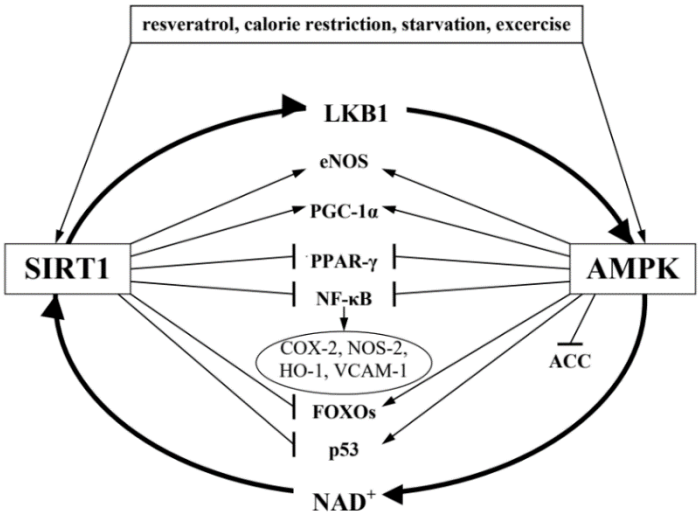


Figure 1. Proposed molecular mechanisms by which SIRT1 and AMPK activate each other and control other regulatory factors associated with metabolism and inflammation (→ activation, ⊥ inhibiton). ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; eNOS (NOS-1), endothelial nitric oxide synthase; FOXOs, forkhead box-containing proteins; HO-1, inducible heme oxygenase; LKB1, liver kinase B1; NAD+, nicotinamide adenine dinucleotide; NOS-2, inducible nitric oxide synthase; PPAR- γ, peroxisome proliferator-activated receptor-γ; PCG-1α, PPAR- γ coactivator-1α; NF-κB, nuclear factor kappa-B; SIRT1, silent information regulator T1; VCAM-1, vascular cell adhesion molecule-1 (Farghali *et* al., 2013).

2. Aims and hypothesis

2.1 Aims

The main goal of the study was to investigate the relationship of SIRT1 and AMPK in the process of hepatotoxicity/hepatoprotection in *in vivo* and *in vitro* animal model of acute drug-induced liver injury.

Objectives:

- To evaluate possible hepatoprotective effect of a natural polyphenolic compound resveratrol and synthetic SIRT1 activator and inhibitor in experimental *in vitro* and *in vivo* models of drug (D-galactosamine/lipopolysaccharides, acetaminophen)-induced hepatotoxicity and to discuss the role of SIRT1 modulation in hepatoprotection.
- To assess in more detail interconnection between SIRT1 and AMPK in primary hepatocytes and to determine whether modulation of SIRT1 and AMPK activity by their synthetic activators and inhibitors can alleviate APAP-induced hepatocyte damage *in vitro*.
- To achieve the above objectives, it was necessary to introduce *in vitro* and *in vivo* rat models of DalN/LPS and paracetamol-induced hepatotoxicity, Western blot method determining the expression of target peptides and brand new SIRT1 deacetylase activity and caspase-3 ELISA assays under experimental conditions at the Institute of Pharmacology 1. LF UK.

2.2 Hypothesis

Liver diseases represent significant cause of morbidity and mortality in man worldwide (Asrani *et* al, 2019). Many herbs have been used to alleviate various liver diseases (Bhaargavi *et* al., 2014). Previous experimental studies, both *in vivo* and *in vitro*, demonstrated that resveratrol is effective in protecting hepatocytes against D-galactosamine/lipopolysacharide-induced hepatotoxicity (Černý et al, 2009; Farghali *et* al., 2009). Resveratrol, polyphenolic compound found in a plant source, could play a key role in cellular physiology in many ways. It supports mitochondrial biogenesis and participates in metabolism through activation of SIRT1 which

can stimulate AMPK. Several reports showed that SIRT1 and AMPK share similar molecular pathways, and activation of SIRT1 by resveratrol could be a consequence of AMPK activation (Nogueiras *et* al., 2012). During our experimental study, we were therefore interested in the involvement of SIRT1 and AMPK as two possible important players in hepatoprotection (Kemelo *et* al., 2014; Farghali *et* al., 2019). We hypothesized that using selective activators and inhibitors of SIRT1 and AMPK in drug-induced hepatotoxic animal models, we would be able to uncover the role of these individual molecules in the process of hepatoprotection and better specify their mutual interconnection or, conversely, independent action.

3. Materials and methods

In our experiments (Kemelo, Wojnarová *et* al., 2014; Wojnarová *et* al., 2015; Njeka Wojnarová *et* al., 2022) we used resveratrol as natural bioactive compound and small synthetic molecules as follows:

- **CAY10591** (2-amino-N-cyclopentyl-1-(3-methoxypropyl)-1H-pyrrolo[2,3-b] quinoxaline-3-carboxamide, CAY, selective activator of SIRT1)
- **EX-527** (6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide; EX, SIRT1 inhibitor)
- **AICAR** (5-aminoimidazole-4-carboxamide ribonucleotide; AMPK activator),
- **Compound C** (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine; CC, AMPK inhibitor).

Hepatotoxicity was induced by single dose of D-galactosamine/lipopolysacharide (D-GalN/LPS) or acetaminophen (APAP) *in vivo* and/or *in vitro*. Some rats/cultured hepatocytes were treated by resveratrol and/or synthetic selective activator or inhibitor of SIRT1 and AMPK. Drug doses were based on a previous experimental studies (Farghali *et* al., 2009; Cerny *et* al., 2011; Lekic *et* al., 2013), literature and MTT/cell viability test (Wojnarová *et* al., 2015; Njeka Wojnarová *et* al., 2022).

1. ***In vivo* experiments** included rat model of D-GalN (400 mg/kg)/LPS (10 μg/kg) and APAP (1g/kg)-induced liver injury affected by intraperitoneal application of resveratrol (2.3 mg/kg; 30 mg/kg) and SIRT1 inhibitor (EX-527, 1 mg/kg) or SIRT1 activator (CAY10591, 0.5 mg/kg).

