A$_3$ adenosine receptor agonist, CF102, protects against hepatic ischemia/reperfusion injury following partial hepatectomy

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Abstract. Ischemia/reperfusion (IR) injury during clinical hepatic procedures is characterized by inflammatory conditions and the apoptosis of hepatocytes. Nuclear factor-κB (NF-κB), nitric oxide and the expression levels of inflammatory cytokines, tumor necrosis factor-α and interleukin-1 were observed to increase following IR and mediate the inflammatory response in the liver. CF102 is a highly selective A$_3$ adenosine receptor (A$_3$AR) agonist, and has been identified to induce an anti-inflammatory and protective effect on the liver via the downregulation of the NF-κB signaling pathway. The present study aimed to determine the effect of CF102 on protecting the liver against IR injury. The potential protective effect of CF102 (100 μg/kg) was assessed using an IR injury model on 70% of the liver of Wistar rats, which was induced by clamping the hepatic vasculature for 30 min. The regenerative effect of CF102 was assessed by the partial hepatectomy of 70% of the liver during 10 min of IR. CF102 reduced the levels of liver enzymes following IR injury. A higher regeneration rate in the CF102 treatment group was observed compared with the control group, suggesting that CF102 had a positive effect on the proliferation of hepatocytes following hepatectomy. CF102 had a protective effect on the liver of Wistar rats subsequent to IR injury during hepatectomy. This may be due to an anti-inflammatory and anti-apoptotic effect mediated by the A$_3$AR.

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Abbreviations: A$_3$AR, A$_3$ adenosine receptor; IL-1, interleukin 1; I/R, ischemia-reperfusion injury; NF-κB, nuclear factor-κB; NO, nitric oxide; PKB/Akt, protein kinase B/Akt; PCNA, proliferation cell nuclear antigen; SGOT, aspartate aminotransferase; SGPT, alanine aminotransferase; TNF-α, tumor necrosis factor-α

Introduction

Ischemia/reperfusion (IR) injury occurs when the cellular damage of a hypoxic organ is increased subsequent to the restoration of oxygen delivery. Warm IR injury may arise clinically in hepatic surgery, liver transplantation, hypovolemic shock and various types of toxic liver injury. The common denominator of the aforementioned conditions is the 'low-flow' state induced by liver hypoxia, which may lead to ischemic hepatitis. The Pringle maneuver has been established as the occlusion of the hepatic artery and the portal vein by cross-clamping the porta hepatis with a vascular clamp. It is frequently used to control bleeding during the repair of extensive liver wounds, hepatic resection and liver transplantation. However, this may result in hepatic IR injury, in the event that the clamping the hepatic vasculature is performed for a long period of time.

Two distinct phases of liver injury have been identified following warm IR injury. The initial phase (<2 h after reperfusion) is characterized by oxidant stress, which directly results in hepatocellular injury. The late phase (6-48 h after hepatic reperfusion) is an inflammatory disorder mediated by recruited neutrophils (1).

Cellular hypoxia has been identified as the key factor that leads to the activation of the transcriptional regulator, nuclear factor-κB (NF-κB), and triggers the release of other inflammatory mediators, including activated Kupffer cells and neutrophils, tumor necrosis factor-α (TNF-α), interleukin (IL)-1 and nitric oxide (NO), possibly in order to mediate the reperfusion injury process (2,3). The production of inflammatory mediators following the activation of Kupffer cells has been determined to be an important component for the pathophysiology of neutrophil-mediated injury during liver IR (4-6). In addition, previous studies have identified that apoptosis in hepatocytes is increased, and a decline in their regeneration during hepatic IR injury has been observed (4,7).

The importance of apoptotic cell death in hepatic IR has been investigated previously. According to previous studies, 40-60% of hepatocytes may undergo apoptosis during IR (8-10).

Ischemic preconditioning is a technique in which organs are exposed to brief periods of IR in order to prepare an organ for the subsequent effects of a longer period of IR exposure. Initially, it was used for coronary ischemia and protection

against myocardial infarction; however, at present, it used in various organs, including the brain, intestine, skeletal muscle and liver (11). The underlying cytoprotective mechanisms remain to be elucidated and may include an increase in the adenosine levels in the cell microenvironment, a decrease in NO levels, a reduction in TNF-α release, changes in energy metabolism and accelerated cell-cycle progression (12).

Previous studies that conducted experimental models of partial liver IR injury by 5-15 min of ischemia followed by 10 min of reperfusion led to hepatoprotective conditions, as a significant reduction in areas of hepatocyte necrosis occurred and the increase in transaminase levels was limited (13,14). A previous study determined that this narrowed hepatoprotective time window for ischemia is defined by the relative tissue concentrations of adenosine and xanthine (15). Additionally, purinergic signaling may have a protective role during hepatic IR injury (16).

