Dissertation Thesis

PHARMACOLOGICAL PERSPECTIVES AND CLINICAL BENEFITS OF SIRT1 AND AMPK ACTIVATORS AND INHIBITOS IN INFLAMMATORY AND OXIDATIVE STRESS IN THE LIVER

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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

Dissertation Thesis

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Prague 2022

Affidavit

I hereby declare that the thesis with title 'Pharmacological perspectives and clinical benefits of SIRT1 and AMPK activators and inhibitors in inflammatory and oxidative stress in the liver' has been composed by myself autonomously. All materials and results that are not original to this work have been fully cited and referenced. This thesis has not been handed in or published before in the same or similar form except of the part describing the effect of D-galactosamine/lipopolysachcaride on sirtuin 1 expression in rats that we shared with MUDr. Mighty Kgalalelo Kemelo, Ph.D. because we contributed to this research to the same extent (Kemelo, Wojnarová *et* al., 2014).

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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).

Obsah

1 List of Author's publications

Publications in impacted journals

- 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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2 List of abbreviations

ACC	Acetyl-CoA carboxylase
AICAR	Analog of adenosine monophosphate
ALF	Acute liver failure
ALT	Alanine aminotransferase
AMPK	Adenosine 5´-monophosphate-activated protein kinase
ANOVA	One-way analysis of variance
APAP	Acetaminophen
APAP-SG	Acetaminophen-glutathione conjugate
AST	Aspartate aminotransferase
CAY10591	Selective sirtuin 1 activator
CC	Compound C, AMPK inhibitor
CD	Conjugated dienes
ChREBP	Carbohydrate-response element-binding protein
D-GalN	D-Galactosamine
DILI	Drug-induced liver injury
DMSO	Dimethyl sulfoxid
DNA	Deoxyribonucleic acid
EX-527	Selective sirtuin 1 inhibitor
FOXO	Forkhead box O
HCC	Hepatocellular carcinoma
HO-1	Heme oxygenase-1
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase (also NOS-2)
JNK	c-Jun N-terminal kinases
LKB1	Liver kinase B1
LPS	Lipopolysaccharide
MPT	Permeability transition pores
NAD^+	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NAPQI	*N*-acetyl-*p*-benzoquinone imine

NF-κB	Nuclear factor-kappa B
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator-1 alpha
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
RES	Resveratrol
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SIRT1	Silent information regulator T1 or sirtuin 1
STACs	SIRT1 activating compounds
SREBP-1c	Sterol regulatory element-binding protein 1
TBARS	Thiobarbituric acid reactive substances
TNF-α	Tumor necrosis factor alpha

3 Introduction

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).

3.1 Liver

The liver is the largest glandular organ in the body, accounting for approximately 2 % to 3% of average body weight with many indispensable functions (Abdel-Misih and Bloomston, 2010). It plays a major role in numerous physiological processes. These include macronutrient metabolism, blood volume regulation, immune system support, endocrine control of growth

signaling pathways, lipid and cholesterol homeostasis. Hepatic lipid uptake and secretion is necessary for absorption of a number of lipid-soluble vitamins. It stores iron and copper. It plays a role in hematology with clotting factor and protein synthesis. In addition, liver is essential for biotransformation of majority of xenobiotics that enter the body, including many current drugs (Trefts, *et* al., 2017; Kalra *et* al., 2021).

3.2 Liver diseases

Liver diseases accounts for approximately 2 million deaths per year worldwide; 1 million due to complications of cirrhosis and 1 million due to viral hepatitis and hepatocellular carcinoma (HCC) (Asrani *et* al,. 2019). There are numerous causes for liver disease, including obesity with the consequent non-alcoholic fatty liver disease (NAFLD), excessive chronic alcohol consumption, immune and cholestatic disorders, inherited metabolic disorders, numerous medications, hemochromatosis, schistosomiasis, fungi infections. Liver disease is a considerable health burden across Europe (Farghali *et* al., 2015; Asrani *et* al. 2019).

3.3 Drug-induced liver injury

Liver injury belongs to the reason for black box warnings, drug non-approval or removing of approved drug from the market and it remains the most common cause of acute liver failure (ALF) in the western world (Katarey and Verma, 2016; Rada *et* al., 2018; McGill and Jaeschke, 2019). Moreover, in preclinical studies, about 50% of candidate compounds present hepatic effects at supra-therapeutic dose and face drug attrition (Chen *et* al., 2015). Population-based studies estimate the incidence to vary between 13.9–19.1 cases per 100,000 people per year (Katarey and Verma, 2016). Drug-induced liver injury (DILI) is classified as either predictable or unpredictable (idiosyncratic). Unfortunately, most of the drugs are associated with no dose dependence idiosyncratic DILI. DILI can also mimic all forms of acute or chronic liver diseases such as acute hepatitis, cholestasis and jaundice, nodular regenerative hyperplasia, or sinusoidal obstruction syndrome, which is often under-recognized due to the complexity of clinical manifestation (Yuan and Kaplowitz, 2013; Iorga *et* al., 2017). Furher classification of DILI could be as immune-mediated (allergic; with latency 1-6 weeks) or non-immune mediated (non-allergic; with latency 1 month – 1 year) (Katarey and Verma, 2016). Multiple risk factors have been found to be associated with an increased susceptibility to idiosyncratic DILI (Katarey and Verma, 2016).

The awareness of potential hepatotoxicity associated with alternative medicines such as herbal preparations and dietary supplements is increasing (European Association for the Study of the Liver, 2019).

3.4 Experimental hepatotoxic models in liver research

Successful development of new drugs and therapy for the liver diseases depends on the availability of *in vitro* (or *ex vivo)* and *in vivo* test model systems for hepatic injury (Farghali *et* al., 2016). Both models are used to evaluate hepatoprotective activity. These systems measure the ability of the drug to prevent or cure hepatic toxicity (induced by different hepatotoxins) in cellular cultures, organs or in experimental animals. Futhermore, oxidative stress plays a pivotal role in many human diseases, therefore, animal models involve reactive oxygen species (ROS) are widely used imitate human disease and to evaluate new therapeutic option (Muriel *et* al., 2017). Most hepatotoxic compounds are initiated by bioactivation of drugs to chemically ROS, which have the ability to interact with cellular macromolecules such as proteins, lipids (lipid peroxidation), nucleic acids (oxidative damage in the DNA), and to reduce of ATP leading to protein dysfunction (Delgado-Montemayor *et* al., 2015; Ahmad and Tabassum, 2012). It can even progress to cell death caused by apoptosis and necrosis.
Drug-induced hepatotoxicity involves metabolic activation of multiple cell types, and perturbation of biochemical pathways involving both hepatocytes and resident macrophages (i.e. Kupffer cells) (Rose *et* al., 2016). Since there are limitations of the outcomes in each model, it is important to combine different methods for confirmation of the findings (Farghali *et* al., 2016).

There are few different preclinically drug-induced hepatotoxicity models to study:

3.4.1 *Ex vivo* and *in vitro* models

In vitro models and *ex vivo* hepatotoxicity methods are routinely used to evaluate hepatotoxicity of drugs/chemicals/bioactive compounds to understand the mechanism(s) and to establish their correlation with *in vivo* hepatotoxicity (Ingawale *et* al., 2014).

a) Fresh isolated hepatocyte in suspension, primary cell cultures, and immortalized cell lines

Cultured liver cells represent the most frequent and best option for the screening and selection of potential hepatoprotective compounds and it is possible to establish action mechanisms at a cellular and molecular level (sberg *et* al., 2002; Delgado-Montemayor *et* al., 2015). These models usually consist of isolated hepatocytes and have been established as valid *in vitro* toxicological models for many years (Farghali *et* al., 2016).

A significant disadvantage of hepatocyte cultures is the absence of organ-specific cell-to-cell interactions (Groneberg *et* al., 2002). Normally in the body, the cells of the liver are organized around the functional structural unit of the liver — the lobule. This consists of chords of hepatocytes organized in a typically hexagonal shape around the central vein. This organization, termed 'metabolic zonation', leads to formation of a number of gradients including different amount of oxygen, hormones, nutrients, and waste products with different metabolic function, metabolic gene expression and functionality (**Fig.1**). Absence of this zonation in cultured hepatocytes affects drug-metabolizing enzymes such as some cytochrome P450 isoenzymes, NADPH-cytochrome c reductase or UDP-glucuronyl transferase enzyme (Trefts *et* al., 2017; Groneberg *et* al., 2002).

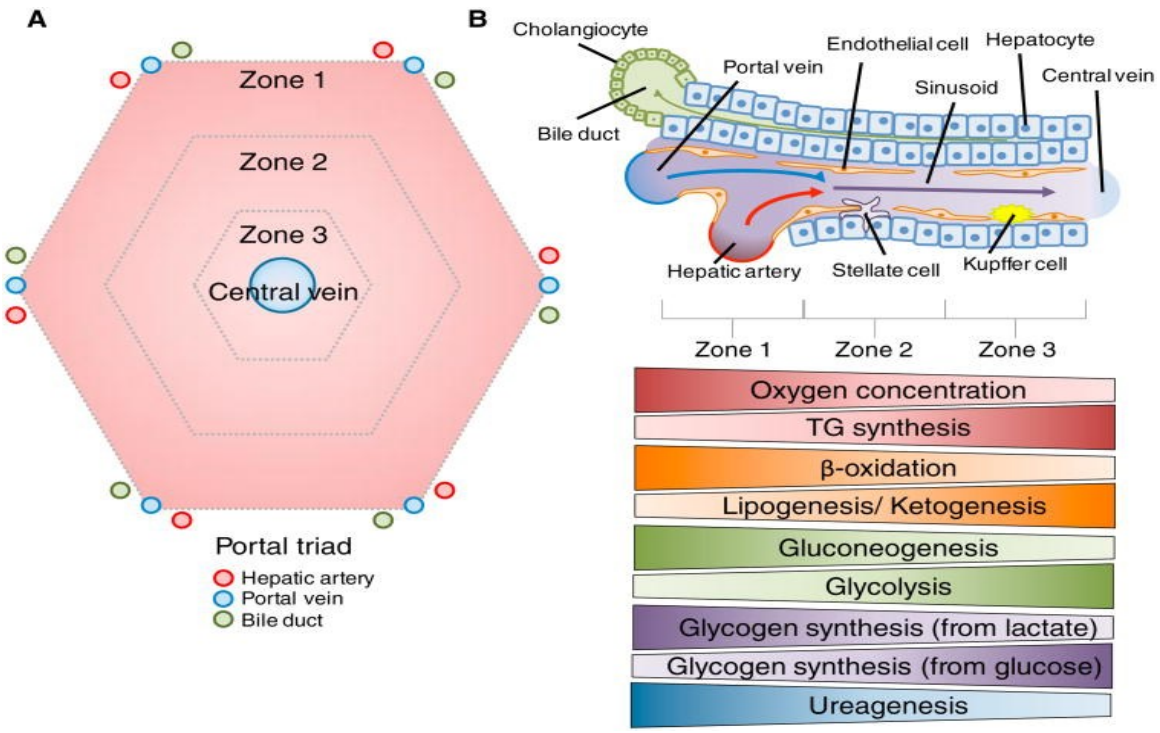

Figure 1. Organization of the liver (Trefts *et* al., 2017)

b) Isolated perfused organs

This model combines *in vitro* characteristics under *in vivo* circumstances (Delgado-Montemayor *et* al 2015). The major advantages of the isolated perfused livers are the preservation of the 3-dimensional organ structure with all its cell-to-cell interactions and the possibility of real-time bile collection and analysis. The major disadvantages are short term studies (2-4 hours' maximum), demanding experimental technology (temperature, perfusate, oxygenation), loss of organ functions (loss of interactions between distinct organs, including metabolic activation, except liver) (Spielmann *et* al., 1998; Groneberg *et* al., 2002).

c) Precision cut liver slices

This model includes e*x vivo* tissue culture which imitates multicellular characteristics of *in vivo* organs (Delgado-Montemayor *et* al 2015). Although the main advantages are represented by the preservation of lobular structures in contrast to cell cultures and the possible application of biochemical and molecular biological methods in contrast to organ perfusions, the main disadvantages are based on the short viability and the missing bile collection (Groneberg *et* al., 2002).

3.4.2 *In vivo* models

In vivo studies are limited by animal ethical guidlines. A toxic dose or repeated doses of various hepatotoxic interventions are administered to induce liver injury in experimental animals (**Fig. 2**) (Farghali *et* al., 2016). The tested potentially hepatoprotective drug/bioactive compound is administered along with, prior or after the toxin treatment. Liver damage and recovery are assessed by quantifying serum marker enzymes (ALT, AST, GPT etc.), bilirubin, bile flow, histopathological changes and biochemical changes in liver (Ahmad and Tabassum, 2012).

a) Experimental model of D-Galactosamine/lipopolysaccharide-induced liver injury

D-Galactosamine (GalN)/lipopolysaccharide (LPS)-induced liver injury is well establish experimental model causing fulminant hepatic failure. The intraperitoneal application of LPS, the outer surface of Gram-negative bacteria, induces oxidative stress in which proinflammatory cytokines such as tumor necrosis factor (TNF) play an important role, e.g. in the pathogenesis of liver injury (Nakama *et* al., 2001; Hamesch *et* al., 2015; Farghali *et* al. 2016). D-GalN disrupts of uridine nucleotide synthesis in the liver resulting in the inhibition

of mRNA and protein synthesis and potentiates the acute toxicity of LPS. The toxicity mechanism of galactosamine causes increase of cell membrane permeability leading to enzyme leakage, enzyme liberation and an increase in intracellular Ca^{2+} concentration and cellular death. In addition, the cholestasis due to galactosamine could be caused by its damaging effects on canalicular membrane, bile ducts or ductules of hepatocytes (Ahmad and Tabassum, 2012; Delgado-Montemayor *et* al, 2015; Farghali *et* al. 2016). Various dose combinations of LPS and D-GalN are used to induce sublethal liver failure which is relevant to clinical situations in viral, drug, alcohol, immune or ischemia reperfusion-induced hepatitis.

b) Model of acetaminophen liver injury

Acetaminophen (N-acetyl-para-aminophenol, paracetamol, APAP) is the most commonly used relatively safe analgesic and antipyretic drug worldwide. However, in high doses, it can cause acute liver damage due to hepatic centrilobular necrosis. It is widely used as an example of DILI and it is frequently utilized as a model hepatotoxic drug to test the hepatoprotective potential of herbal and other compounds (McGill *et* al., 2012; Delgado-Montemayor *et* al, 2015; Lee *et* al., 2017).