2. **Primary rat hepatocyte isolation for *in vitro* experiments and treatments.** Hepatocytes were isolated from 12-14 week old untreated Wistar rats using two phase collagenase perfusion method. Cells were seeded on collagen-coated polystyrene Nunclon™ dishes and treated with fresh complete medium alone or with APAP and/or resveratrol, SIRT1 and AMPK modulators at concentrations listed in the **Table 1**.
3. **Hepatocyte integrity** and function were assessed by customized kits as plasma and medium alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin levels.
4. **Determination of** sample nitrite (NO_2^-), the stable end-product of nitric oxide (NO) oxidation, was detected spectrophotometrically by using Griess reagent.
5. **Evaluation of markers of lipidperoxidation**. The measurement of thiobarbituric acid reacting substances (TBARS) and conjugated dienes (CD) in liver or hepatocyte homogenates was done.
6. **MTT/cell viability test** was used both to assess the optimal non-toxic concentration of some drugs and to measure cell viability at the end of *in vitro* experiments.
8. **Caspase-3 ELISA commercial assay** (LifeSpan BioSciences, Inc, Seattle, USA) was used for detection of caspase-3 proenzyme in hepatocyte lysates.
9. **Histological evaluation –** liver sections were stained with hematoxylin and eosin and examined by light microscope.
10. **Optimalized Western blot analysis** of cell and tissue lysates was used to detect SIRT1, phosporylated and total AMPK proteins.
7. **SIRT1 deacetylase activity** was evaluated in liver and cell lysates according to company instruction of fluorometric SIRT1 Assay Kit (Sigma-Aldrich)**.**
11. **Data sampling and statistical analysis.** Data were expressed as means ± SEM (standard error of mean). All experiments were performed in means of 6 animals per group for *in vivo* experiments and at least of 3 independent *in vitro* experiments (Kemelo, Wojnarová et al., 2014; Wojnarová et al., 2015; Njeka Wojnarová *et* al., 2022). The significance of differences between the groups was assessed by one-way analysis of variance (ANOVA) followed by Turkey-Kramer or Bonferroni multiple comparison test (Graph-Pad Prism 4.03, Graph Pad Software, San Diego, CA, USA). P-value less than 0.05 was reckoned to indicate a statistically significant difference.

Table 1. Concentration of drugs used in primary rat hepatocyte cultures.

Drug Treatment	**Concentration**
APAP (acetaminophen)	5 or 12.5 mM
Resveratrol (RES)	20 μM
AICAR (AMPK activator)	50 μM
CC ("Compound C" – AMPK inhibitor)	10 μM
CAY10591 (SIRT1 activator)	30 μM
10 μM EX-527 (SIRT1 inhibitor)	10 μM

4. Results

In our first study (Kemelo, Wojnarová *et* al., 2014) we investigated and discussed the role of SIRT1 in D-GalN/LPS model of acute liver failure. Treatment of animals with D-GalN/LPS induced liver injury by a significant increase in transaminases and bilirubin levels (**Fig. 2**) and oxidative stress markers (**Fig. 3**) relative to the negative control groups. There was over 20-fold increase in ALT levels and slightly less with AST and bilirubin. Furthermore, we repeatedly confirmed the hepatoprotective effect of resveratrol (RES). Resveratrol pretreatment attenuated D-GalN/LPS-induced hepatotoxicity, significantly reduced all observed markers of liver damage (ALT, AST, bilirubin) and oxidative stress (conjugated dienes, TBARS). EX-527, on the other hand, blocked the effects of resveratrol and significantly increased the ALT and bilirubin levels. These finding provides a clear indication that the catalytic activity of SIRT1 is required for the cytoprotective effects of resveratrol. In addition, D-GalN/LPS treatment was able to dramatically decrease of SIRT1 protein levels in liver tissue (**Fig. 3**) (Kemelo, Wojnarová *et* al., 2014).

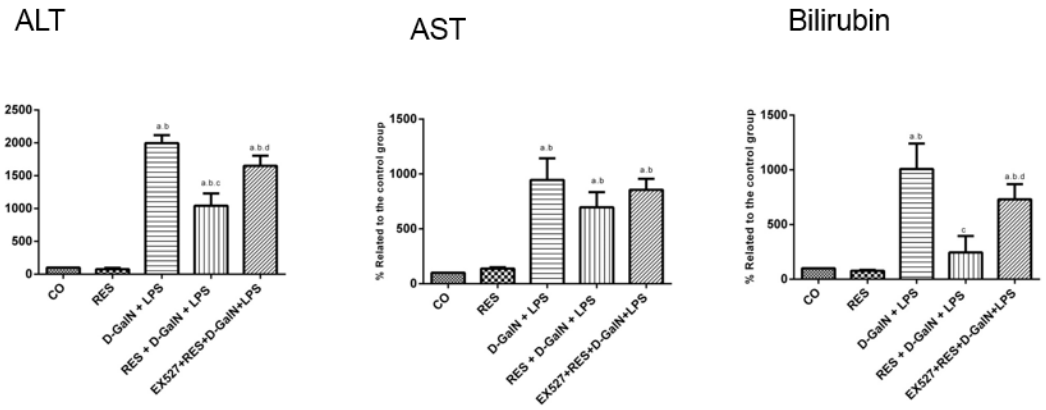


Figure 2. *Data are expressed as means ± SEM (n=6). [a]P<0.05 versus CO, [b]P<0.05 versus RES, [c]P<0.05 versus D- D-GalN+LPS, [d]P<0.05 versus RES+D-GalN+LPS.*