Adenosine accumulates in the extracellular space following ischemia by binding to selective G-protein-associated membrane receptors, termed A_1, A_{2A}, A_{2B} and A_3, and has been established to promote cytoprotection. In a model of IR injury in mice, adenosine administration was observed to induce a protective effect on the liver (17). Additionally, the activation of the A_3 adenosine receptor (A_3AR) has been identified to exert cardioprotection, neuroprotection and pulmonoprotection against IR injury (18-22).

Upon activation of the A_3AR, specific signal transduction pathways are modulated, including the Wnt and NF-κB signaling pathways (23,24). The activation of A_3AR in inflammatory cells and tissues may lead to deregulation of the NF-κB signaling pathway and may result in reduced expression levels of phosphoinositide 3-kinase (PI3K), protein kinase B/Akt (PKB/Akt), IκB kinase, IκB and NF-κB, resulting in an anti-inflammatory effect (24-26). Additionally, apoptosis of inflammatory cells was observed downstream of PKB/Akt modulation, leading to upregulation of caspase 3 expression levels (27).

It is of note that activation of the A_3AR resulted in increased differentiation of tumor cells compared with normal cells (28). CF102, a selective agonist to A_3AR, induced the differentiation of normal and tumor cells and inhibited their proliferation, along with the induction of apoptosis in inflammatory cells, whereas it had a protective effect on normal cells (29).

Based on the aforementioned previous studies, the present study aimed to determine the ability of CF102 to protect the liver against IR injury.

Materials and methods

Reagents. The A_3AR agonist, CF102, a compound also termed 2-chloro-N6-(3-iodobenzyl)-adenosine-5'-N-methyl-uronamide (Cl-IB-MECA),was synthesized for Can-Fite BioPharma (Petah Tikva, Israel) by Albany Molecular Research, Inc. (Albany, NY, USA). A stock solution of 5 mg/ml was prepared in dimethylsulphoxide and further diluted in phosphate-buffered saline (PBS).

Animals. Animal handling was conducted according to the guidelines of the National Institutes of Health (Bethesda, MD, USA) and the Association for Assessment and Accreditation of Laboratory Animal Care (Frederick, MD, USA).

Male Wistar rats (n=76; age, 10 weeks; Harlan Laboratories, Inc., Jerusalem, Israel) were used in the present study. The body weights of the rats ranged from 275 to 300 g. The animals were provided with a nutritionally balanced rodent diet and water *ad libitum*. They were maintained under constant environmental conditions, temperatures range was 18-24°C and relative humidity range was 30-70% with 12 h light and 12 h dark cycle. The present study was performed following approval by the Can-Fite Biopharma, Institutional Ethic committee (Petah Tikva, Israel) , and in compliance with the Israel Animal Welfare Act, approval nos. 029_a123 and 029_a128.

IR injury procedure. A total of 28 male Wistar rats (275-300 g) were used for the IR injury procedure in the following groups: i) Naïve (n=4), laparatomy was performed without IR procedure; ii) control (n=12) no treatment or surgery; and iii) CF102 (n=12) laparatomy and IR were performed, followed by treatment with CF102. Rats were fasted 12 h prior to surgery, then anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg). Laparatomy was performed via a subcostal, bilateral incision. The main portal pedicle to the total liver was clamped for a period of 30 min, and the blood flow was restored by de-clamping. Subsequently, the rats were injected subcutaneously with 100 μg/kg CF102 (in 0.1 ml PBS) during reperfusion, and the compound was orally administered three times per day thereafter. The control rats received normal saline of the identical volume. All animals received 5 ml 5% glucose and 0.2 ml penicillin subcutaneously immediately following the operation. Postoperatively, the animals were maintained on a standardized pelleted diet and were supplied with water *ad libitum*. The present study was conducted over the course of 48 h.

The rats were anesthetized using isoflurane (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) 48 h after the operation, 2 ml blood samples were collected from the inferior vena cava and were centrifuged at 24, 000 x g for 15 min at 25°C. The supernatant was collected and serum aspartate aminotransferase (SGOT) and alanine aminotransferase (SGPT) levels were analyzed in the Medical Center Laboratories (Herzelia, Israel) to evaluate the extent of liver injury. The rats were sacrificed using CO_2 in a gas chamber. The livers were removed and fixed in 10% buffered formalin for pathological analysis and staining.

