

# Sirtuin 1 Modulation in Rat Model of Acetaminophen-Induced Hepatotoxicity

L. WOJNAROVÁ<sup>1</sup>, N. KUTINOVÁ CANOVÁ<sup>1</sup>, H. FARGHALI<sup>1</sup>, T. KUČERA<sup>2</sup>

<sup>1</sup>Institute of Pharmacology, First Faculty of Medicine, Charles University, Prague, Czech Republic,

<sup>2</sup>Institute of Histology and Embryology, First Faculty of Medicine, Charles University, Prague, Czech Republic

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## Summary

Sirtuin 1 (SIRT1) is involved in important biological processes such as energy metabolism and regulatory functions of the cell cycle, apoptosis, and inflammation. Our previous studies have shown hepatoprotective effect of polyphenolic compound resveratrol, which is also an activator of SIRT1. Therefore, the aim of our present study was to clarify the role of SIRT1 in process of hepatoprotection in animal model of drug-induced liver damage. Male Wistar rats were used for both *in vivo* and *in vitro* studies. Hepatotoxicity was induced by single dose of acetaminophen (APAP). Some rats and hepatocytes were treated by resveratrol or synthetic selective activator of sirtuin 1 (CAY10591). The degree of hepatotoxicity, the activity and expression of the SIRT1 were determined by biochemical, histological and molecular-biological assessments of gained samples (plasma, liver tissue, culture media and hepatocytes). Resveratrol and CAY attenuated APAP-induced hepatotoxicity *in vivo* and *in vitro*. Moreover, both drugs enhanced APAP-reduced SIRT1 activity. Our results show that modulation of the SIRT1 activity plays a role in hepatoprotection. Synthetic activators of SIRT1 would help in understanding the role of SIRT1 and are therefore a major boost towards the search for specific treatment of liver disease.

## Key words

SIRT1 • Resveratrol • Acetaminophen • CAY10591 • Liver

## Corresponding author

L. Wojnarová, Institute of Pharmacology, First Faculty of Medicine, Charles University, Albertov 4, 128 00 Prague 2, Czech Republic. E-mail: lea.wojnarova@lf1.cuni.cz

## Introduction

Drug-induced liver injury (DILI) is the most frequent cause of hepatic dysfunction and it is the most common cause of acute liver failure in the United States. Over 1000 medications and herbal products have been associated in the development of DILI. In many studies, acetaminophen-induced liver injury is commonly used as a standard hepatotoxicity model in rodents to test the hepatoprotective effects of herbal and other compounds. Acetaminophen (APAP, paracetamol) belongs to the most widely used analgetic and antipyretic drugs. Overdose can cause hepatotoxicity in experimental animals and humans (Brune *et al.* 2015, Ingawale *et al.* 2014, Jaeschke *et al.* 2013, McGill *et al.* 2012). But what is a maximum safe dose of APAP? The safety of this drug and its toxic reactions are influenced by a large number of factors, both known and unknown (Kaplowitz *et al.* 2004). It is well established that APAP is detoxified by three major pathways. The majority of APAP dose is conjugated with glucuronate and sulfate and remaining part of APAP especially in its excess is metabolized by an alternative pathway known as cytochrome P450 oxidase enzyme system (mainly CYP2E1). The latter metabolic pathway leads to the formation of a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI). NAPQI is detoxified especially by glutathione (GSH) to form APAP-SG conjugate (Kučera *et al.* 2011, Roušar *et al.* 2009). In the case of GSH depletion, this metabolite covalently binds to cysteine groups on protein, forming APAP-protein adducts in the cell and in mitochondria (Jaeschke *et al.* 2013, Wang *et al.* 2015). It results in

conformational and functional changes in cellular proteins, increased reactive oxygen and reactive nitrogen species production (ROS, RNS), lipoperoxidation, mitochondrial permeability and transition pore opening. These are key events leading to cell death (Bajt *et al.* 2006, Du *et al.* 2015, Jaeschke *et al.* 2013, Karthivashan *et al.* 2015, Roušar *et al.* 2009). Despite the processes mentioned above, the detailed understanding of the mechanism of APAP toxic effect still remains unknown (Kaplowitz *et al.* 2004, Roušar *et al.* 2009).

Our previous studies have shown hepatoprotective effect of resveratrol in lipopoly-saccharide/D-Galactosamine (LPS/D-GAIN) model of hepatotoxicity (Černý *et al.* 2009, Farghali *et al.* 2014). Resveratrol pretreatment led to the overall improvement in hepatotoxic markers and morphology after the hepatic insult. Several studies have also highlighted the hepatoprotective properties of resveratrol (Bishayee *et al.* 2010). Resveratrol (trans-3,4',5-trihydroxystilbene) is a polyphenolic compound found in a plant sources such as peanuts, grapes and red wine and it's one of the extensively studied natural product, with wide ranging biological activity and clinical potentials. Many studies have suggested that resveratrol possess many pharmacological actions including anti-aging, anticarcinogenic, anti-inflammatory, and antioxidant properties (Wang *et al.* 2015). Moreover, resveratrol is also the most widely investigated activator of sirtuin 1 (SIRT1, silent information regulator T1) (Baur 2010, Kutinová Canová *et al.* 2012, Ruderman *et al.* 2010, Silva and Wahlestedt 2010).