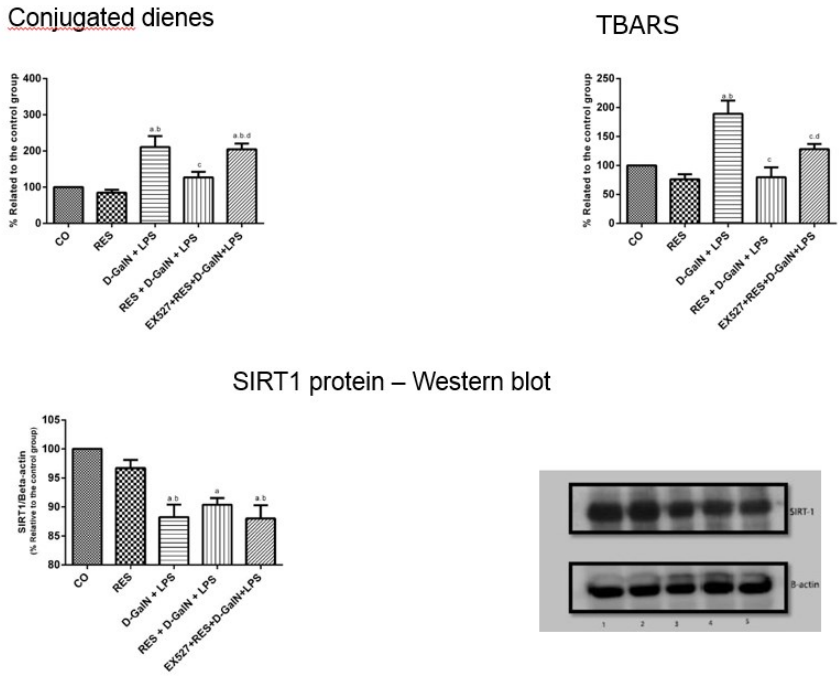


Figure 3. *Conjugated dienes and TBARS: Data are expressed as mean ± SEM (n=6). [a]P<0.05 versus CO (control), [b]P<0.05 versus RES (resveratrol), [c]P<0.05 versus D-GalN+LPS, [d]P<0.05 versus RES+D-GalN+LPS.*

Western blot: Data are expressed as mean ± SEM (n=6). [a]P<0.05 versus CO, [b]P<0.05 versus RES. (b) Representative Western blot images lanes: 1) CO, 2) RES, 3) D-GalN+LPS, 4) RES+D-GalN+LPS, 5) EX-527+RES + D-GalN+LPS.

Our second study (Wojnarová *et* al., 2015) described that APAP significantly increased ALT and total bilirubin levels with the same trend *in vivo* as *in vitro*. RES and CAY were administrated after pretreatment with APAP, and both RES and CAY slightly attenuated APAP-induced hepatotoxicity both *in vivo* and *in vitro* **(Figs. 4 and 5)**. Moreover, both drugs enhanced APAP reduced SIRT1 activity but not SIRT1 expression (**Fig. 5**). Histology evaluation of liver sections of APAP-treated rats revealed only slight increase in the appearance and number of apoptotic hepatocytes. Liver parenchyma had normal morphology after application of APAP followed by CAY or resveratrol to rats (*data not shown*).

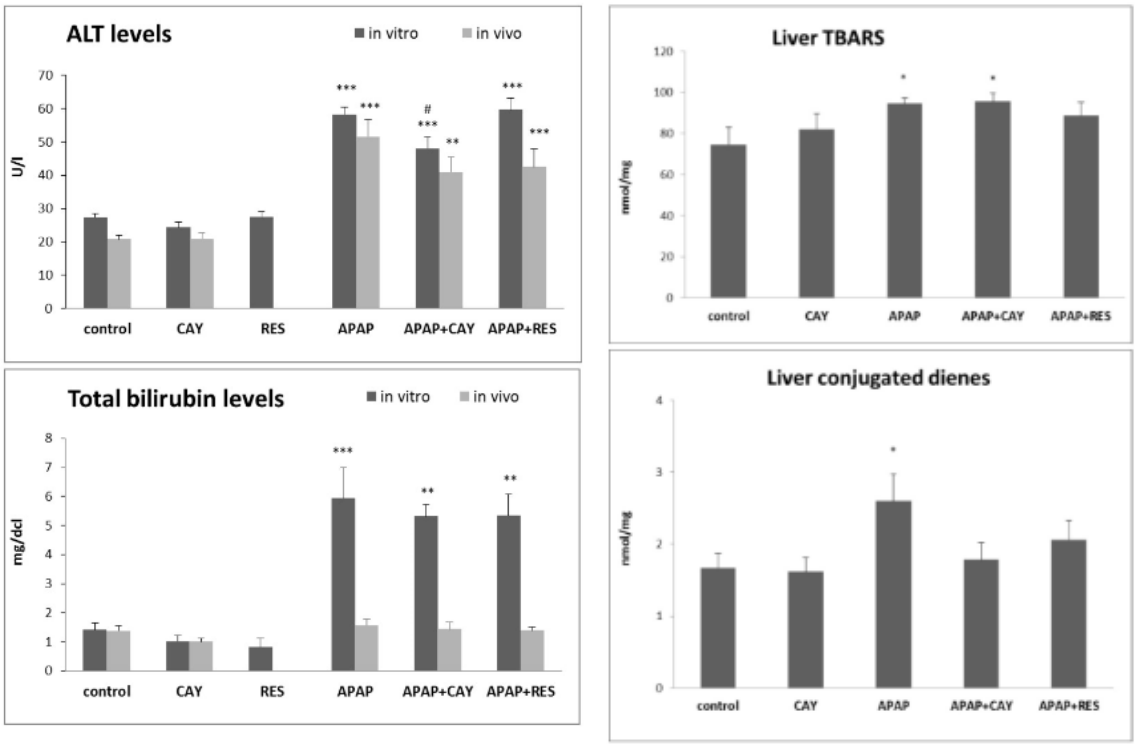


Figure 4. *Plasma or medium ALT and total bilirubin levels, liver TBARS and Conjugated dienes: Data are expressed as means ± SEM (n=6): * P<0.05, ** P<0.01, *** P<0.001 vs. respective control; # P<0.05 vs. APAP alone in vitro.*