In therapeutic doses, the majority of APAP is conjugated in the liver with glucuronide or sulphate and minor fraction of APAP (5-15%) is metabolized by cytochrome P450 (mainly the CYP2E1 isoform) to form a reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI is detoxified especially by glutathione (GSH) to form non-toxic acetaminophen-glutathione conjugate (APAP-SG) (James, Mayeux and Hinson 2003; Kučera *et* al. 2011; Roušar *et* al. 2009). In the case of glutathione depletion, this metabolite covalently binds to proteins via cysteine residues forming acetaminophen-protein adducts in the cell and mitochondria. Mitochondria are critical targets for drug toxicity follow APAP toxicity, binding to mitochondria causes ROS generation and inhibition of mitochondrial respiration and ATP depletion. The initial ROS formation activate kinases like apoptosis signal-regulating kinase 1 (ASK1) and the c-Jun N-terminal kinases (JNK), which then exacerbates the mitochondrial oxidative stress (James *et* al., 2003; Jaeschke *et* al., 2013; Ghanem *et* al., 2016; Wang *et* al. 2015; Yan *et* al., 2018).

APAP-induced mitochondrial oxidant stress and peroxynitrile formation can lead to structural alternations of proteins and mitochondrial DNA or to opening of mitochondrial membrane permeability transition pores (MPT). MPT pores opening can lead to collapse of the mitochondrial membrane potential and ATP synthesis arresting. Releasing of intermembrane

proteins, apoptosis-inducing factors and endonucleases G and their transport to the nucleus can induce nuclear DNA fragmentation and following necrotic cell death. On the other hand, releasing of cytochrome C and pro-apoptotic factors from mitochondria can support caspase activation and apoptotic cell death (Jaeschke *et* al., 2012).

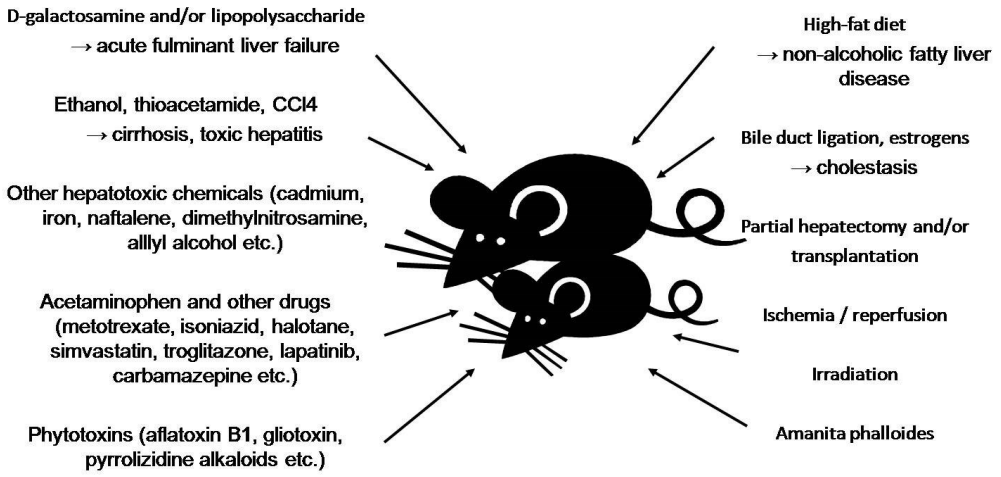

Figure 2. Common *in vivo* models of liver damage. (Farghali *et* al., 2016)

3.5 SIRT1 and AMPK

3.5.1 SIRT1

Sirtuins (silent information regulators) are a family of highly conserved NAD-dependent class III histone deacetylases (Ma *et* al., 2019). A common feature of the activity of sirtuins is their dependence on intracellular levels of NAD (nicotinamide adenine dinucleotide) in its oxidized (NAD^+) or reduced form (NADH). As epigenetic modulators, they play important functions in numerous biological processes (Ding *et* al., 2017). Seven sirtuins have been identified in mammalian cells. SIRT3 - SIRT5 are mitochondrial proteins and play a role in oxidative stress and lipid metabolism, whilst SIRT1, SIRT6 and SIRT7 are predominantly nuclear enzymes (Wątroba *et* al., 2017). All these sirtuins are epigenetic modulators controling the transcription silencing of genes which means that they influence the gene expression without modification of the DNA sequence. In fact, deacetylation of histone proteins increases the positive charge on the histone proteins leading to their increased affinity for DNA and repression transcription (Ma *et* al., 2019). One of the most studied is sirtuin 1 (SIRT1, silent information regulator T1).

3.5.1.1 Physiological/pathological role of SIRT1 in organism

SIRT1 called “anti-aging protein” plays important role in many physiological functions, including gene transcription, energy metabolism, oxidative stress, cell apoptosis/survival and senescence (Meng *et* al., 2017; Wang *et* al., 2020). SIRT1 also plays beneficial roles in regulating hepatic lipid metabolism, controlling hepatic oxidative stress and mediating hepatic inflammation through deacetylating some transcriptional regulators against the progression of fatty liver diseases (Farghali *et* al., 2019). These includes for example carbohydrate-response element-binding protein (ChREBP), sterol regulatory element-binding protein 1 (SREBP-1c), peroxisome proliferator-activated receptor alpha (PPARα), peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1α), nuclear factor-κB (NF-κB). Moreover, defects in the pathways controlled by SIRT1 are known to result in various metabolic disorders (Nogueiras *et* al., 2012). Interestingly, increasing evidences indicate that SIRT1 plays a complex role in tumorigenesis with functions in both tumor promoting and tumor suppressing. SIRT1 can negatively regulate multiple pathways including both tumor suppressors (FOXO, p53) and oncogenic proteins (Survivin, NF-κB, β-catenin). Strong evidence that SIRT1 function as tumor promoter comes from the findings that SIRT1 expression is significantly increased in several murine and human cancer cells. SIRT1 is

consistently overexpressed in acute myeloid leukemia, mouse and human prostate cancer cells, colon carcinoma cells etc. Therefore, SIRT1 inhibitors could be the idea targets for developing potential anti-cancer drugs (Li and Luo, 2011).

3.5.1.2 SIRT1 activators

Resveratrol

In 2003, a screen for small molecule activators of SIRT1 identified 21 different SIRT1-activating molecules; the most potent was resveratrol (Price *et* al, 2012). Resveratrol (trans-3,40,5-trihydroxystilbene, **Fig. 3a**) is a polyphenolic compound found in a plant sources such as phytochemical berries, grapes and wine. It is one of the most extensively studied natural product with wide ranging biological activity and vast clinical potentials.

Many studies have suggested that resveratrol has anti-aging, anticarcinogenic, anti-inflammatory, and antioxidant properties that might be relevant to chronic diseases and/or longevity in humans. It was found that resveratrol protects liver cells by suppressing oxidative stress and apoptosis, inhibits liver tumor growth and angiogenesis and decreases fibrosis (Černý *et* al., 2009; Farghali *et* al., 2014).

Resveratrol, among others, has been recently described as the most potent natural activator of SIRT1 that increases adenosine-5´-monophosphate-activated protein kinase (AMPK) phosphorylation and reduces the oxidative damage biomarkers during aging in laboratory settings (Farghali *et* al., 2013). After resveratrol administration in mice, there was observed increased mitochondrial function and elevated levels both AMPK and NAD^+ whereas mice with SIRT1 knockouts didn´t have any of these phenomens (Price *et* al., 2012).

Metformin

Metformin (**Fig. 3b)** is the first-line anti-diabetic drug for type 2 diabetes (Guo *et* al. 2021). Moreover, it was demonstrated that metformin attenuates APAP-induced liver injury by not otherwise specified antioxidant properties or probably through inhibition of JNK signaling along with stimulation of hemeoxygenase (HO)-1 expression, resulting in hepatoprotection against oxidative stress (Saeedi Saravi *et* al., 2016; Tripathy *et* al., 2019; Jaeschke *et* al., 2020). Although the fundamental mechanism of metformin has not been fully clarified its dual activating effect on SIRT1/AMPK should be taken into the consideration. It was demonstrated that metformin decreases liver gluconeogenesis and ketosis-conveyed inflammatory response through activation of AMPK protein expression leading to SIRT1

induction in mice and porcine hepatocytes, respectively (Caton *et* al., 2010, Xu *et* al., 2021). Other works rather, however, showed that metformin reduces lipid accumulation by SIRT1 stimulation independently of AMPK (Song *et* al., 2015) or by acting primarily through AMPK independently of SIRT1 when increasing SIRT1 activity simultaneously (Nelson *et* al., 2012).

Selective synthetic SIRT1 activators (STACs)

Since 2003, synthetic molecules have been discovered with much higher affinity for SIRT1 and better pharmacokinetics properties then resveratrol and similarly structured polyphenols (Schultz *et* al., 2019; Farghali *et* al., 2019).

The first synthetic STACs were derivatives of an imidazothiazole scaffold (e.g. SRT1460, SRT1720, and SRT2183). They were synthesized at *Sirtris Pharmaceuticals* (Milne *et* al., 2007). Molecules such as SRT1720 (**Fig. 3c**) were shown to activate SIRT1 via the same K_m-lowering mechanism as that of resveratrol but with a much lower EC_{50}, the concentration required to increase activity by 50% (Hubbard *et* al., 2014). Further class of chemically distinct STACs (STAC-5, STAC-9, STAC-10, CAY10591 – **Fig. 3d**) was based on benzimidazole and urea scaffolds (Farghali *et* al., 2019).

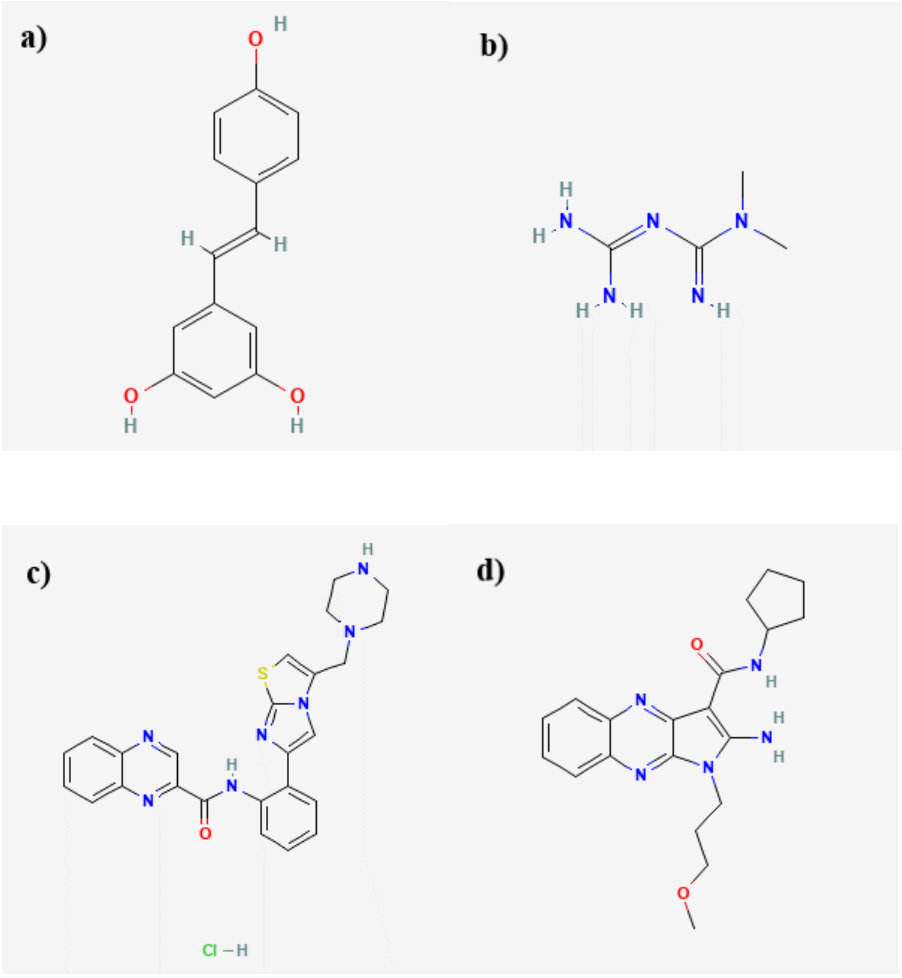

Figure 3. Example of SIRT1 activators: **a)** resveratrol (C14H12O3), **b)** metformin (C4H11N5), **c)** SRT1720 (C25H24ClN7OS), **d)** CAY10591 (C20H25N5O2) PubChem (nih.gov).