SIRT1 is the evolutionarily conserved NAD<sup>+</sup>-dependent histone/protein deacetylase whose function is linked to cellular metabolism. It plays a role in metabolism of numerous tissues, including liver, muscle, adipose tissue, heart, and endothelium (Chang and Guarente 2014). Moreover, an important target for SIRT1 is nuclear factor-kappa B (NF-κB), a regulator of many processes, including the cell cycle, proliferation, apoptosis, and inflammation (Yang *et al.* 2007). Several small-molecule SIRT1 activators, among others e.g. CAY1059 and SRT1720, were synthesized with higher pharmacologic potency compared with the traditionally studied SIRT1 activator resveratrol (Feige *et al.* 2008, Nayagam *et al.* 2006).

The main goal of the present study was to clarify the role of SIRT1 in the process of hepatotoxicity/hepatoprotection in animal model of APAP-induced acute liver injury and to investigate SIRT1 activation and

protein expression under treatment with natural SIRT1 activator resveratrol and selective synthetic SIRT1 activator CAY10591 (CAY) both *in vivo* and *in vitro*.

## Materials and Methods

### Materials

CAY10591 was obtained from Cayman Pharma (Neratovice, Czech Republic) and collagenase type IV from Sevapharma (Prague, Czech Republic). Bio-Rad protein DC assay dye reagent was from Bio-Rad (Prague, Czech Republic). Acetaminophen, resveratrol (3,4',5-trihydroxy-trans-stilbene, 5-[(1E)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol ≥99 % GC), SIRT1 assay kit, PEG400, saline, tris-HCl, Nonidet P40, dimethyl sulfoxide (DMSO), isopropyl alcohol, Tween 20, 2-thiobarbituric acid, tetraethoxypropane, trichloroacetic acid, sodium dodecyl sulphate, ammonium persulfate, methanol, glycine, N,N,N',N''-tetramethylethylenediamine, NaCl, KCl, Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, filter paper, nitrocellulose membrane, anti-mouse IgG (whole molecule) peroxidase antibody and mouse monoclonal anti-beta-actin antibody, protease and phosphatase inhibitor cocktails, and other standard chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic). SirT1 (1F3) mouse mAb antibody was from Cell Signaling Technology through BioTech (Prague, Czech Republic). Non-fat dry milk was also from BioTech. Water for injection 100 % w/v was obtained from Baxter (Czech Republic, Prague).

### Animals

Outbred male Wistar rats (Velaz-Lysolaje, Czech Republic) of 200-350 g body weight were used throughout the present studies. 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 protocol was approved by the Faculty Ethical Committee.

### Drug treatments

The rats were divided randomly into five groups of six animals each and treated as follows: 1. Control – polyethyleneglycol 400 (40 %) + DMSO, 2. CAY – CAY 0.5 mg/kg in DMSO, 3. APAP – acetaminophen 1 g/kg in PEG 400 (40 %), 4. APAP+CAY – acetaminophen 1 g/kg

in PEG 400 (40 %) + CAY 0.5 mg/kg in DMSO, 5. APAP+RES – acetaminophen 1 g/kg in PEG 400 (40 %) + RES 30 mg/kg in DMSO.

To induce acute liver injury in rats, 1 g/kg of APAP was injected intraperitoneally from a 0.2 g/ml solution in 40 % PEG 400 (PEG 400 in saline). It was followed after one hour by intraperitoneal injection of CAY10591 or resveratrol, both dissolved in DMSO of total volume of 1.5 ml/kg. 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 alanine aminotransferase (ALT), total bilirubin and nitric oxide (NO) as nitrite ( $\text{NO}_2^-$ ) levels. Their liver samples were immediately homogenized in cooled Tris-HCl buffer or snap frozen in liquid nitrogen for Western blot analysis. The liver homogenates were used for determination of total lipid peroxidation assessed as thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD).

#### *Isolation and culture of primary rat hepatocytes*

Hepatocytes were isolated from untreated 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 hepatocyte cell viability was greater than 85 %. Cells were seeded on collagen-coated polystyrene Nunclon™ dishes at density of 104 000 viable cells/cm<sup>2</sup>. 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<sub>2</sub> throughout the study. Unattached hepatocytes were removed 3 h after seeding and remaining hepatocytes further cultured in fresh complete medium overnight. Hepatocytes were then treated with fresh medium alone or with acetaminophen (APAP, 5 mM). Thirty minutes later, vehicle with DMSO (0.1 % of final concentration), resveratrol (RES, 20 μM) or CAY10591 (CAY, 30 μM) were added to respective hepatocyte cultures. After 24 h, 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 and lysed in RIPA buffer containing protease inhibitor cocktail. The homogenates were centrifuged at 14,000 g for 10 min at 4 °C and thereafter used for SIRT1 activity measurement.