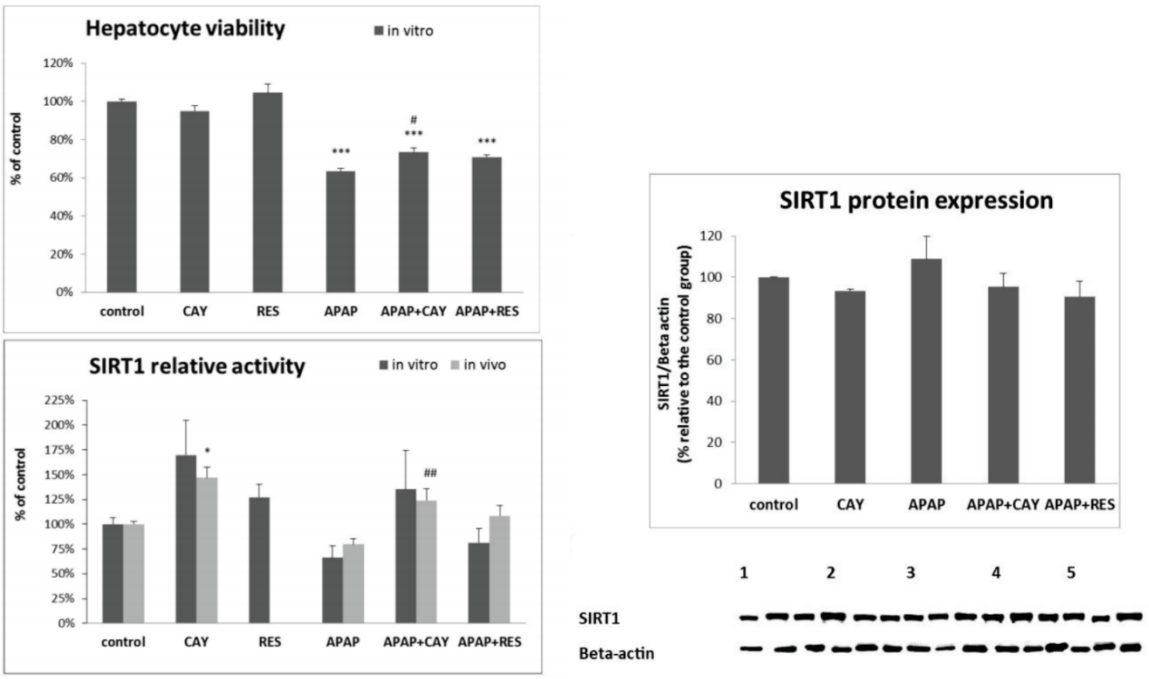


Figure 5. *Hepatocyte viability, SIRT1 relative activity: Data are expressed as means ± SEM (n=6 for MTT test and in vivo SIRT1 activity, n=3 for in vitro SIRT1 activity): * P<0.05, *** P<0.001 vs. respective control; # P<0.05, ## P<0.01 vs. respektive APAP group.*
SIRT1 protein expression: Data are expressed as means ± SEM (n=3). b) Western blot images are shown as three samples of each treated group: 1. Control, 2. CAY, 3. APAP, 4. APAP+CAY, 5. APAP+RES.

Our third study (Njeka Wojnarová *et* al., 2022) demonstrated that the toxic effect of APAP on primary rat hepatocytes in drug-induced liver injury (**Figs. 6 and 7**) is associated with significantly reduced AMPK activity (**Fig. 8**), SIRT1 activity and protein expression (**Fig. 9)**, and increased oxidative stress. Our experiments have shown that the AMPK activator (AICAR) does not alleviate the potent hepatotoxic effect of acetaminophen (APAP) whereas administration of AMPK inhibitor (Compound C, CC) significantly aggravated APAP toxicity. On the contrary, the addition of AICAR or SIRT1 activator (CAY10591) significantly suppressed the negative hepatotoxic effects of the combination of APAP+CC. In addition, AICAR in contrast to CAY10591 did not attenuate the toxic action of APAP in combination with SIRT1 inhibitor (EX-527) (Njeka Wojnarová *et* al., 2022).