3.5.2 AMPK

AMPK is a serine/threonine protein kinase complex consisting of a catalytic α-subunit (α1 and α2), a scaffolding β-subunit (β1 and β2) and a regulatory γ-subunit (γ1, γ2 and γ3). Generally, AMPK is an enzyme which works as a sensor of cellular energy and it is activated by increased levels of AMP/ATP and/or ADP/ATP (Ke *et* al., 2018). The effect of AMPK is also based on ability to phosphorylate and inactivate acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase which play a key role in lipid biosynthesis (Hardie *et* al., 2014).

3.5.2.1 Physiological/pathological role of AMPK in organism

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. It is a big therapeutical player in many metabolic diseases such as diabetes or obesity, and in tumorigenesis (Kim *et* al., 2016; Liang *et* al., 2007). AMPK participates in tumor-suppresing effects of liver kinase B1 (LKB1). Inductors of AMPK such as metformin or fenformin can delay the onset of tumorigeneses. In addition, AMPK activation has been described to cause G1 cell cycle arrest, which is associated with activation of p53, followed by induction of the cell cycle inhibitor protein (p21). p21 is one of these factors that promote cell cycle arrest in response to a variety of stimuli (Karimian *et* al., 2016; Kim *et* al., 2016; Liang *et* al., 2007).

3.5.3 Relationship between SIRT1 and AMPK

The relationship between AMPK and SIRT1 under the present experimental conditions will be addressed to illuminate the regulatory influence on each other and common target molecules, if any (Ruderman *et* al., 2010). These findings and the concurrent demonstration by many laboratories of common activators, actions, and target molecules of SIRT1 and AMPK led to an examination of a possible linkage between SIRT1 and the primary upstream AMPK kinase, LKB1 (**Fig. 4**). For example, the evidence for a SIRT1/LKB1/AMPK signaling mechanism was reported by Hou *et al.* (2008) who demonstrated that the ability of polyphenols (resveratrol, SI 17834) to activate AMPK in cultured HepG2 cells and mouse liver *in vivo* required the presence of both SIRT1 and LKB1. Likewise, in studies carried out predominantly in HepG2 cells, Suchankova *et* al. (2009) noted that incubation with 25 vs. 5 mM glucose (6 hours) or the SIRT1 inhibitor nicotinamide (10 mM, 2 hours) down-regulated the activity of both SIRT1 and AMPK (indicated by increased PGC-1α acetylation), whereas incubation with pyruvate and the SIRT1 activator quercetin increased both of their activities. Similar effects of pyruvate and glucose on SIRT1 had been described previously in primary hepatocytes, suggesting that they are not unique to HepG2 cells (Rodgers *et* al., 2005).

Another important target for SIRT1 and AMPK is NF-κB, a regulator of many processes, including cell cycle, apoptosis, and inflammation. SIRT1 down-regulates NF-κB-mediated pro-inflammatory effects by deacetylating the RelA/p65 subunit of NF-κB (Yang *et* al., 2007; Yeung *et* al., 2004). It is well-documented that chronic overfeeding, by increasing circulating

fatty acids, might lead to inflammation, insulin resistance and injury in the liver (Mollica *et* al., 2011). SIRT1-overexpressing transgenic mice have decreased hepatic NF-κB activity, which protects from high-fat diet/lipid-induced hepatic inflammation, glucose intolerance, and NAFLD (Pfluger *et* al., 2008). Another study by Lee *et al.* (2009) finds that SIRT1-mediated attenuation of NF-κB signaling prevents cytokine-induced pancreatic β-cell damage. NF-κB is considered as a major regulator of the oxidative stress and inflammatory response (as described above) due to its ability to regulate the transcription of genes involved in the establishment of immune and inflammatory response (IL-6, TNF-*α* and iNOS). It has been described that the activated AMPK could inhibit NF-*κ*B signaling through its downstream target molecules such as SIRT1, PGC-1*α*, Forkhead box O (FOXO) and reduce the expression of inflammatory factors (Zhu *et* al., 2018; De Gregorio *et* al., 2020).

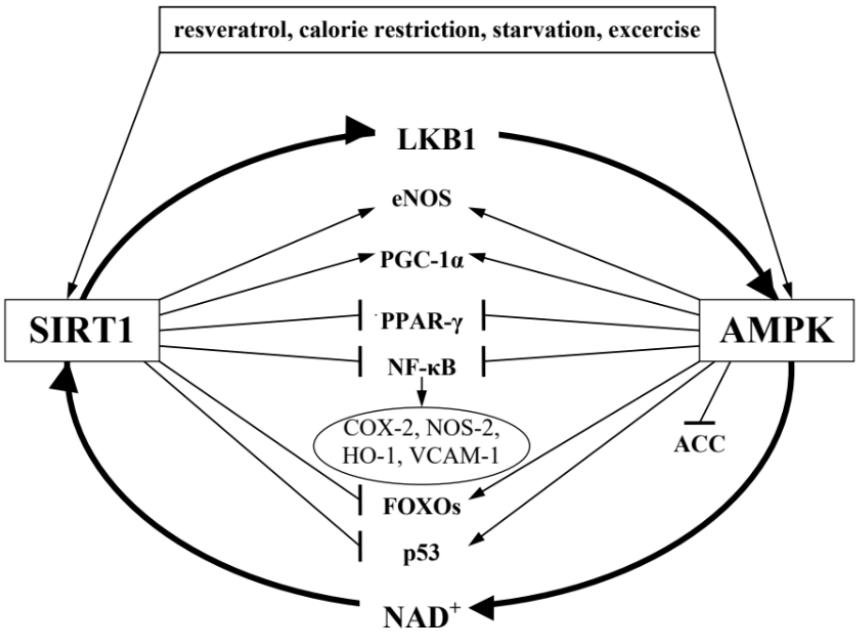

Figure 4. Proposed molecular mechanisms by which SIRT1 and AMPK activate each other and control other regulatory factors associated with metabolism and inflammation (→ activation, ⊥ inhibiton). ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; eNOS (NOS-1), endothelial nitric oxide synthase; 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).

4 Aims and hypothesis

4.1 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/lipopolysaccharide, 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 D-GalN/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.

4.2 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-GalN/LPS -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 silent information regulator T1 (SIRT1) which can stimulate adenosine-5′-monophosphate-

activated protein kinase (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. 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.

5 Description of experimental methods

5.1 Animals

Outbred male Wistar rats were obtained from Velaz-Lysolaje, Czech Republic. Rats were allowed to tap water and standard granulated diet *ad libitum* and were maintained under standard light (12/12h light/dark), temperature (22±2 °C) and relative humidity (50±10 %) conditions. All rats received humane care in compliance with the general guidelines of the First Faculty of Medicine, Charles University in Prague. The study protocols were approved by the Faculty Ethical Committee and by the Ministry of Agriculture and Ministry of Education, Youth and Sports of the Czech Republic (Kemelo, Wojnarová *et* al., 2014; Wojnarová et al., 2015; Njeka Wojnarová et al., 2022). Rats having body weight of 200-350 g were used for experiments.

5.2 Drug treatments

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 a 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 tests (Wojnarová *et* al., 2015; Njeka Wojnarová *et* al., 2022).

5.3 *In vivo* experimental studies

5.3.1 D-Galactosamine/lipopolysacharide rat model of hepatotoxicity

In the first experimental study (Kemelo, Wojnarová *et al.* 2014), the rats were randomly divided into five groups according to application of tested drugs (**Tab. 1**).

Table 1. Experimental goups of the first *in vivo* study[#]

Group	***Treatment***
Group 1 (control)	DMSO + physiologic solution
Group 2	Resveratrol (2.3 mg/kg)
Group 3	D-GalN (400 mg/kg) + LPS (10 μg/kg) in one injection
Group 4*	Resveratrol (2.3 mg/kg) + D-GalN (400 mg/kg) + LPS (10 μg/kg)
Group 5**	EX-527 (1 mg/kg) + Resveratrol (2.3 mg/kg) + D-GalN (400 mg/kg) + LPS (10 μg/kg)

**Rats were pretreated with resveratrol 60 minutes before induction of hepatic failure by D-GalN/LPS combination.*

***Rats were pretreated with EX-527 30 minutes before resveratrol and 90 minut before induction of hepatic failure by D-GalN/LPS combination.*

[#]Kemelo MK, Wojnarová L, Kutinová Canová N, Farghali H (2014) D-galactosamine/lipopolysaccharide-induced hepatotoxicity downregulates sirtuin 1 in rat liver: role of sirtuin 1 modulation in hepatoprotection. Physiol Res 63, 615-23.

Resveratrol and EX-527 were dissolved in dimethyl sulfoxid (DMSO). To induce acute liver injury, combination of D-GalN/LPS in physiological solution was injected intraperitoneally. At the end of the treatment period (6 hours later), the animals were anesthetized with *diethylether* and then euthanized by exsanguination. Their blood samples were promptly collected into heparinized tubes for biochemical investigations (assessment of ALT, AST, total bilirubin, nitric oxide as NO_2^-). The liver homogenates were used for determination of total lipid peroxidation assessed as thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD) or snap-frozen in liquid nitrogen for Western blot studies (Kemelo, Wojnarová *et* al., 2014).

5.3.2 Acetaminophen (APAP) rat model of hepatotoxicity

In the second experimental study (Wojnarová *et* al., 2015), acute liver injury was induced by acetaminophen (APAP). APAP (1 g/kg) was injected intraperitoneally from a 0.2 g/ml solution in 40% polyethyleneglycol 400 (PEG 400 in saline). CAY10591 and resveratrol were both dissolved in DMSO. The rats were divided randomly into five groups of six animals each and treated as described in **Table 2**.

Table 2. Experimental goups of the second *in vivo* study[#]

Group	***Treatment***
Group 1 (Control)	PEG 400 (40 %) + DMSO
Group 2	CAY (0.5 mg/kg)
Group 3	RES (30 mg/kg)
Group 4	APAP (1 g/kg)
Group 5*	APAP (1 g/kg) + CAY (0.5 mg/kg)
Group 6*	APAP (1 g/kg) + RES (30 mg/kg)

** Rats were treated with resveratrol or CAY10591 60 minutes after APAP application.*

[#]Wojnarová L, Kutinová Canová N, Farghali H, Kučera T (2015) Sirtuin 1 modulation in rat model of acetaminophen-induced hepatotoxicity. Physiol Res 64, S477-S487.

Twenty-four hours after APAP application, the animals were anesthetized with *diethylether* and then euthanized by exsanguination. Blood samples were collected into heparinized tubes. Plasma was immediately isolated by centrifugation at 4,000 rpm for 10 min and used for assessment of ALT, total bilirubin, and nitric oxide as NO_2^-. Rat liver samples were immediately homogenized or snap frozen in liquid nitrogen for Western blot analysis. The liver homogenates were used for determination of total lipid peroxidation assessed as TBARS and conjugated dienes (Wojnarová *et* al., 2015).

5.4 *In vitro* experimental studies

Isolation and culture of primary rat hepatocytes

Hepatocytes were isolated from 12-14 week old untreated Wistar rats using the standard two phase collagenase perfusion method (Berry *et* al., 1991). Separated hepatocytes were counted and cell viability was assessed by trypan blue exclusion method. The viability of freshly isolated hepatocytes was greater than 85%. Cells were seeded on collagen-coated polystyrene Nunclon™ dishes at density of 104 000 viable cells/cm^2. They were incubated in complete medium (William´s medium E, penicillin/streptomycin 1%, glutamine 1%, insulin 0.06%, FBS-fetal bovine serum 5%) at 37 °C in a humid atmosphere with 5% CO_2 throughout the study. Unattached hepatocytes were removed 3 hours after seeding and remaining hepatocytes further cultured in fresh complete medium overnight. Hepatocytes were then treated with fresh medium containing 0.1% of solvent (DMSO) or with acetaminophen or SIRT1 and AMPK modulators at concentrations listed in the **Table 3** and **Table 4**. After 4 or 24 hours, medium samples were collected for biochemical analysis and hepatocyte viability was assessed by MTT test.

At the end of experiments, some cultured hepatocytes were washed by cooled phosphate buffered saline (PBS) and lysed in RIPA buffer containing protease and phosphatase inhibitor cocktail. The homogenates were centrifuged at 14,000 g for 10 min at 4 °C and thereafter used for SIRT1 activity measurement and protein expression by Western blot analysis (Wojnarová *et* al., 2015; Njeka Wojnarová *et* al., 2022).

Table 3. Experimental goups of the first *in vitro* study[#]

Group	***Treatment***
Group 1 (Control)	DMSO (0.1%)
Group 2	CAY (30 μM)
Group 3	RES (20 μM)
Group 4	APAP (5 mM)
Group 5*	APAP (5 mM) + CAY (30 μM)
Group 6*	APAP (5 mM) + RES (20 μM)

** Hepatocytes were treated with resveratrol or CAY10591 30 minutes after APAP application.*

[#]Wojnarová L, Kutinová Canová N, Farghali H, Kučera T (2015) Sirtuin 1 modulation in rat model of acetaminophen-induced hepatotoxicity. Physiol Res 64, S477-S487.