#### *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 suitable for our study and to measure hepatocyte viability at the end of *in vitro* experiments following test description by Kutinova Canova *et al.* (2008).

#### *Biochemical analysis*

ALT and bilirubin concentrations in the medium or plasma samples were measured using customized diagnostic kits according manufacturer's instruction (Vian Diagnostics, Prague, Czech Republic).

Medium  $\text{NO}_2^-$ , the stable end-product of NO oxidation, was determined spectrophotometrically by using Griess reagent (1 % sulfanilamide, 0.1 % naphthylethylenediamine, 2.5 % trihydrogenphosphoric acid). The absorbance at 540 nm was recorded and the  $\text{NO}_2^-$  levels were calculated from  $\text{NaNO}_2$  standard curve.

#### *Evaluation of oxidative stress parameters*

The measurement of homogenate levels of TBARS and conjugated dienes were carried out according to Farghali *et al.* (2009).

#### *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 manual instruction of 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).

#### *Histological evaluation*

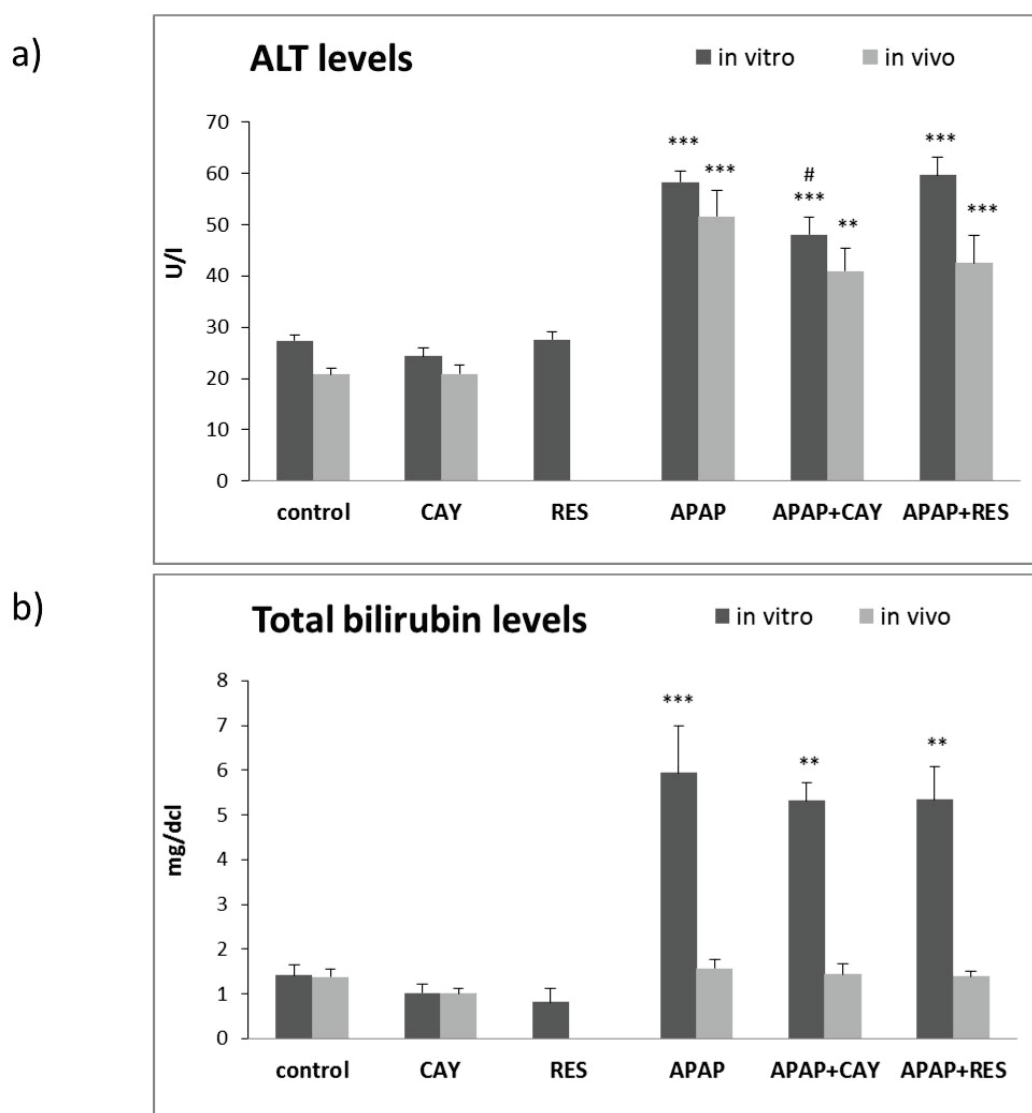
After the excised liver (1 cm<sup>3</sup>) fixation in 4 % paraformaldehyde in PBS, thin tissue paraffin sections (5 μm) were cut by microtome, stained with hematoxylin and eosin and examined by light microscope.