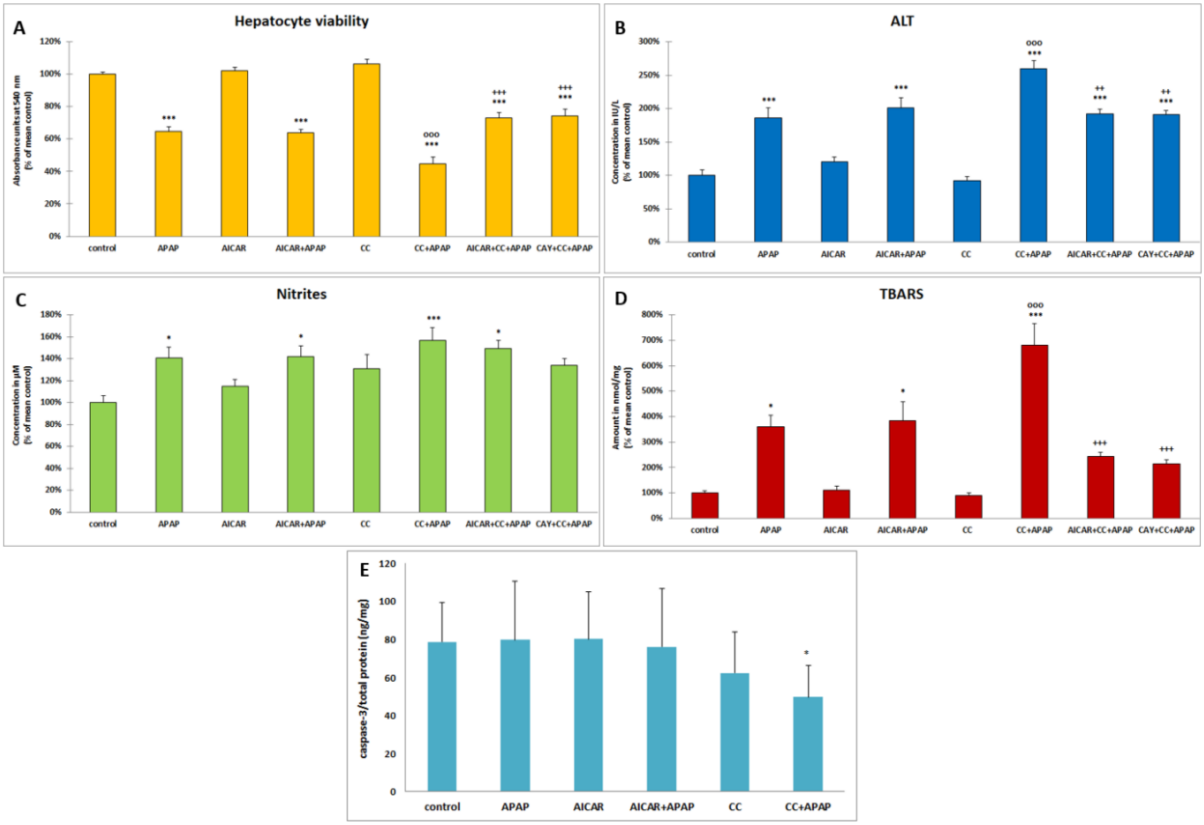


Figure 6. *Data are expressed as means ± SEM (n = 7-16 for A-D and n = 3 for E): *P < 0.05, **P < 0.01, ***P < 0.001 vs. control; ^{ooo}P < 0.001 vs. APAP; $^{++}$P < 0.01, $^{+++}$P < 0.001 vs. CC+APAP combination.*

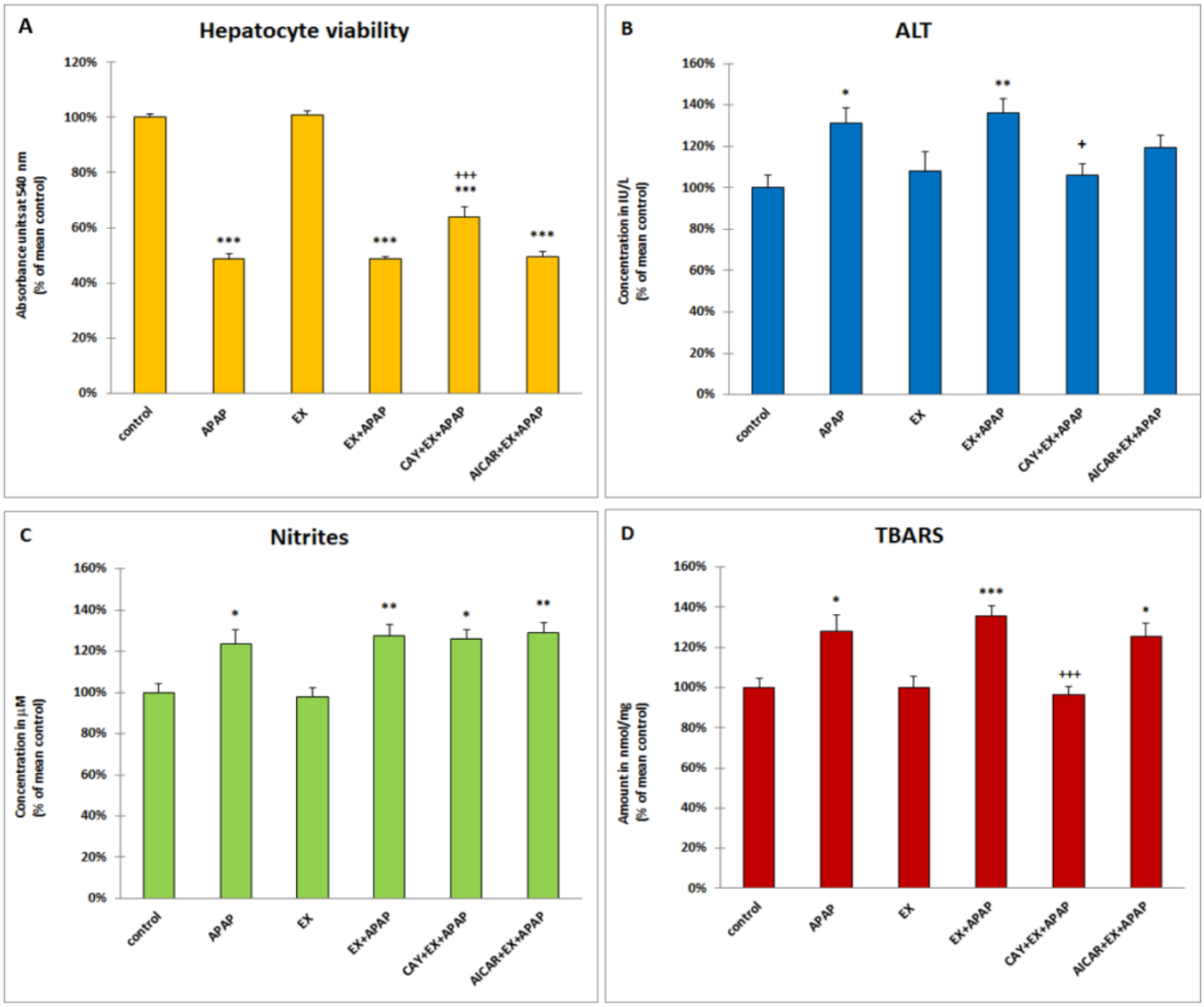
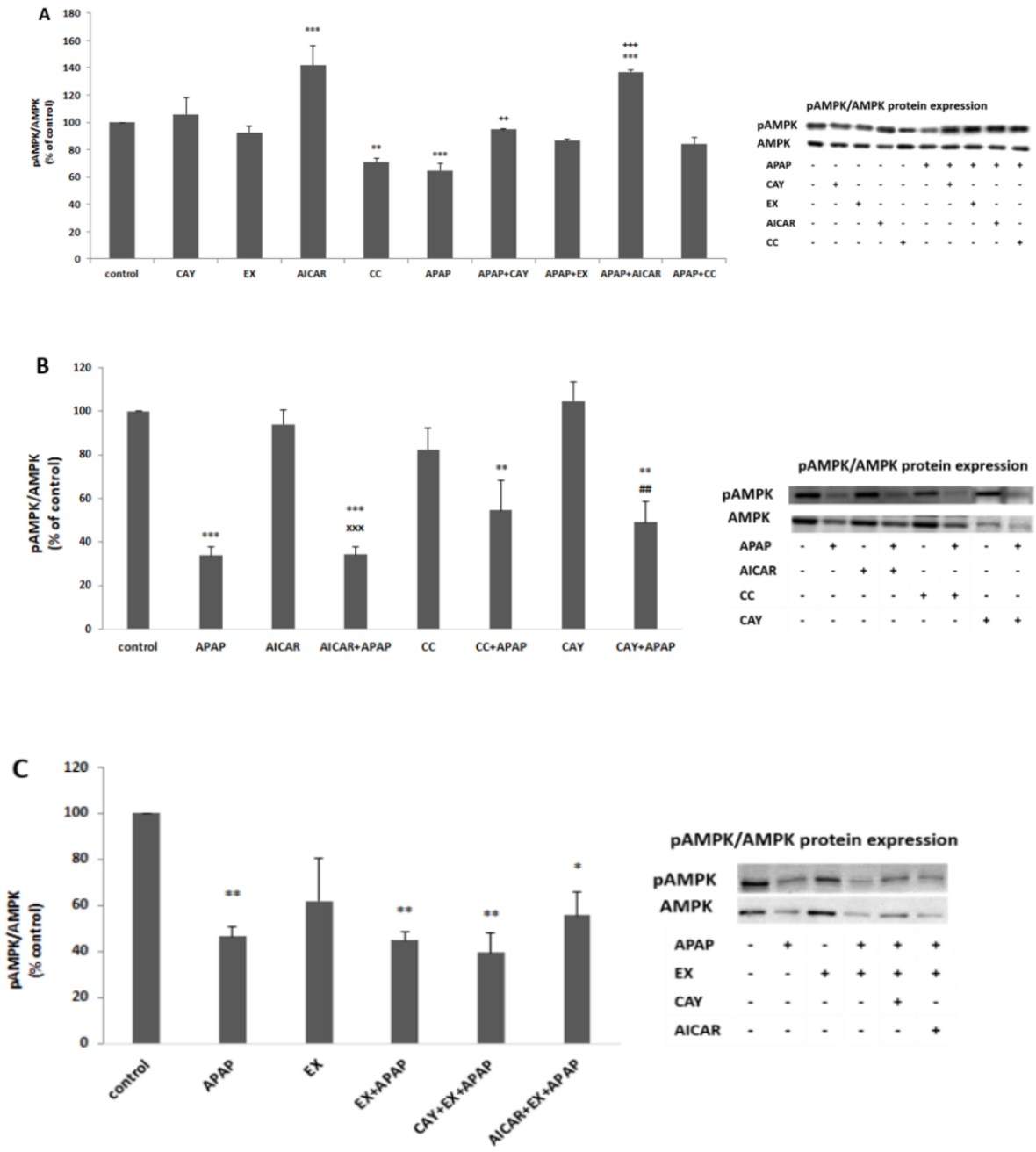