Table 4. Experimental goups of the second *in vitro* study[#]

Group	***Treatment***
Group 1 (Control)	DMSO (0.1%)
Group 2	APAP (12.5 mM)
Group 3	AICAR (50 μM)
Group 4*	AICAR (50 μM) +APAP (12.5 mM)
Group 5	CC (10 μM)
Group 6*	CC (10 μM) + APAP (12.5 mM)
Group 7*	AICAR (50 μM) + CC (10 μM) + APAP (12.5 mM)
Group 8*	CAY (30 μM) + CC (10 μM) + APAP (12.5 mM)
Group 9	EX (10 μM)
Group 10*	EX (10 μM) + APAP (12.5 mM)
Group 11*	CAY (30 μM) + EX (10 μM) + APAP (12.5 mM)
Group 12*	AICAR (50 μM) + EX (10 μM) + APAP (12.5 mM)

**Hepatocytes were pretreated with fresh medium containing either DMSO or SIRT1 and AMPK modulators 30 minutes before addition of APAP to hepatocyte cultures.*

Concentrations were determined by MTT test in 96-well plates.

[#]Njeka Wojnarová L, Kutinová Canová N, Arora M., Farghali H (2022) Differentiated modulation of signaling molecules AMPK and SIRT1 in experimentally drug-induced hepatocyte injury. (Acceptted for publication in Biomedical Papers after revision).

5.5 Histological evaluation

After the excised liver (1 cm^3) fixation in 4% paraformaldehyde in PBS, thin tissue paraffin sections (5 μm) were cut by microtome, stained with hematoxylin and eosin and examined by light microscope (Wojnarová *et* al., 2015).

5.6 Hepatocyte integrity and function (AST, ALT and total bilirubin)

Damaged hepatocytes release their contents including ALT and AST into the extracellular space (Ozer *et* al., 2008).

The alanine aminotransferase **(ALT)** enzyme, also known as serum glutamic-pyruvic transaminase (SGPT), catalyzes the transfer of amino groups from L-alanine to α-ketoglutarate, and the converted products are L-glutamate and pyruvate. The aspartate

aminotransferase (**AST)** enzyme, also known as aspartate transaminase and glutamate-oxaloacetate transaminase (GOT), catalyzes the transfer of an alpha amino group from aspartate to alpha-ketoglutarate, producing glutamate and oxaloacetic acid.

Elevation of bilirubin concentrations can be induced by numerous causes and hence, it is a nonspecific marker of liver dysfunction. In the hyperacute stage of ALF, bilirubin concentration is relatively low as compared to the substantial elevation of aminotransferase levels in plasma. However, in the subacute stage, the situation reverses. In this case, elevated levels of bilirubin in plasma are an indicator of poor prognosis and mortality (Ruiz *et* al., 2021).

Hepatocyte integrity was assessed as plasma and medium ALT, AST, and total bilirubin levels by commercially available diagnostic kits from Vian Diagnostics (Prague, Czech Republic) (Wojnarová *et* al., 2015). The results were expressed either in IU/l (international units per liter) and mg/dcl for ALT and total bilirubin, respectively, or as a percentage of average control values.

5.7 Determination of nitrite levels

Large amount of nitric oxide (NO) released due to stimulation of iNOS induces cytotoxic effects in hepatocytes either directly or via peroxynitrite anions producing oxidative stress (Černý *et* al., 2009). Therefore, medium nitrite (NO_2^-), the stable end-product of NO oxidation, was detected spectrophotometrically by using Griess reagent (1% sulfanilamide, 0.1% naphtylethylendiamine, 2.5% trihydrogenphosphoric acid). The absorbance at 540 nm was recorded and the NO_2^- values were subtracted from $NaNO_2$ standard curve (Černý *et* al., 2009).

5.8 TBARS and conjugated dienes analysis

Liver homogenates were used for determination of TBARS and conjugated dienes as markers of lipid peroxidation.

- The analysis of **thiobarbituric acid reactive substances (TBARS)** in hepatocyte lysates was carried out according to Farghali *et* al. (2009). This method uses the

reaction of lipid peroxidation products, especially malondialdehyde (MDA) and thiobarbituric acid (TBA), which leads to the formation of MDA-TBA2 adducts named TBARS. TBARS (red-pink color) is determined spectrophotometrically (De Leon and Borges, 2020). The results were calculated as the molar amount per 1 mg of lysate protein (assessed by Bio-Rad protein DC assay) (Njeka Wojnarová *et* al., 2022).

- **Conjugated dienes (CD)**, were extracted from the liver homogenate or plasma and assessed using 233 nm compared to heptane as blank. Test tube contained 2 ml heptane and 2 ml isopropylalcohol (to counteract further lipidperoxidation, reaction mixture contained 0.1 ml of 0.2 M EDTA only for plasma, not for homogenate). This mixture was shortly mixed (2 s) and incubated 20 min at 20 °C. Then 1 ml 0.01 M HCl and 2 ml heptane were added, mixed and incubated 30 min at 20 °C. Pipetted supernatant was used for measurement. The results were expressed in nmol/ml of plasma or nmol/mg protein in liver tissues (Farghali *et* al., 2009).

5.9 Caspase-3 ELISA assay

The instructions by the manufacturer of Rat Caspase 3 ELISA Kit (LifeSpan BioSciences, Inc, Seattle, USA) were followed to detect caspase-3 proenzyme in hepatocyte lysates (Njeka Wojnarová *et* al., 2022).

5.10 Immunoblotting

The cell lysates were mixed (1:1) with sample buffer (2 x Laemmli buffer 950µL+50µL of β-mercaptoethanol) and then heated for 10 min at 90 °C. Proteins (assessed by Bio-Rad protein DC assay) from the cell samples were separated on 10% SDS-acrylamide gel (TGX™ FastCast™ Acrylamide Solutions by Bio-Rad) and transferred to a nitrocellulose membrane (Hybond ECL, Cytiva, Prague, Czech Republic) by electrophoresis. Membranes were blocked for 2 hours with 5% non-fat milk or 5% BSA in 10x Tris Buffered Saline with Tween 20 (TBST). Membranes were then washed in TBST washing buffer and incubated with either mouse primary antibody against SIRT1 (1:1,000), beta actin primary antibody (3:15,000), rabbit primary antibody against AMPK (1:1,000) or pAMPK (1:1,000) and followed with corresponding secondary rabbit antibody anti-mouse or anti-rabbit IgG HRP conjugate (3:20,000 or 1:80,000). For visualization, chemiluminescence labelling with Super Signal

West Pico Chemiluminescent Substrate (GeneTiCA, Prague, Czech Republic) was used. Bands were detected with the use of Molecular Imager VersaDoc™ MP 5000 System and analysed by Quantity One 1-D Analysis Software (Bio-Rad, Prague, Czech Republic). Optical densities of SIRT1, pAMPK/AMPK and beta actin bands were normalized by the corresponding loading control and then to the mean of the appropriate control group (Kemelo, Wojnarová *et* al., 2014; Wojnarová *et* al., 2015; Njeka Wojnarová *et* al., 2022).

5.11 MTT (cell viability test)

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was used both to assess the optimal non-toxic concentration of drugs (resveratrol, SIRT1 and AMPK modulators) suitable for our study and to measure hepatocyte viability at the end of *in vitro* experiments.
The MTT assay depends on the cellular reduction of tetrazolium salts to their formazan crystals by viable cells (Kutinová Canová *et* al., 2008).

5.12 SIRT1 deacetylase activity assay

SIRT1 deacetylase activity was evaluated in 5 µl of the whole liver lysate as well as cultured hepatocyte lysate according to instructions of commertial fluorometric SIRT1 Assay Kit (Sigma-Aldrich). The measured fluorescence was directly proportional to deacetylation activity of the SIRT1 enzyme in the sample. All measurements were performed in duplicate and the results were reported as arbitrary units of relative fluorescence per 1 mg of lysate protein (assessed by Bio-Rad protein DC assay) (Wojnarová *et* al., 2015).

5.13 Statistical analysis

The statistical significance of differences of mean scores was determined using 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 considered to be statistically significant. 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).

6 Results

6.1 D-Galactosamine/Lipopolysaccharide-Induced Hepatotoxicity

D-Galactosamine/Lipopolysaccharide-Induced Hepatotoxicity Downregulates Sirtuin 1 in Rat Liver: Role of Sirtuin 1 Modulation in Hepatoprotection

ALT, AST and bilirubin

Measurement of the levels of ALT, AST and bilirubin in plasma (**Fig. 5)**. There was over 20 - fold increase in ALT levels and slightly less with AST and bilirubin. Resveratrol pretreatment in D-GalN/LPS rats significantly lowered the ALT and bilirubin levels. There was also the same trend with AST, despite the statistical non-significance. Resveratrol was effective in attenuating D-GalN/LPS-induced hepatotoxicity. EX-527 blocked the effects of resveratrol and significantly increased the ALT and bilirubin levels (Kemelo, Wojnarová *et* al., 2014).

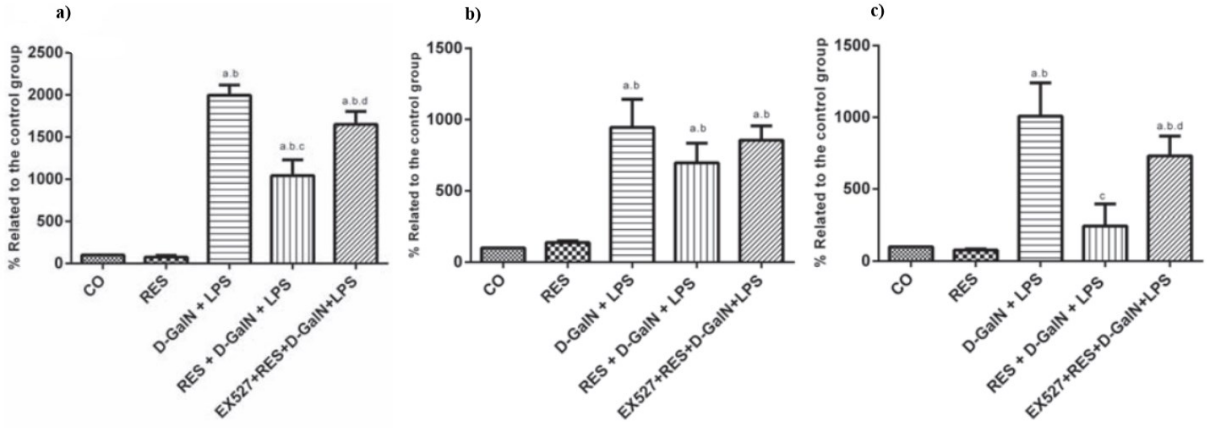

Figure 5. *Effects of resveratrol and EX-527 pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (D-GalN/LPS) on plasma levels of alanine aminotransferase ALT (a), aspartate aminotransferase AST (b) and bilirubin (c). CO, control group; RES, 2.3 mg/kg resveratrol; D-GalN + LPS, 400 mg/kg D-galactosamine with 10 µg/kg lipopolysaccharide; RES + D-GalN + LPS, 2.3 mg/kg resveratrol + D-GalN + LPS; EX-527 + RES + D-GalN + LPS, 1 mg/kg EX-527 plus combination of previous substances. 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.*

TBARS and conjugated dienes

Measurement of lipid peroxidation by determination of TBARS and CD in homogenate (**Fig. 6).** Both CD and TBARS were significantly enhanced after D-GalN/LPS treatment reflecting increased peroxidation. Resveratrol pretreatment reduced the levels of both markers by more than a fold. The anti-peroxidative effects of resveratrol were blocked by EX-527 as evidenced by a significant increase in both the TBARS and CD levels (Kemelo, Wojnarová *et* al., 2014).

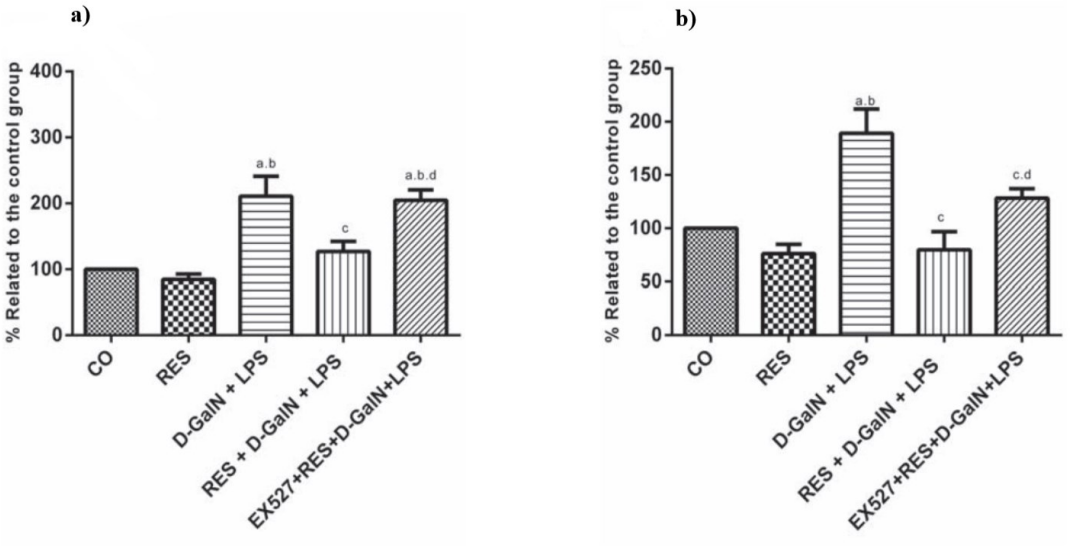

Figure 6. *Effects of resveratrol pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (LPS/D-GalN) on the formation of (a) Conjugated dienes (CD) and (b) Thiobarbituric acid reactive substances (TBARS) in liver homogenate. CO, control group; RES, 2.3 mg/kg resveratrol; D-GalN + LPS, 400 mg/kg D-galactosamine with 10 µg/kg lipopolysaccharide; RES + D-GalN + LPS, 2.3 mg/kg resveratrol + D-GalN + LPS; EX-527 + RES + D-GalN + LPS, 1 mg/kg EX-527 plus combination of previous substances. Data are expressed as mean ± 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.*

Western blot analysis

A Western blot analysis (**Fig. 7).** Resveratrol alone, did not have any statistically significant effect on the total endogenous amount of SIRT1. However, treatment with D-GalN/LPS dramatically decreased SIRT1 expression levels. In spite of an increasing trend on the blot, resveratrol pretreatment of D-GalN/LPS rats did not have any statistical significance on SIRT1 expression. There was no significant change in SIRT1 expression levels in response to EX-527 pretreatment (Kemelo, Wojnarová *et* al., 2014).