#### *Immunoblotting*

Isolated liver samples were lysed with RIPA buffer (2 M TRIS, 5 M NaCl, 0.5 M EDTA, NP-40, NAF) and homogenized with an electric homogenizer. The samples were then centrifuged for 20 min at 12,000 rpm at 4 °C and supernatant was collected. The

cell lysates were mixed (1:1) with sample buffer (2 x Laemmli buffer 950  $\mu$ l+50  $\mu$ l of  $\beta$ -mercaptoethanol) and then heated for 10 min at 90 °C. Proteins (assessed by Bio-Rad protein DC assay) from the tissue samples were separated on 10 % SDS-acrylamide gel and transferred to a nitrocellulose membrane by electrophoresis overnight at 4 °C. Membranes were blocked for 2 h with 5 % non-fat milk in Tris-buffered solution, at 4 °C. Membranes were then washed in a washing buffer (NaCl, KCl, Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, Tween, H<sub>2</sub>O). They were then incubated with mouse primary antibody against SIRT1 (1:1,000) or beta-actin primary antibody (1:5,000) and followed with

corresponding secondary rabbit antibody anti-mouse IgG HRP conjugate (1:80,000). For visualization was used chemiluminescence labeling with Super Signal West Pico Chemiluminescent Substrate (GenTiCA, Prague, Czech Republic). Bands were detected with the use Molecular Imager VersaDoc™ MP 5000 System and analyzed by Quantity One 1-D Analysis Software (Bio-Rad, Prague, Czech Republic). Optical densities of SIRT1 and beta-actin bands were normalized by the corresponding loading control and then to the mean of the corresponding control group (Lekić *et al.* 2013).



**Fig. 1.** 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 **a)** Alanine aminotransferase ALT and **b)** Bilirubin. Control (24 hour-vehicle treated hepatocytes or rats); CAY (CAY10591: 30  $\mu$ M *in vitro*, 0.5 mg/kg *in vivo*); RES (Resveratrol: 20  $\mu$ M *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  $\pm$  SEM (n=6); \*\* P<0.01, \*\*\* P<0.001 vs. respective control; # P<0.05 vs. APAP *in vitro*.

### Statistical analysis

The statistical significance of differences of mean scores was determined using one-way analysis of variance (ANOVA) followed by Bonferroni Multiple Comparisons 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  $\pm$  SEM (standard error of mean). All experiments were performed in means of 6 animals per group for *in vivo* experiments and 6 hepatocyte cultures per group for 3 independent *in vitro* experiments.

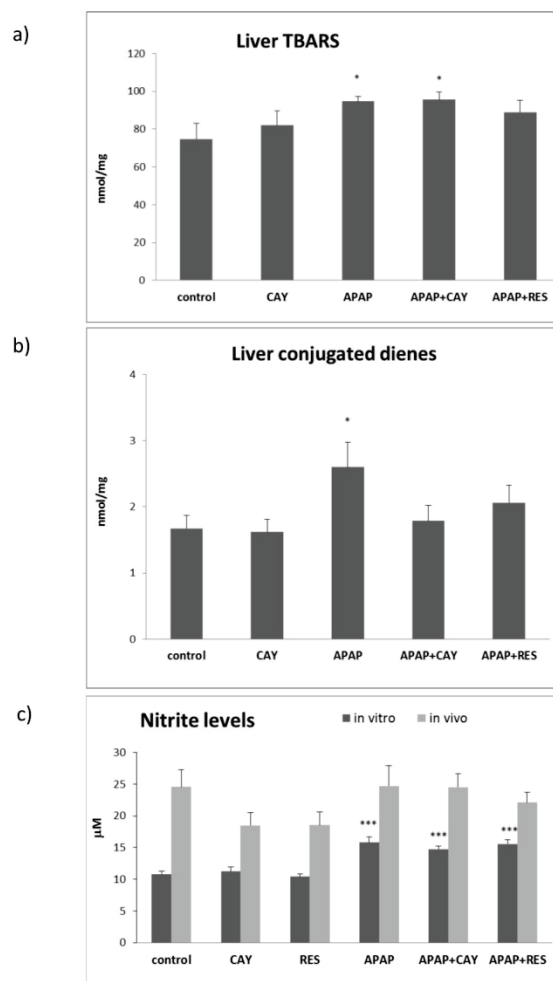
## Results

### Effects of CAY in comparison with RES treatment on liver function in APAP treated rats *in vitro* and *in vivo*

We measured the levels of ALT and bilirubin in plasma to detect the extent of hepatotoxicity (Fig. 1a). 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 significantly lowered the ALT parameters *in vivo*. Interesting difference was in *in vitro* experiments, where only CAY treatment slightly 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. 1b).