Figure 7. *Data are expressed as means ± SEM (n = 9-16): *P < 0.05, ** P < 0.01, ***P < 0.001 vs. respective control; $^{+}P < 0.05$, $^{+++}P < 0.001$ vs. EX+APAP combination.*

Figure 8 - the picture follows on the next page. *Effects of acetaminophen (APAP), specific modulators of AMPK (activator – AICAR and inhibitor - Compound C, CC) and SIRT1 (activator - CAY10591/CAY and inhibitor EX-527/EX) on AMPK activity in cultured primary rat hepatocytes after 4 hours (A) and 24 hours (B, C). Activity of AMPK was calculated as pAMPK/AMPK ratio of protein expression. Quantitative data of optical band densitometry (graphs) and representative Western blot images are presented. Data are expressed as means ± SEM (n = 3-5): *P < 0.05, **P < 0.01, ***P < 0.001 vs. respective control; $^{+}P < 0.05$; $^{++}P < 0.01$, $^{+++}P < 0.001$ APAP in combination vs. APAP alone; $^{XXX}P < 0.001$ vs. AICAR; $^{\#\#}P < 0.01$ vs. CAY.*



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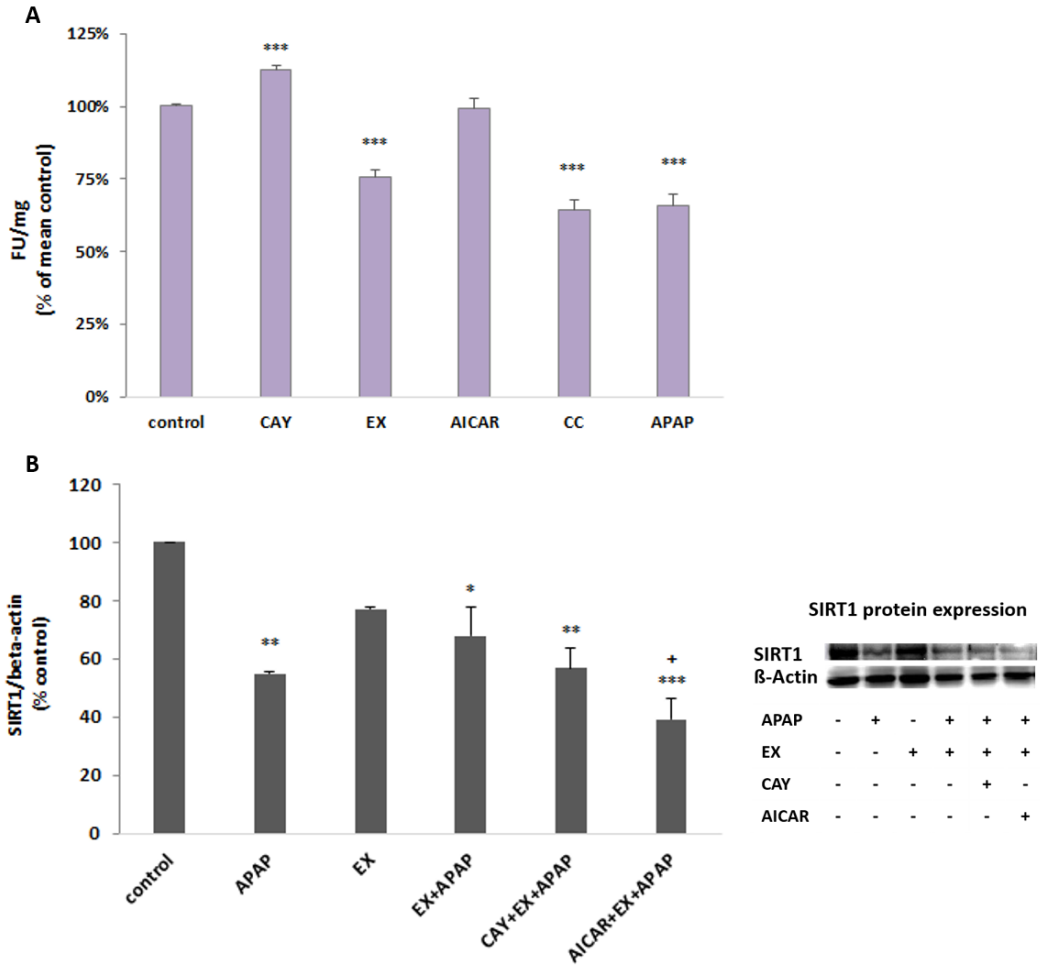