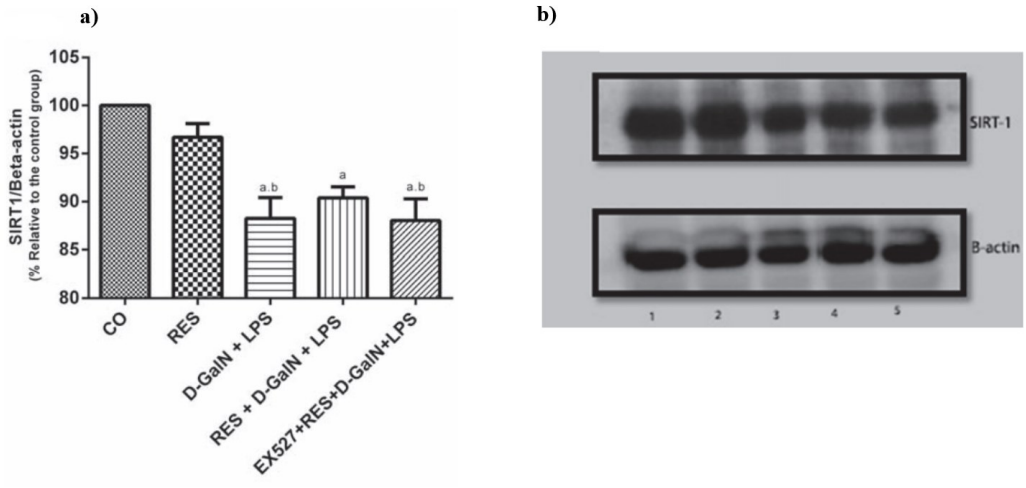

Figure 7. *Effects of resveratrol and EX-527 pretreatment on SIRT1 expression. (a) Quantification of SIRT1 expression levels by densitometry. Band intensity measurements were done using Bradford software. In each panel, the intensity of a given band was normalized to the intensity of the corresponding β-actin band. CO, control group; RES, 2.3 mg/kg resveratrol; D-GalN + LPS, 400 mg/kg D-galactosamine with 10 μg/kg lipopolysaccharide; RES + D-GalN + LPS, 2.3 mg/kg resveratrol + D-GalN + LPS; EX-527 + RES + D-GalN + LPS, 1 mg/kg EX-527 plus combination of previous substances. 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.*

6.2 Acetaminophen-induced Hepatotoxicity (*In vitro* and *in vivo*)

Sirtuin 1 Modulation in Rat Model of Acetaminophen-Induced Hepatotoxicity

ALT and bilirubin in vivo and in vitro

Measurement of ALT and bilirubin in plasma and culture medium (**Fig. 8).** The APAP treatment in rats produced significant two-fold increase of ALT release ($P < 0.001$) compared to the control group both *in vitro* and *in vivo*. Moreover, treatment with RES and CAY after induction of hepatotoxicity slightly lowered the ALT parameters *in vivo*. Difference was in *in vitro* experiments, where only CAY treatment significantly reduced ALT, whereas RES did not influence the resulting values. Significant increase of bilirubin, fivefold higher, was observed in hepatocyte cultures compared to the control. There was a tendency of resveratrol and CAY to reduce APAP-increased total bilirubin levels, both *in vitro* and *in vivo* (**Fig. 8**) (Wojnarová *et* al., 2015).

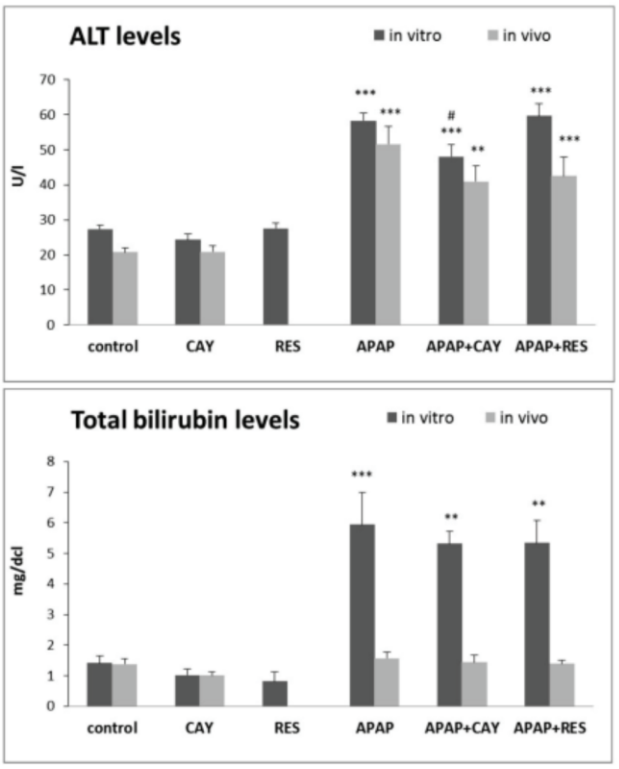

Figure 8. *Effect of specific SIRT1 activator, CAY10591, and resveratrol treatments on hepatocyte function in acute APAP-induced hepatocyte/liver injury in vitro and in vivo expressed as medium or plasma levels of Alanine aminotransferase (ALT) and Bilirubin. Control (24 hour-vehicle treated hepatocytes or rats); CAY (CAY10591: 30 µM in vitro, 0.5 mg/kg in vivo); RES (Resveratrol: 20 µM in vitro, 30 mg/kg in vivo); APAP (Acetaminophen: 5 mM in vitro, 1 g/kg in vivo); APAP + CAY (combination of Acetaminophen and CAY10591 in the stated doses); APAP + RES (combination of Acetaminophen and Resveratrol in the stated doses). Data are expressed as means ± SEM (n=6): ** P<0.01, *** P<0.001 vs. respective control; # P<0.05 vs. APAP in vitro.*

TBARS and conjigated dienes

Measurement of TBARS and conjugated dienes (CD) **(Fig. 9). Figure 9** demonstrates that APAP treatment produced moderate increase of lipid peroxidation as evidenced by the formation of TBARS and CD. Single APAP treatment significantly increased both TBARS and CD ($P<0.05$) in liver homogenate. CAY following APAP treatment slightly reduced only CD levels in contrast with RES, which reduced the levels of both markers. APAP significantly increased medium NO_2^- levels ($P<0.001$) *in vitro*. On the other hand, NO_2^- plasma levels were not significantly affected by any treatment even though CAY and resveratrol slightly reduced it (Wojnarová *et* al., 2015).

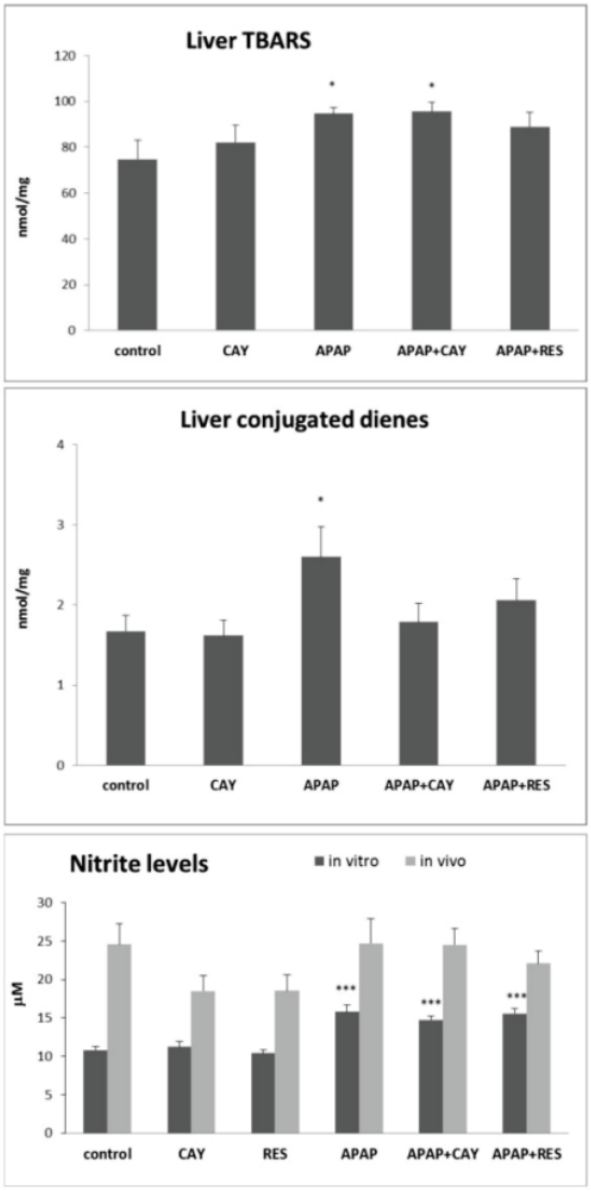

Figure 9. *Effect of specific SIRT1 activator, CAY10591 in comparison with resveratrol treatment in APAP-induced hepatocyte/liver injury on the formation of Thiobarbituric acid reactive substances (TBARS) and Conjugated dienes (CD) in vivo, and NO_2^- production in vitro and in vivo. Control (24 hour-vehicle treated hepatocytes or rats); CAY (CAY10591: 30 μM in vitro, 0.5 mg/kg in vivo); RES (Resveratrol: 20 μM in vitro, 30 mg/kg in vivo); APAP (Acetaminophen: 5 mM in vitro, 1 g/kg in vivo); APAP + CAY (combination of Acetaminophen and CAY10591 in the stated doses); APAP + RES (combination of Acetaminophen and Resveratrol in the stated doses). Data are expressed as means ± SEM (n=6): * $P<0.05$, *** $P<0.001$ vs. respective control.*

Hepatocyte viability and SIRT1 activity

Measurement of hepatocyte viability in *in vitro* experiments and **relative SIRT1 activity** both *in vitro* and *in vivo* are shown in **Figure 10.** APAP treatment significantly reduced viability (P< 0.001) of cultured hepatocytes in comparison to the untreated control group and the group treated only by RES or CAY. MTT test showed that RES and CAY alone did not have toxic effect on hepatocytes in cell culture and that CAY more potently increased APAP-reduced hepatocyte viability. APAP also markedly reduced SIRT1 enzyme activity (by 34 % *in vitro* and 20 % *in vivo*). RES and especially CAY increased SIRT1 activity compared to control and APAP treatments. The same trend was observed as *in vitro* as *in vivo*.

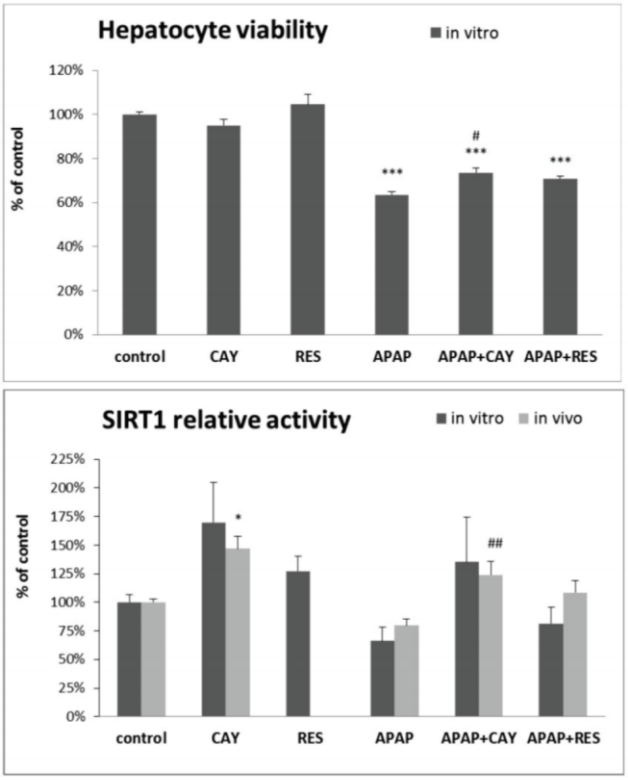

Figure 10. *Effect of specific SIRT1 activator, CAY10591, in comparison with resveratrol treatment in APAP-induced hepatocyte/liver injury on hepatocyte viability in in vitro experiments and SIRT1 relative activity in in vitro and in vivo experiments. Control (24 hour-vehicle treated hepatocytes or rats); CAY (CAY10591: 30 μM in vitro, 0.5 mg/kg in vivo); RES (Resveratrol: 20 μM in vitro, 30 mg/kg in vivo); APAP (Acetaminophen: 5 mM in vitro, 1 g/kg in vivo); APAP + CAY (combination of Acetaminophen and CAY10591 in the stated doses); APAP + RES (combination of Acetaminophen and Resveratrol in the stated doses). 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. respective APAP group.*

SIRT1 expression

There were no significant changes on the liver SIRT1 expression in Western blot analysis (**Fig. 11).** According to our analysis, single dose treatment with APAP, RES and CAY and their combinations had no effect on the total endogenous amount of SIRT1 expression.