### Effects of CAY in comparison with RES treatment on $\text{NO}_2^-$ *in vitro* and *in vivo* and oxidative stress parameters in APAP treated rats *in vivo*

Figures 2a and 2b demonstrate that APAP treatment produced moderate increase of lipid peroxidation as evidenced by the formation of TBARS and conjugated dienes (CD), respectively, as a result of oxidative stress in liver. Single APAP treatment significantly increased both TBARS and CD ( $P < 0.05$ ) in homogenate. CAY following APAP treatment slightly reduced only CD levels in contrast with RES, which reduced the levels of both markers. Moreover, APAP significantly increased medium  $\text{NO}_2^-$  levels ( $P < 0.001$ ) *in vitro*. On the other hand,  $\text{NO}_2^-$  plasma levels were not significantly affected by any treatment even though CAY and resveratrol slightly reduced it (Fig. 2c).



**Fig. 2.** Effect of specific SIRT1 activator, CAY10591 in comparison with resveratrol treatment in APAP-induced hepatocyte/liver injury on the formation of **a)** Thiobarbituric acid reactive substances (TBARS) and **b)** Conjugated dienes (CD) *in vivo*, and **c)**  $\text{NO}_2^-$  production *in vitro* and *in vivo*. Control (24 hour-vehicle treated hepatocytes or rats); CAY (CAY10591: 30  $\mu\text{M}$  *in vitro*, 0.5 mg/kg *in vivo*); RES (Resveratrol: 20  $\mu\text{M}$  *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  $\pm$  SEM ( $n=6$ ): \*  $P < 0.05$ , \*\*\*  $P < 0.001$  vs. respective control.

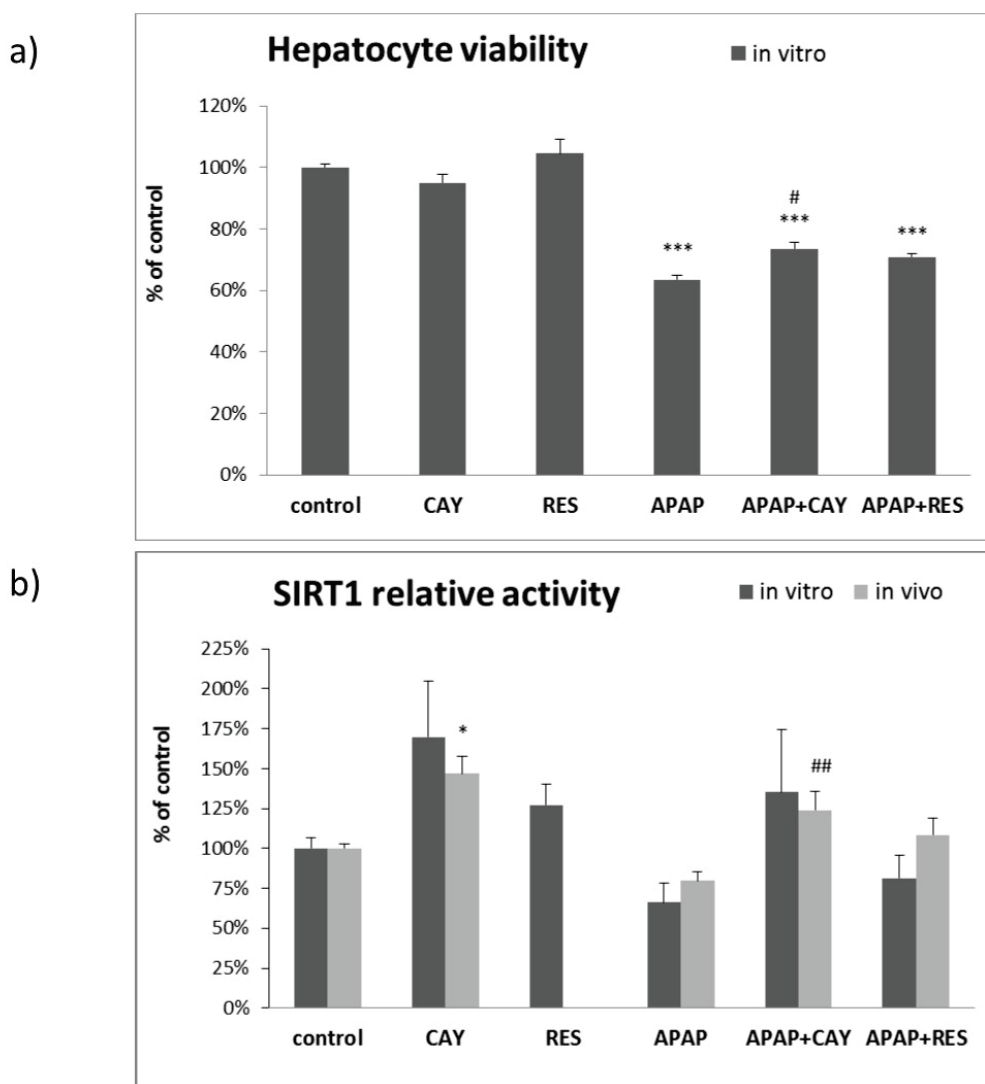
### Effects of CAY in comparison with RES treatment on hepatocyte viability *in vitro* and relative SIRT1 activity *in vitro* and *in vivo*