Figure 9. *A) SIRT1 activity after 4 hours, and B) SIRT1 protein expression after 24 hours; both in cultured primary rat hepatocytes. Quantitative data of fluorescence activity (A) and optical band densitometry (B) are expressed in graphs as means ± SEM (n = 5 and 3, respectively): **P < 0.01, *P < 0.05, ***P < 0.001 vs. control; $^{+}$P < 0.05 vs. EX+APAP. Representative Western blot image is also presented.*

5. Discussion

In most cases, liver diseases are associated with inflammation and oxidative stress that can lead to the destruction of liver parenchyma, fibrosis, and cirrhosis with loss of liver function (Del Campo *et* al., 2018; Wang *et* al., 2020). Many studies observed that oxidation stress has been presented in many liver diseases however therapeutical interventions to reactive oxygen species (ROS) show limited clinical efficiency (Ramachandran *et* al., 2018). Earlier study of our research group have shown that less specific SIRT1 activator, resveratrol, can effectively attenuate acute liver injury, as demonstrated by the quantification of serum ALT/AST levels, oxidative stress parameters, and via histological examination (Farghali *et* al., 2009).

In our first presented study we used D-Galactosamine (D-GalN) and lipopolysaccharide (LPS) model of acute liver injury (Kemelo, Wojnarová *et* al., 2014). Various dose combinations of LPS and D-GalN were used to produce sublethal liver failure which is relevant to clinical situations in viral, drug or alcohol-induced, immune-induced or ischemia reperfusion hepatitis. We reported that treatment of animals with D-GalN/LPS was able to induce hepatotoxicity as evidenced by a significant increase in transaminases and bilirubin levels together with elevated markers of oxidative stress and lipid peroxidation (TBARS, conjugated dienes). Moreover, resveratrol alleviated hepatotoxicity in all parameters. In addition, D-GalN/LPS-induced hepatotoxicity downregulated SIRT1 protein expression in rat liver. The precise mechanism by which D-GalN/LPS treatment represses SIRT1 expression was not investigated. However, several studies suggest that generation of ROS plays a key role in the cytotoxic effects of this model. Some prevoius studies (Yamakuchi, 2012; Choi and Kemper, 2013) have shown that microRNAs such as miR-34a can downregulate SIRT1 expression in response to oxidative stress and therefore augment liver damage. Our data suggest that SIRT1 expression is decreased to some extent by the degree of oxidative stress (Kemelo, Wojnarová *et* al., 2014).

For our further two studies, we chose acute rat APAP intoxication (*in vivo* and *in vitro*) followed or preceded by SIRT1 and/or AMPK modulators to investigate their connection in process of hepatoprotection or hepatotoxicity. The liver impairment was much lower after APAP treatment than after LPS/D-GAlN and did not lead to fulminant hepatic failure (Wojnarová *et* al., 2015). APAP-induced oxidative stress and mitochondrial dysfunction plays the central role in the pathogenesis of acute APAP-induced liver injury (Ramachandran and Jaeschke, 2019). We used this model of mild hepatic impairment because it more resembles the human APAP-

induced liver injury with potentially following pharmacological intervention (Wojnarová *et* al., 2015). Despite it was shown that rats are resistant to APAP hepatotoxicity in compare to mice (hepatotoxic dose is 400-600 mg/kg for mice with contrast of 1-2 g/kg for rats) (McGill *et* al., 2012). Some recent publications found that human hepatocytes are much more resistant to APAP toxicity than hepatocytes originated from other species (Wojnarová *et* al., 2015).
Our results also reported that the cell death induced by APAP is related to necrosis rather than apoptosis because total caspase-3 was not increased (Njeka Wojnarová *et* al., 2022) which is in line with comments in publications of Ramachandran and Jaeschke (2019), Jaeschke and Ramachandran (2020), describing that not apoptosis but oncotic necrosis is a relevant mode of cell death during APAP-induced liver injury both *in vitro* and *in vivo*.

For investigation the role of SIRT1 and AMPK in hepatoprotection we used combination of small synthetic molecules - CAY10591 (CAY, activator of SIRT1), EX-527 (EX, SIRT1 inhibitor), AICAR (AMPK activator), and Compound C (CC, AMPK inhibitor) and evaluated effects of their original mutual combinations in primary rat hepatocytes cultured with APAP. We revealed that pretreatment with either AMPK activator AICAR (aminoimidazole-4-carboxamide riboside, adenosine analogue that selectively activates AMPK) or SIRT1 synthetic activator CAY10591 significantly increased the AMPK activity already after 4 hours of hepatocyte incubation with APAP. However, AMPK activity was not influenced or only slightly increased with AICAR and CAY10591, respectively, after 24 hours. One of explanations of this time-dependent trend could be short half-life of AICAR in cells and maybe also similar for CAY10591 (Njeka Wojnarová *et* al., 2022). The explanation for CAY10591-enhanced AMPK activation could be that SIRT1 deacetylates the AMPK kinase LKB1 (liver kinase B1), leading to increased increased phosphorylation and activation of AMPK (Hou *et* al., 2008). Another of the important and shared targets is the nuclear factor kappa B (NF-κB), a key mediator of proinflammatory signaling pathways triggered by cytokines. The relationship between NF-κB and SIRT1 is antagonistic, decreased nuclear SIRT1 level/activity increases NF-κB RelA/p65 activity and amplifies proinflammatory gene expression as reported by Wang *et* al. (2020) and De Gregorio et al. (2020). Interestingly, this relationship was confirmed in publication of Rada et al. (2018) which revealed that *in vivo* administration of the NF-κB inhibitor protected from APAP-mediated acute hepatotoxicity (Price *et* al., 2012; Scudiero *et* al., 2016; Njeka Wojnarová et al., 2022).