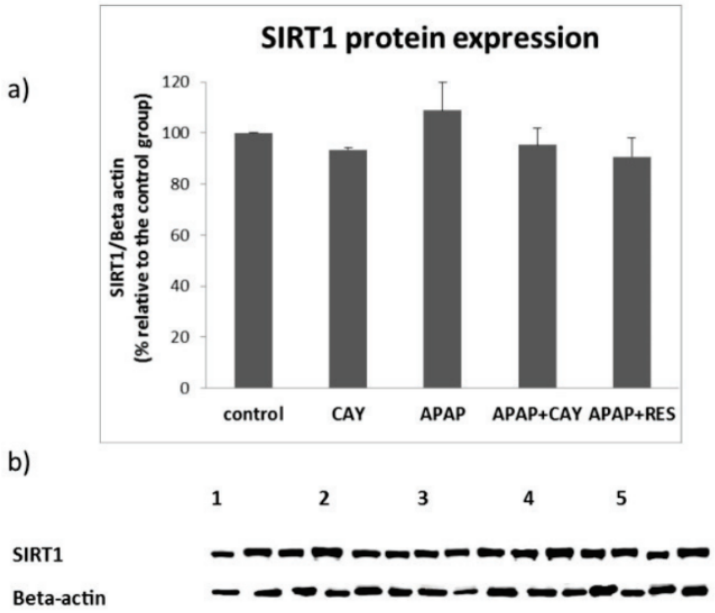

Figure 11. *Effect of specific SIRT1 activator, CAY10591, in comparison with resveratrol treatment in APAP-induced liver injury in vivo on a) quantification of SIRT1 expression levels by densitometry. The intensity of each panel was normalized to the intensity of corresponding beta-actin band. Control (24 hour-vehicle treated rats); CAY (CAY10591: 0.5 mg/kg); APAP (Acetaminophen: 1 g/kg); APAP + CAY (combination of Acetaminophen and CAY10591 in the stated doses); APAP + RES (combination of Acetaminophen 1 g/kg and Resveratrol 30 mg/kg). 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.*

Morfological analysis

Histological observations (Fig. 12). No signs of steatosis, inflammation (except of rare small mononuclear infiltrates), hepatocellular necrosis or fibrosis were observed in the rat liver of control (**Fig. 12a)** and CAY (**Fig. 12b)**. However, there were visible several mitotic hepatocytes in otherwise normal liver parenchyma of these rats, especially in CAY treated ones (**Fig. 12c)**. Histological changes in the liver induced by APAP were not significant (**Fig. 12d)**. APAP caused slight increase in the appearance and number of apoptotic hepatocytes (**Fig. 12e)** and apoptotic bodies surrounded by a mononuclear infiltrate (**Fig. 12f)**. No mitotic hepatocytes were found in the liver of APAP treated rats. Liver parenchyma had normal morphology after application of APAP followed by CAY (**Fig. 12g)** or resveratrol (**Fig. 12h)** to rats.

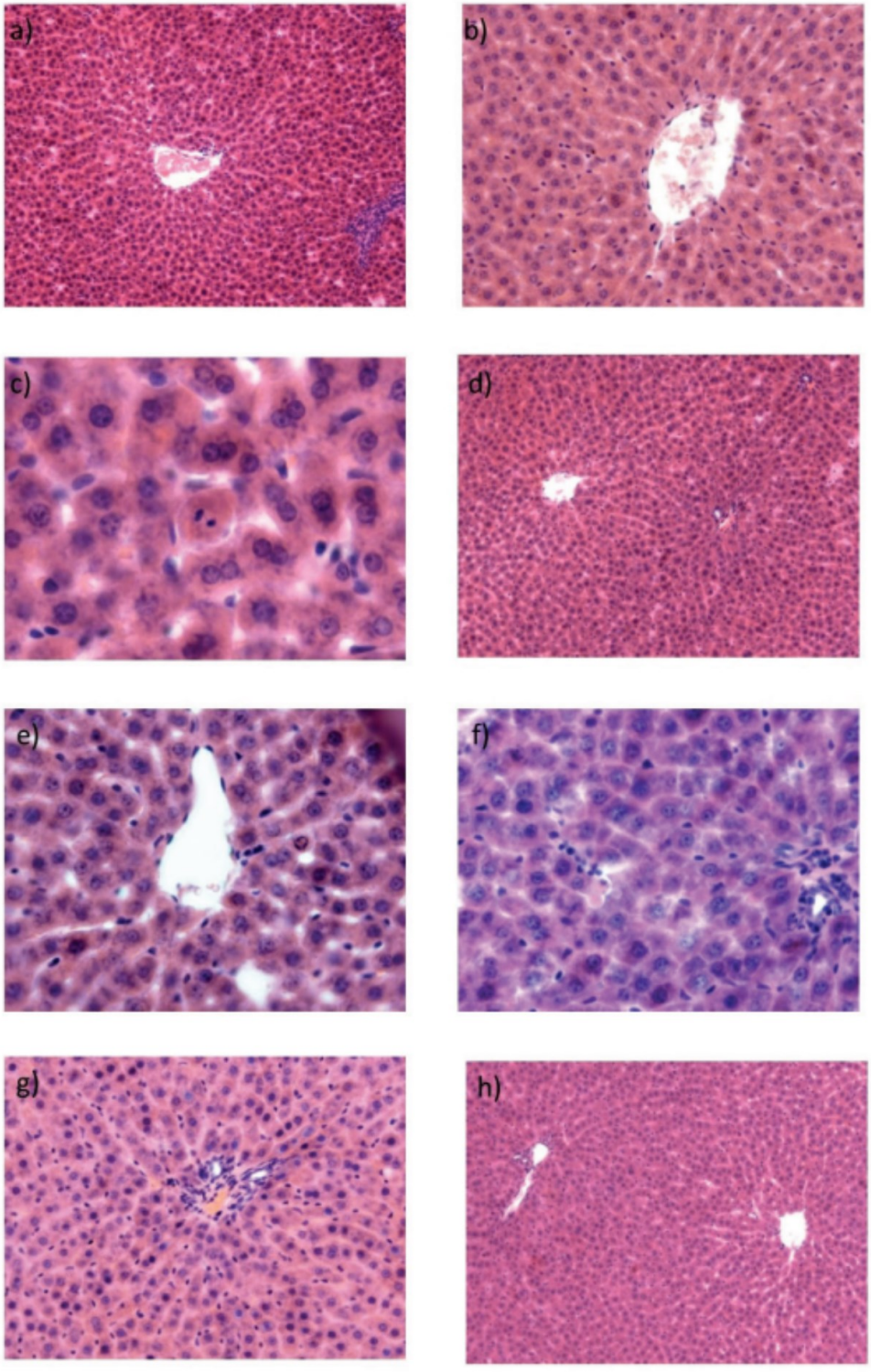

Figure 12. *Representative histopathological samples of livers taken from* ***(a)*** *control rats and animals treated with* ***(b, c)*** *CAY (CAY10591);* ***(d,e,f)*** *APAP (Acetaminophen);* ***(g)*** *APAP + CAY; and* ***(h)*** *APAP + RES (combination of Acetaminophen and Resveratrol) for 24 hours. Hematoxylin and eosin staining (magnification x 200 with a detailed view in* ***c, e, f).***

Differentiated modulation of signaling molecules AMPK and SIRT1 in experimentally drug-induced hepatocyte injury

Hepatocyte viability, ALT, TBARS, nitrites, caspase-3

Cell viability (**Fig. 13A)**, ALT release from hepatocytes to cultivation medium **(Fig. 13B)**, and oxidative stress markers like the end products of inducible nitric oxide synthesis represented by medium NO_2^- levels (**Fig. 13C)** and TBARS formed in hepatocytes (**Fig. 13D)** were evaluated.

Single treatment with APAP at higher dose (12.5 mM) significantly reduced the viability of cultured hepatocytes and increased ALT, nitrite, and TBARS levels (**Fig. 13)**. AMPK inhibitor, Compound C (CC), significantly amplified APAP-induced hepatotoxic effect in all observed parameters. Interestingly, AICAR and CAY10591 pretreatments remarkedly lowered the hepatotoxic and pro-oxidative effects of the APAP+CC combination. The cell death induced by APAP was related to necrosis rather than apoptosis as evidenced by unaffected caspase-3 levels (**Fig. 13E)**. Interestingly, CC in combination with APAP significantly decreased caspase-3 proenzyme suggesting cleavage of inactive pro-caspase-3 to active caspase-3 and induction of apoptosis (Njeka Wojnarová *et* al. 2022).

Pretreatment with EX-527, an inhibitor of SIRT1, slightly enhanced APAP toxicity (**Fig. 14).** The addition of CAY10591 significantly decreased the toxic effect of combination EX-527+APAP. AICAR mildly lowered the ALT release (**Fig. 14C)** from cultured hepatocytes induced by the combination of EX-527+APAP but did not increase hepatocyte viability (**Fig. 14A)**.

TBARS levels **(Fig. 14D)** imitated previously mentioned results on cell viability (**Fig. 14A)** and ALT levels (**Fig. 14C)**. Mainly, CAY10591 but not AICAR pretreatment significantly suppressed the formation of TBARS markedly induced by the combination of EX-527+APAP. Neither CAY10591 nor AICAR had a significant effect on the highly increased nitrite production by hepatocytes after EX-527+APAP application **(Fig. 14B)** (Njeka Wojnarová *et* al. 2022).

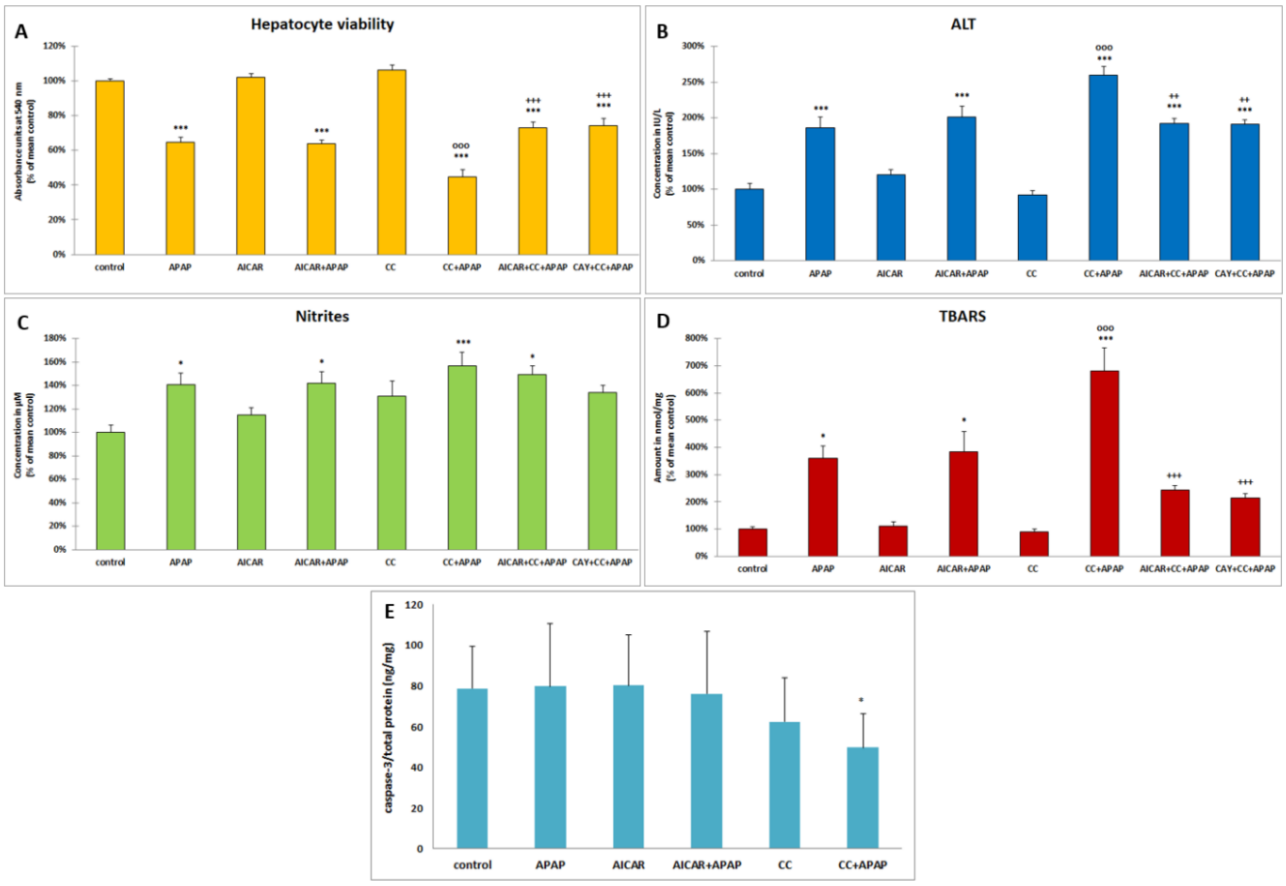

Figure 13. *Effects of specific AMPK modulators (activator – AICAR and inhibitor - Compound C, CC) and specific activator of SIRT1 (CAY10591/CAY) in in vitro acetaminophen (APAP)-induced hepatotoxicity on hepatocyte viability (A) and levels of: medium alanine aminotransferase (ALT) (B), medium nitrites (NO_2^-) (C), cell lysate thiobarbituric acid reactive substances (TBARS) (D), and caspase-3 proenzyme (E) after 24 hours of treatment. 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.*