Measurement of hepatocyte viability (using MTT test) in *in vitro* experiments and relative SIRT1 activity both *in vitro* and *in vivo* are shown in Figure 3. 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 did not have toxic effect on hepatocytes in cell culture and that CAY more

potently increased APAP-reduced hepatocyte viability (Fig. 3a). APAP also markedly reduced SIRT1 enzyme activity (by 34 % *in vitro* and 20 % *in vivo*) as shown in Figure 3b. Furthermore, RES and especially CAY increased SIRT1 activity compared to control and APAP treatments. The same trend was observed as *in vitro* as *in vivo*. In summary, the relationship between activity and viability was indirectly proportional.

#### Effect of RES, CAY and APAP on SIRT1 expression levels in rats

In comparison with SIRT1 activity mentioned above (Fig. 3b), there were no significant changes on the SIRT1 expression in Western blot analysis (Fig. 4). According to our analysis, single dose treatment with APAP, RES and CAY had no effect on the total endogenous amount of SIRT1 expression.



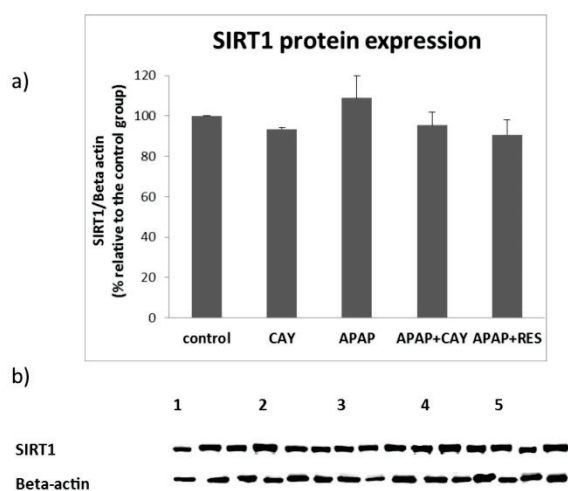
**Fig. 3.** Effect of specific SIRT1 activator, CAY10591, in comparison with resveratrol treatment in APAP-induced hepatocyte/liver injury on **a)** Hepatocyte viability in *in vitro* experiments and **b)** SIRT1 relative activity in *in vitro* and *in vivo* experiments. Control (24 hour-vehicle treated hepatocytes or rats); CAY (CAY10591: 30  $\mu$ M *in vitro*, 0.5 mg/kg *in vivo*); RES (Resveratrol: 20  $\mu$ M *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  $\pm$  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.

#### Histological observations

The general structure of the liver parenchyma of all rats was well-preserved. Liver trabecules were arranged radially around the central vein and portal

spaces were well visible (Fig. 5). No signs of steatosis, inflammation (except of rare small mononuclear infiltrates), hepatocellular necrosis or fibrosis were observed in the rat liver of control (Fig. 5a) and CAY

(Fig. 5b). However, there were visible several mitotic hepatocytes in otherwise normal liver parenchyma of these rats, especially in CAY treated ones (Fig. 5c). Histological changes in the liver induced by APAP were not significant (Fig. 5d). APAP caused slight increase in the appearance and number of apoptotic hepatocytes (Fig. 5e) and apoptotic bodies surrounded by a mononuclear infiltrate (Fig. 5f). 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. 5g) or resveratrol (Fig. 5h) to rats.