Compound C, also known as dorsomorphin, is the only small AMPK inhibitor that has been widely used to study AMPK signaling pathway. Our experiments have shown that AMPK inhibitor, CC, significantly amplified APAP-induced hepatotoxic effect in all observed parameters. Interestingly, AICAR and CAY10591 pretreatments lowered the hepatotoxic and pro-oxidative effect of APAP+CC combination (Njeka Wojnarová *et* al., 2022).

To investigate what and how important role SIRT1 plays in the process of hepatoprotection we performed also experiments with EX-527. EX-527 is widely used as a SIRT1 inhibitor both *in vitro* and *in vivo* with high potency and significant isoform selectivity.
Our results implied that pretreatment with EX-527 only slightly enhanced APAP toxicity (Njeka Wojnarová *et* al., 2022). Our Western blot data showed that EX-527 down-regulated SIRT1 expression. Besides that, combination of SIRT1 inhibitor and APAP treatment slightly aggravated SIRT1 protein levels regardless addition of SIRT1 activator – CAY and especially AMPK activator – AICAR. Above that, addition of CAY10591 significantly decreased the toxic effect of combination EX+APAP suggesting that primarily a change in catalytic activity rather than SIRT1 protein expression plays a role in the hepatoprotective action of SIRT1 against APAP-induced hepatotoxicity. We hypothesized that addition of AICAR in combination of EX-527+APAP could increase expression of SIRT1 protein which was not confirmed. Despite downregulation of SIRT1 expression, administration of this combination (e.g. AICAR+EX+APAP) was not accompanied by worsening of hepatotoxicity, nonetheless it would be interesting to determine whether decreased SIRT1 expression was preceded by its increased activity induced by AICAR as negative feedback loop. One of the possible mechanisms how the AMPK could activate the SIRT1 is through an indirect increase in cellular NAD^+ levels. Furthermore, the question that arises, is whether some *in vivo* protective effect is not paradoxically associated with the sirtuin inhibitor EX-527 as mentioned in Huang et al. (2017). They revealed that *in vivo* treatment with the selective SIRT1 inhibitor EX-527 alleviated LPS-induced systemic inflammation and acute lung injury. Their results indicated that the anti-inflammatory effects of the SIRT1 inhibitor might be partially attributed to the suppression of mTOR (Price *et* al., 2012; Huang *et* al., 2017; Njeka Wojnarová *et* al., 2022).

Although our preliminary results (Wojnarová *et* al. 2015) showed no significant changes on the SIRT1 protein expression in APAP model of liver injury compared with reduced SIRT1 enzyme activity after APAP treatment, our later experiments with increased dose of APAP revealed association of the hepatotoxic effect of APAP with simultaneous decrease in SIRT1 activity

and protein expression and enhanced oxidative stress and hepatocytes damage *in vitro* (Njeka Wojnarová *et* al.,2022). This relationship was confirmed also in publication of Rada *et* al. (2018). After APAP overdosing, the levels of SIRT1 was decreased in both human and mice hepatocytes. There was shown a mouse which kept higher level of SIRT1 after APAP injection was more protected against hepatotoxicity due to antioxidants system and restrained inflammatory responses (Rada *et* al., 2018).

All these events and new findings give an impression that the cytoprotective effects of SIRT1 occur within a limited range of its expression. The catalytic activity of SIRT1 is equally important in the hepatoprotective effects of SIRT1 modulators (Farghali *et* al., 2019).
As the results above illustrated AMPK and SIRT1 signaling possess a close relationship in liver injury and it might be evidence of complicated interaction of these two signaling pathways. Moreover, pharmacologic modulation of SIRT1 and AMPK could be a future major step in the treatment of DILI (Njeka Wojnarová *et* al., 2022).

6. Conclusion

According to our results, downregulation of SIRT1 protein expression is involved in the cytotoxic effects of D-GalN/LPS model and SIRT1 activity contributes to the cytoprotective effects of resveratrol in the liver. Similarly, resveratrol and specific SIRT1 activator, CAY10591 (CAY), attenuates APAP-induced hepatotoxicity *in vivo* and in *vitro*. The toxic effect of acetaminophen (APAP) on primary rat hepatocytes is associated with significantly reduced AMPK activity, SIRT1 activity and protein expression, and increased oxidative stress. Our experiments have shown that the AMPK activator (AICAR) does not alleviate the potent hepatotoxic effect of APAP whereas administration of AMPK inhibitor (Compound C, CC) significantly aggravated APAP toxicity. On the contrary, the addition of AICAR or SIRT1 activator (CAY10591) significantly suppressed the negative hepatotoxic effects of the combination of APAP+CC. In addition, AICAR in contrast to CAY10591 did not attenuate the toxic action of APAP in combination with SIRT1 inhibitor (EX-527). Taken together, our results from *in vitro* experiments suggest that hepatoprotective effects of SIRT1 against APAP toxicity could be at least partially independent of AMPK activity.
Thus suggesting modulation of SIRT1 and AMPK activity by synthetic small molecules with higher pharmacologic and specific potency compared with natural polyphenolic compounds could provide an interesting and novel therapeutic option for hepatocyte injury in the future.

7. References

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8. List of Author's publications

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- Kemelo MK*, **Wojnarová L***, Kutinová Canová N, Farghali H. D-galactosamine/lipopolysaccharide-induced hepatotoxicity downregulates sirtuin 1 in rat liver: role of sirtuin 1 modulation in hepatoprotection. Physiol Res. 2014;63(5):615-23; IF: 1.88 (2021-2022). * *The authors contributed to the publication to the same extent.*

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