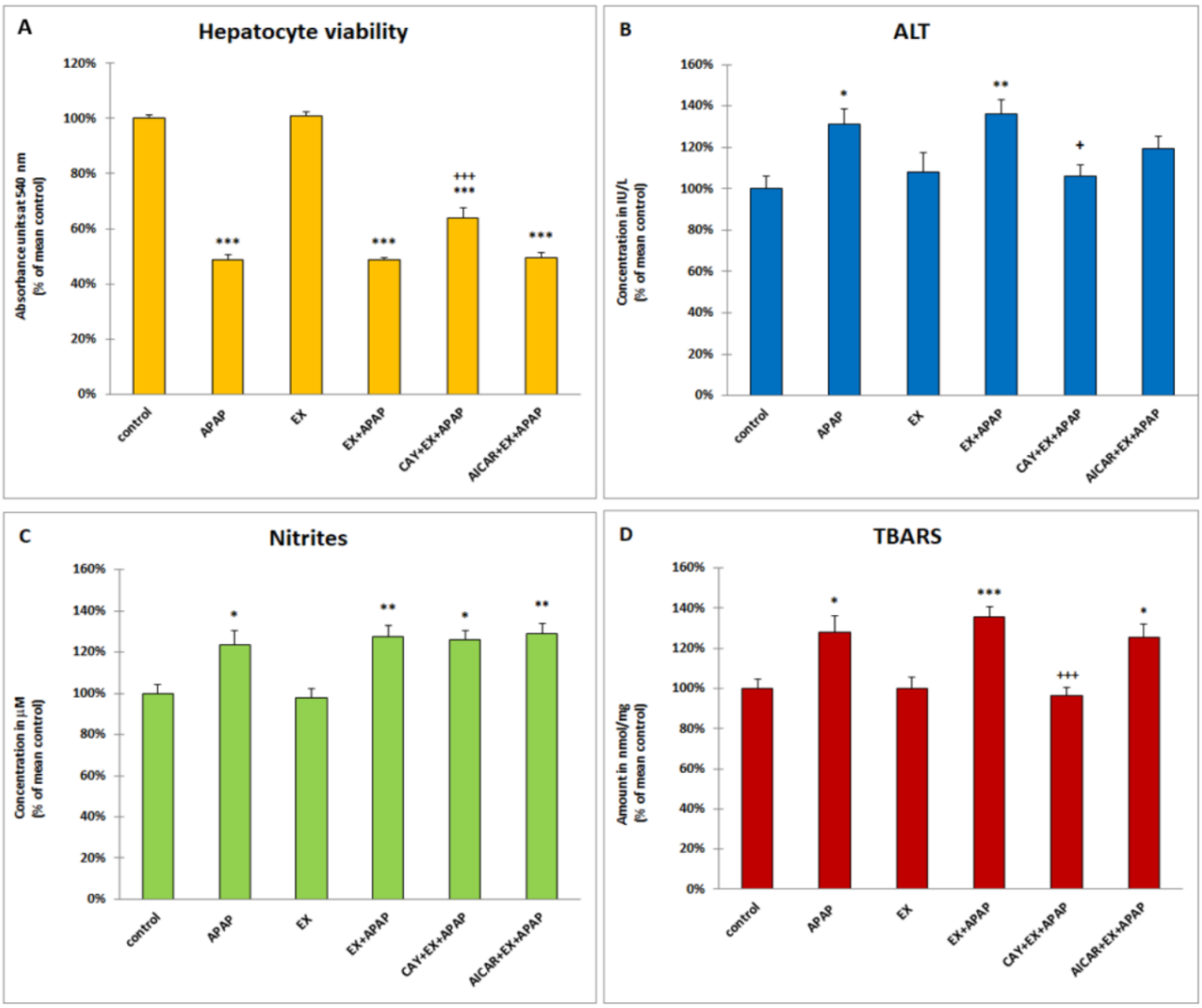

Figure 14. *Effects of specific SIRT1 modulators (activator – CAY10591/CAY and inhibitor – EX-527/EX) and AMPK activator (AICAR) in acetaminophen (APAP)-induced hepatotoxicity on A) hepatocyte viability, B) alanine aminotransferase (ALT) release, C) nitrite (NO_2^-) production, and D) thiobarbituric acid reactive substances (TBARS) formation in in vitro experiments after 24 hours. 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.*

AMPK activity

Results from *in vitro* experiments demonstrate that the hepatotoxic effect of APAP was coupled with a significant decrease in AMPK activity (**Fig. 15)**. This was apparent already after 4 hours of hepatocyte incubation when AICAR and CAY10591 in combination with APAP significantly increased AMPK activity and CC alone decreased it as suggested (**Fig. 15A)**. However, the suppression of AMPK activity by APAP was not further influenced by neither AMPK nor SIRT1 modulators after 24 hours **(Fig. 15B, C)** (Njeka Wojnarová *et* al. 2022).

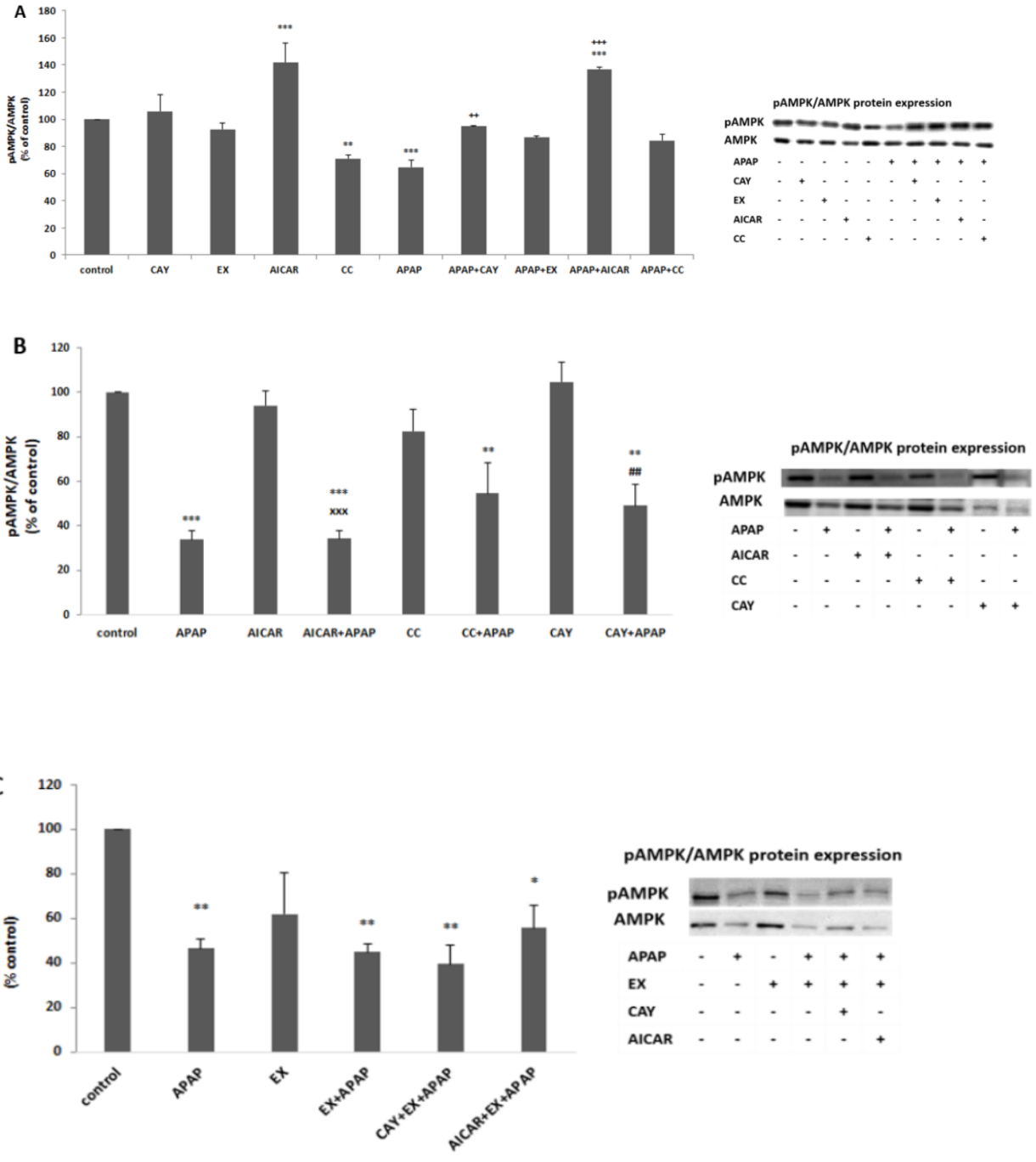

Figure 15. *Effects of acetaminophen (APAP), specific modulators of AMPK (activator – AICAR and inhibitor - Compound C, CC) and SIRT1 (activator - CAY10591/CAY and inhibitor EX-527/EX) on AMPK activity in cultured primary rat hepatocytes after 4 hours (A) and 24 hours (B, C). Activity of AMPK was calculated 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.*

SIRT1 activity and expression

APAP significantly inhibited the deacetylase activity and expression of SIRT1 in cultured hepatocytes incubated for 4 and 24 hours, respectively **(Fig. 16**). CAY10591 but not AICAR significantly increased SIRT1 activity, whereas EX-527 and even AMPK inhibitor (Compound C) markedly decreased it (**Fig. 16A**). The addition of AICAR to EX+APAP further decreased SIRT1 protein levels (**Fig. 16B**) (Njeka Wojnarová *et* al. 2022).

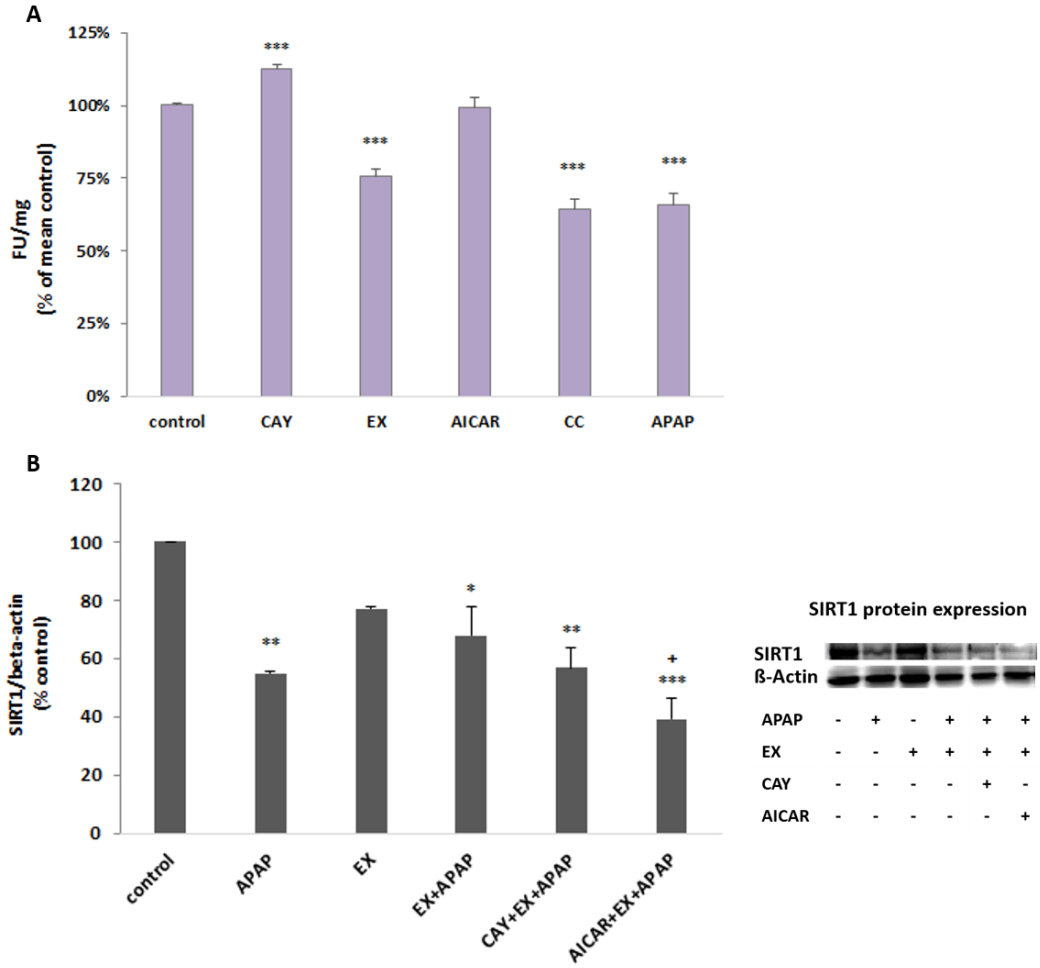

Figure 16. *Effects of acetaminophen (APAP), specific modulators of SIRT1 (activator – CAY10591/CAY and inhibitor – EX-527/EX) and AMPK activator (AICAR) on: A) SIRT1 activity after 4 hours, and B) SIRT1 protein expression after 24 hours; both in cultured primary rat hepatocytes. Quantitative data of 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.*

7 Disscussion

Liver diseases involve a wide range of liver pathologies from fatty liver, hepatitis, and fibrosis to cirrhosis and hepatocellular carcinoma (Li *et* al. 2018) and represent a significant cause of morbidity and mortality worldwide (Asrani et al. 2019). In most cases liver diseases are associated with inflammation and oxidative stress leading to destruction of liver parenchyma with loss of liver function (Wang *et* al.*,*2020). In this context, current evidence describes the beneficial properties associated to polyphenols that own a variety of beneficial effects for the liver (Simón *et* al. 2020). Earlier experimental studies of natural polyphenolic compounds such as silymarin, curcumin and resveratrol have shown strong hepatoprotective potential probably due to their anti-inflammatory, antioxidant and liver regenerative capabilities (Farghali *et* al., 2015).