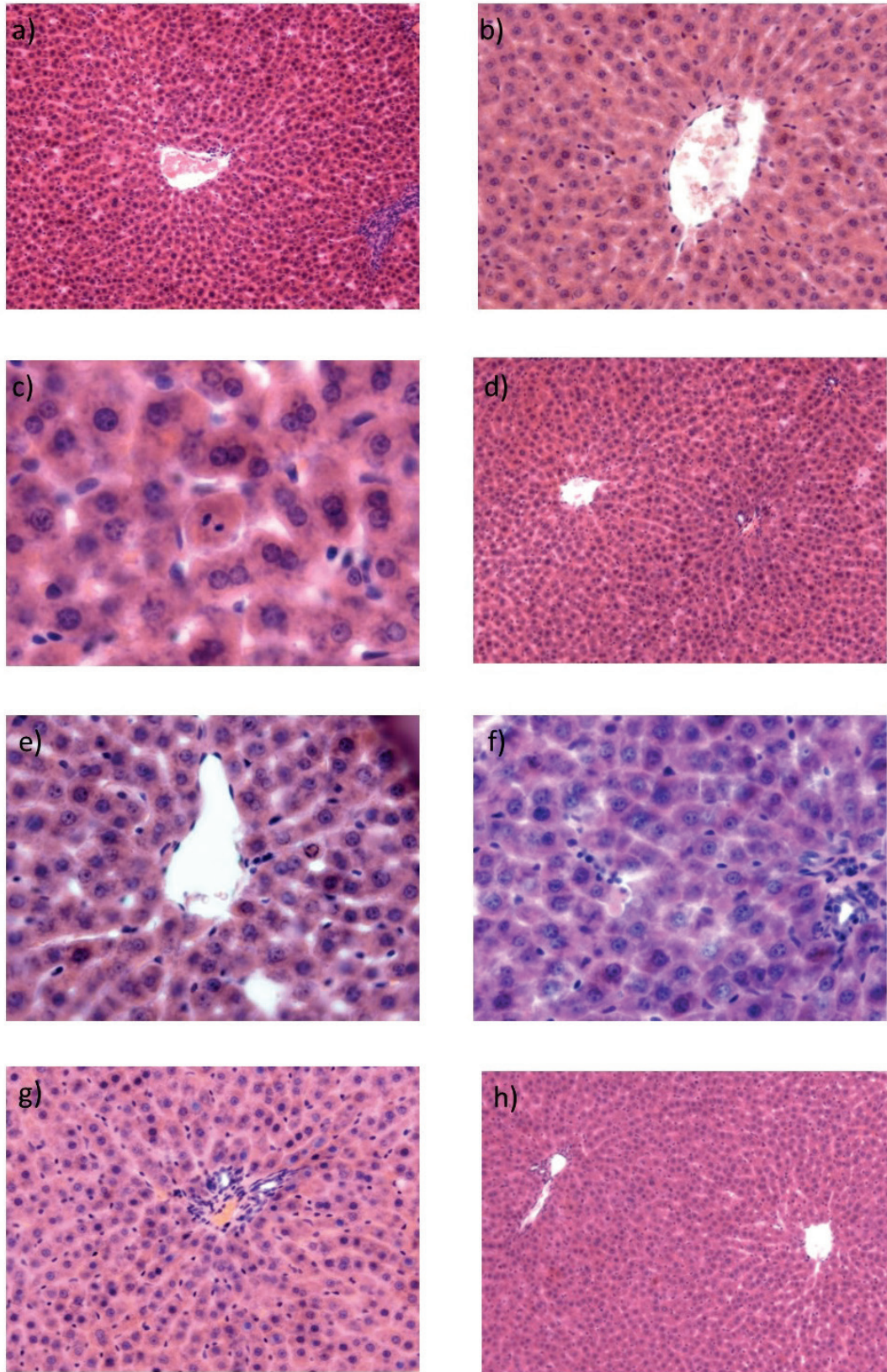
**Fig. 4.** Effect of specific SIRT1 activator, CAY10591, in comparison with resveratrol treatment in APAP-induced liver injury *in vivo* on **a)** quantification of SIRT1 expression levels by densitometry. The intensity of each panel was normalized to the intensity of corresponding beta-actin band. 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  $\pm$  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.

## Discussion

Our and other previous studies demonstrated that small polyphenolic molecules with antioxidant properties such as resveratrol reduced parameters of liver injury in fulminant hepatic failure induced by LPS/D-GAIN combination (Farghali *et al.* 2014, Kemelo *et al.* 2014, Lekić *et al.* 2013). We chose acute rat APAP intoxication followed by potentially hepatoprotective compounds RES and CAY for our present study. The liver impairment was much lower after APAP treatment than after

LPS/D-GAIN 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. 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 (Jemnitz *et al.* 2008). Many factors (e.g. interspecies variability in expression and/or activity of metabolizing enzymes and membrane transporters, formation of NAPQI adducts, oxidative stress, disruption of mitochondrial function, protein nitrosylation, DNA damage among others) contribute to processes leading to APAP-induced liver damage. The electrophilic metabolite NAPQI, which is mainly produced by CYP2E1, is conventionally thought to initiate the toxic processes in liver. Surprisingly, some of CYP2E1 activity data do not support that only formation of reactive metabolite alone can explain interspecies variability after APAP intoxication (Jemnitz *et al.* 2015). Moreover, liver regeneration, which is final outcome of APAP-induced sublethal liver injury, begins at about 12 h and continue until about 72 h post-dosing and can thus attenuate the final biochemical and histological parameters of hepatotoxicity evaluated from samples collected on a single late time point (e.g. 24 h) (Jaeschke *et al.* 2013).