D-Galactosamine (D-GalN) and lipopolysaccharide (LPS) is an ideal *in vivo* model causing acute liver injury in experimental animals where oxidative stress plays a major role. The combined effects of these two agents produce more severe form of liver injury (Nakama *et* al., 2001; Kutinová Canová *et* al., 2008; Ingawale *et* al., 2014). In our first study, D-GalN and LPS markedly increased the plasma levels of transaminases confirming that acute liver injury occurred. Likewise, D-GalN/LPS treatment exacerbated lipid peroxidation by increase in the TBARS and conjugated dienes. Moreover, resveratrol alleviated hepatotoxicity in all parameters and EX-527 reversed its hepatoprotective effects suggesting role SIRT1 catalytic activity in hepatoprotection. In addition, D-GalN/LPS-induced hepatotoxicity downregulated SIRT1 protein expression in rat liver. The exact 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. As an illustration, LPS may execute induction of iNOS and subsequent peroxynitrite anion which can oxidize a wide array of molecules within cells including DNA and lipids. One of the explanation could be that the miRs suppress directly expression of SIRT1 in response to oxidative stress. This may worsen liver damage (Kemelo, Wojnarová *et* al., 2014; Konovalova *et* al., 2019).

For our further 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 (Wojnarová *et* al., 2015; Njeka Wojnarová *et* al., 2022). The liver impairment was much lower after APAP treatment than after LPS/D-GalN and did

not lead to fulminant hepatic failure. 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).

Mild APAP-induced hepatic impairment caused slight increase in number of apoptotic hepatocytes without any signs of necrosis in our *in vivo* study (Wojnarová *et* al., 2015), which may suggest that apoptosis precede necrosis or can lead to necroptosis or pogrammed necrosis (Jaeschke *et* al., 2018). APAP-induced oxidative stress and mitochondrial dysfunction plays the central role in the pathogenesis of acute APAP-induced liver injury. APAP toxicity consists of multi-stages and multi-signaling pathways, including APAP metabolism, oxidative stress, endoplasmic reticulum stress, autophagy, sterile inflammation, microcirculatory dysfunction, and compensatory liver repair and regeneration (Ramachandran and Jaeschke, 2019). APAP is mainly converted to NAPQI by CYP2E1. The excess NAPQI depletes GSH, resulting in the covalent attachment of excess NAPQI to sulfhydryl groups in other proteins, particularly in mitochondrial proteins. This leads to mitochondrial dysfunction, which produces oxidative stress and excess superoxide free radicals, ultimately resulting in mitochondrial dysfunction, DNA fragmentation and finally hepatocyte necrosis (Gao *et* al., 2020; Jaeschke *et* al., 2020). Some studies support the hypothesis that APAP hepatotoxicity is mediated by necrosis or programmed necrosis and doesn´t involve apoptosis (Jaeschke *et* al., 2018). Evidence of APAP-induced liver failure show that there was not activation of caspase after overdosing APAP but there was possibility that electrophilic metabolites of acetaminophen may inactivate the caspases (Lawson *et* al., 1999). Really, Gujral *et* al. (2002) compared the level of hepatocytes with criteria of apoptosis and with criteria for necrosis after the APAP administration. The first cell fraction was about 1% of all parenchyma cells and the second one involved 40-60% of all hepatocytes. Therefore, there is confirmed the thesis that oncotic necrosis is a principal mechanism of hepatocytes death after APAP poisoning (Gujral *et* al., 2002). This is in line with our *in vitro* results that the cell death induced by APAP is related to necrosis rather than apoptosis because caspase-3 levels were not affected (Njeka Wojnarová *et* al., 2022).

Although results from our pilot study showed no significant changes on the SIRT1 protein expression in APAP model of liver injury compared with reduced SIRT1 enzyme activity (by 34 % *in vitro* and 20 % *in vivo*) after APAP treatment (Wojnarová *et* al., 2015), 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 *in vitro* (Njeka Wojnarová *et* al., 2022). The same trend was also reported in publication of Rada *et* al. (2018) where SIRT1 protein levels were decreased in the liver of humans and mice in APAP-induced liver injury. Above that, they observed the retained protein levels of SIRT1 in the liver of APAP-treated SIRT1-Tg mice (mice with moderate overexpression of SIRT1) were associated with reduced liver injury as assessed by histology and other biomarkers. These differences in the capture of SIRT1 protein expression could be due to various factors and conditions of the above-mentioned experiments, e.g. fulminant liver failure (D-GalN/LPS) versus drug-induced liver injury (APAP), 3 and 6 hours versus 24 hours from the beginning of the administration of harmful substances till liver/hepatocyte sampling, rat versus mouse model etc. (Njeka Wojnarová *et* al., 2022).

In addition, APAP-reduced SIRT1 activity was accompanied by simultaneous enhanced oxidative stress as evidence from increased TBARS, CD, and nitrite levels and pronounced liver and hepatocyte injury both *in vivo* and *in vitro* (Wojnarová *et* al., 2015; Njeka Wojnarová *et* al.,2022). It could be explained by involvement of NF-κB in process of APAP-induced inflammation (Rada *et* al., 2018). The relationship between NF-κB and SIRT1 is antagonistic, decreased nuclear SIRT1 level/activity increase NF-κB RelA/p65 activity and amplify proinflammatory gene expression (Wang *et* al*.,* 2020; De Gregorio *et* al., 2020). Rada *et* al. (2018) revealed that *in vivo* administration of the NF-κB inhibitor protected from APAP-mediated acute hepatotoxicity.

As mentioned above, by targeting many different molecules such as p53, PGC-1α, FOXO/ manganese superoxide dismutase (MnSOD) pathway, and NF-κB, SIRT1 is capable to regulate numerous vital signaling pathways, including DNA repair and apoptosis, cell proliferation, damage repair, muscle and fat differentiation, neurogenesis, mitochondrial biogenesis, glucose and insulin homeostasis, hormone secretion, cell stress responses, and circadian rhythms (Hwang *et* al., 2013; Huang *et* al., 2017; Jaeschke *et* al., 2020). In addition, earlier studies reported that liver specific SIRT1 deficiency caused an increase in ROS production (Yan *et* al., 2019). Moreover, SIRT1 regulates the levels of inflammation and protects against oxidative stress which plays a key role in the pathogenesis of DILI where the overproduction of ROS, including free radicals, and reactive nitrogen species (RNS) can lead to damage of cellular components (Wojnarová *et* al., 2015). Recent studies revealed that SIRT1 protected cells against oxidative stress by regulating FOXO-mediated transcription and inhibiting NADPH oxidase activation (Wang *et* al., 2016). In accordance with that are our

results demonstrating that pretreatment with the activator of SIRT1 significantly suppressed oxidative stress (e.g. the formation of TBARS) induced by APAP alone and in combination with SIRT1 inhibitor (Njeka Wojnarová *et* al.,2022).

To better understand 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.
Our experiments demonstrated that the hepatotoxic effect of APAP is associated with a significant decrease in AMPK activity throughout *in vitro* experiments. It was really found that APAP-induced liver injury causes hepatocyte depletion of ATP by inhibiting mitochondrial function. Hwang *et* al. (2015) observed that APAP administration declines active phosphorylation of the Thr172 active site of AMPK, following a model resembling to that observed with ATP loss. They hypothesized that ATP depletion caused by APAP might correlate with inhibition of AMPK activity. Further explanation could be the duration of time after administration of APAP (Njeka Wojnarová *et* al., 2022). As it was mentioned above, deficiency of ATP is significantly related to hepatic cell death induced by APAP which is primary caused by mitochondrial dysfunction. It was reported that fall in active phosphorylation of AMPK in response to APAP cooperate with decrease ATP levels *in vivo* so there was a hypothesis that induction of production of ATP via AMPK stimulation can ameliorate APAP-induced liver failure (Hwang *et* al., 2015). Really, inductor of AMPK protected mice liver against APAP induced liver injury via ATP synthesis by anaerobic glycolysis. Although administration of AMPK inductor prevented the loss of intracellular ATP, APAP-induced reduction of mitochondrial dysfunction was not improved (Hwang *et* al., 2015). AMPK is also a nutrient and energy sensor, and AMPK activation inhibits the formation of ROS by NADPH oxidase (Wang *et* al., 2016). Moreover, it has been reported that the activated AMPK could inhibit NF-κB signaling through its downstream target molecules such as SIRT1, FOXO, and PGC-1α (Hwang *et* al., 2015; Zhu *et* al., 2018).

We revealed that pretreatment with either AICAR or CAY10591 significantly increased the AMPK activity only after 4 hours of hepatocyte incubation with APAP. 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).

Compound C (6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine) also known as dorsomorphin, is the only small AMPK inhibitor that has been broadly utilized to study the AMPK signaling pathway (Njeka Wojnarová *et* al., 2022). It was shown that inhibition of AMPK activity by CC or by transfection with a dominant negative form of AMPK near entirely suppressed autophagy in hepatocytes. It can be explained by the direct effect of AMPK on the mammalian target of rapamycin (mTOR). The activation of AMPK inhibits mTOR and thus increasing autophagy (Morita et al. 2015). Autophagy is induced in response to cellular stressors such as starvation, hypoxia, nutrient and growth factor deprivation, and oxidative injury. Autophagy promotes cell survival through its basic function of degrading intracellular components. The diverse cellular functions of autophagy suggest that the liver is highly dependent on autophagy for both normal function and pathophysiological states with its ability to prevent or promote the development of different hepatic disease including toxin-, drug- and ischemia/reperfusion-induced liver injury, fatty liver, viral hepatitis and hepatocellular carcinoma or liver cirrhosis (Czaja et al. 2013). When autophagy was enhanced by treatment with rapamycin, APAP-induced necrosis was significantly inhibited in cultured primary hepatocytes and mouse liver (Ni *et* al., 2012). As autophagy and apoptosis are interrelated and play important role in liver injury (Wang *et* al., 2015), we can hypothesize that inhibition of autophagy by CC due to AMPK inhibition could lead to caspase-3 activation with consequent apoptosis and the intensification of APAP-induced hepatotoxicity in our study. The role of AMPK in this process can be supported by the fact that the AMPK activator, AICAR, reversed the detrimental effect of CC on APAP-induced hepatotoxicity (Njeka Wojnarová *et* al., 2022).

To futher investigate what and how important role does SIRT1 plays in the process of hepatoprotection/hepatotoxicity we performed also experiments with EX-527 *in vitro*. Our data implied that pretreatment with EX-527 only slightly enhanced APAP toxicity. Western blot data surprisingly showed that EX-527 down-regulated SIRT1 expression. Besides that, the 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, the addition of CAY 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 (Njeka Wojnarová *et* al., 2022). This highly resembles the results of our first *in vivo* study with combination of EX+RES+D-GalN/LSP (Kemelo, Wojnarová *et* al., 2014). Therefore, the catalytic activity of SIRT1 is equally important in the hepatoprotective effects of SIRT1 modulators. Moreover, these events and other findigs of our research group give an impression that the cytoprotective effects of SIRT1 occur within a limited range of its expression (Farghali *et* al., 2019).

The above discussion illustrates that AMPK and SIRT1 pathways are at some extent interrelated. Hence, pharmacologic modulation of AMPK and SIRT1 activity could be a future major step in the understanding of DILI.

8 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, 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.

In addition, 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.

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10 APPENDIX (ORIGINAL MANUSCRIPTS RELATED TO THE THESIS)

Publication 1:

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.*

Publication 2:

Wojnarová L, Kutinová Canová N, Farghali H, Kučera T. Sirtuin 1 modulation in rat model of acetaminophen-induced hepatotoxicity. Physiol Res. 2015;64(Suppl 4): S477-S487; IF: 1.88 (2021-2022).

Publication 3:

Njeka Wojnarová L, Kutinová Canová N, Arora M., Farghali H (2022) Differentiated modulation of signaling molecules AMPK and SIRT1 in experimentally drug-induced hepatocyte injury. *(Accepted for publication in Biomedical Papers after revision);* IF: 1,245 (2020).