In the present study, APAP significantly increased ALT and total bilirubin (specific markers of liver injury) levels with the same trend *in vivo* as *in vitro*. RES and CAY were administrated after pretreatment of APAP, and both RES and CAY significantly reduced ALT and also total bilirubin levels in *in vivo* experiments and enhanced APAP-reduced hepatocyte viability *in vitro*. As expected and in accordance with other studies (Kučera *et al.* 2012, McGill *et al.* 2012), we did not observe marked hepatocyte necrosis in liver of APAP treated rats as evidenced from histological findings. Interestingly, there were visible several mitotic hepatocytes in otherwise normal liver parenchyma of CAY treated rats. It can be explained by induction of hepatocyte proliferation due to increased SIRT1 activation leading to regulation SIRT1/p53 signaling pathway (Braidly *et al.* 2011, Hwang *et al.* 2013, Jaeschke *et al.* 2013, Wang *et al.* 2015).



**Fig. 5.** 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 h. Hematoxylin and eosin (magnification x 200 with a detailed view in c,e,f).



In the case of RES, its hepatoprotective effect may be due to its well-known antioxidant effects. Moreover, Wang *et al.* (2015) have found that repeated RES pretreatment of mice significantly increased SIRT1 protein expression and prevented APAP-induced hepatotoxicity revealed as decreased overall necrosis, possibly by inhibition of CYP-mediated APAP bioactivation, and regulation of SIRT1/p53 signaling pathway. RES can by this way promote hepatocyte proliferation and thus facilitate liver regeneration after APAP-induced liver injury. In the case of CAY, which is a selective activator of SIRT1, it can be hypothesized that its hepatoprotective effect may be due to the same SIRT1/p53 signaling pathway as RES. In contrast with study of Wang *et al.* (2015), there were no significant changes on the SIRT1 expression in Western blot analysis. This difference can be explained by only one application of lower dose (30 mg/kg) of RES in our experimental settings instead of several day lasting repeated high dose RES pretreatments in the study of Wang *et al.* (2015) that could induce SIRT expression. However, the SIRT1 activity was highly increased by RES and CAY addition to APAP both *in vivo* and *in vitro* that was accompanied by reduction of oxidative stress markers and hepatocyte/liver injury in our study. Furthermore, APAP reduced SIRT1 activity was accompanied simultaneous enhanced oxidative damage as evidences from increased TBARS, CD, and NO<sub>2</sub><sup>-</sup> levels and pronounced liver and hepatocyte injury both *in vivo* and *in vitro*. Indeed, some recent studies have shown that SIRT1 is a redox sensitive molecule that can be inactivated by oxidative stress (Braid *et al.* 2011, Hwang *et al.* 2013).

SIRT1 itself has multiple biological activities including gene silencing, stress resistance, apoptosis, regulation of cell proliferation, aging, inflammation, and acts as molecular sensor of nutritional status and protects against oxidative stress (Hwang *et al.* 2013, Kutinová Canová *et al.* 2011). However, it should be kept in mind that SIRT1 expression and activity can be modulated at different levels. It was shown that the age-related increasing SIRT1 expression in rat liver and other organs

is accompanied by a simultaneous reduction of SIRT1 activity (Braid *et al.* 2011). Moreover, RES is thought to activate SIRT1 allosterically (Farghali *et al.* 2013). It binds to the non-catalytic N-terminus of SIRT1 to cause a conformational change that lowers its Michaelis constant (Howitz *et al.* 2003). SIRT1 in turn deacetylates and suppresses transcription factors such as NF-κB responsible for induction of proinflammatory cytokines and pro-apoptotic factors (Yeung *et al.* 2004, Kemelo *et al.* 2014). Moreover, SIRT1 is also involved in regulation of SIRT1/p53 and other antiapoptotic (e.g. SIRT1/p66shc) signaling pathways (Shan *et al.* 2015, Wang *et al.* 2015). It can explain the almost complete absence of apoptotic hepatocytes in liver tissue sections of RES+APAP and CAY+APAP treated rats connected with enhanced SIRT activity unlike APAP alone.

In conclusion, resveratrol and CAY attenuated APAP-induced hepatotoxicity *in vivo* and *in vitro* in our study. Moreover, both drugs enhanced APAP-reduced SIRT1 activity but not SIRT1 expression. Therefore, our results show that modulation of the SIRT1 activity plays a role in hepatoprotection. To better understand the role of SIRT1 in hepatoprotection helped us novel specific SIRT1 activator CAY10591. Several other small molecules are being synthesized with higher pharmacologic potency compared with the polyphenolic SIRT1 activator resveratrol. In addition, class III histone deacetylases (sirtuins) are becoming increasingly recognized as important epigenetic drug targets in various diseases (Huber *et al.* 2011) thus suggesting modulation of sirtuin activity could provide an interesting and novel therapeutic option.

### Conflict of Interest

There is no conflict of interest.

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