Partial hepatectomy. A total of 48 male Wistar rats (300-350 g) were used in the present study: The control group contained 22 individuals, the CF102 group had 22 and the naive group had 4 rats. Rats were fasted 12 h prior to surgery and anesthetized by intraperitoneal injection of ketamine (45 mg/kg) and xylazine (5 mg/kg). Laparatomy was performed via a subcostal, bilateral incision. The primary portal pedicle to the liver was identified and clamped prior to the hepatectomy procedure. A period of 10 min total liver ischemia was initiated, during which a 70% hepatectomy was performed. This was achieved by resection of the median and left lateral lobes, according to the technique previously described by Higgins and Anderson (30). Following 10 min of ischemia and

completion of 70% hepatectomy, blood flow to the remaining liver was restored by de-clamping. All animals received 5 ml 5% glucose and 0.2 ml penicillin subcutaneously immediately following the operation. Postoperatively, the animals were maintained on a standardized pelleted diet and were supplied with tap-water. Rats were injected subcutaneously with 100 µg/kg CF102 (in 0.1 ml PBS) during reperfusion, and the compound was administered orally three times per day thereafter. The control rats received normal saline at the identical volume. At 2, 4 and 48 h after surgery, rats were anesthetized with isoflurane and blood samples (2 ml) were collected from the rats' tails for analysis of SGOT and SGPT levels. Rats were sacrificed using CO_2 after 24 and 48 h, and the liver tissues were resected and weighed in order to calculate the hepatic regeneration rate, with subsequent fixing in 10% buffered formalin for further pathological analysis and staining.

Immunohistochemical staining and histology. Liver tissue specimens were fixed in 10% formalin, embedded in paraffin and cut into 5 mm thick sections.

Hematoxylin and eosin staining was performed in order to obtain a mitotic index (MI) by dividing the number of cells undergoing mitosis by the total cell number in 30 high-power fields. Additionally, the presence of hepatic injury and necrosis was also observed.

Apoptosis was examined by observation of DNA fragmentation using the ApopTag Peroxidase in Situ Detection kit (Chemicon; EMD Millipore, Billerica, MA, USA) for a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay following the manufacturer's protocol. Next, the sections were deparaffinized, re-hydrated and endogenous peroxidase was quenched with 20% hydrogen peroxidase in methanol for 20 min. The slides were then pretreated with proteinase K (BioVision, Inc., Milpitas, CA, USA) for 15 min at room temperature, and treated with an equilibration buffer for 10 s and terminal deoxynucleotidyl transferase enzyme (Sigma-Aldrich; Merck Millipore) for 1 h. Sections were incubated with an anti-digoxigenin conjugated antibody (cat. no. ab119345; 1:1,000; Abcam, Cambridge, UK), washed with PBS and developed with 3,3'-diaminobenzidine, counterstained with hematoxylin and eosin and then mounted.

To calculate the proliferation cell nuclear antigen (PCNA) labeling index, 5 mm sections were incubated with anti-PCNA antibody (cat. no. ab29; 1:1,000; Abcam). The proliferation of hepatocytes was calculated as the number of cells in the S-phase. PCNA-positive cells exhibited brownish-yellow or yellow nuclei. If brown particles were observed in the nucleus, the cells were considered to be positive.

Hepatic regeneration rate. The growth of residual liver lobes was assessed using the following equation: Hepatic regeneration rate (%) = C-(A-B) / A x100, where A is the estimated total liver weight prior to the hepatectomy (A was considered to be 3.4% of a rat's total weight) (31), B is the weight of the resected liver during the hepatectomy and C is the weight of the regenerated liver at the final resection (32).

Statistical analysis. The results were evaluated using Student's t-test. Comparisons between the mean values of the different
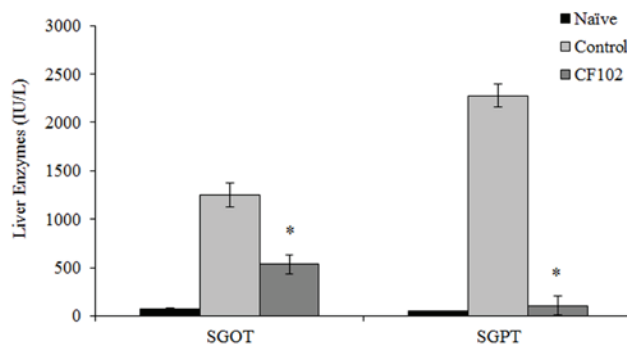

Figure 1. CF102 protects the liver following ischemia/reperfusion injury. Liver ischemia was performed on 70% of the liver for 30 min following reperfusion. Oral treatment with CF102 (100 µg/kg) or saline were administered three times daily until the rats were sacrificed. The liver enzymes, SGOT and SGPT, decreased upon CF102 treatment 48 h after reperfusion. *P<0.05. SGOT, aspartate aminotransferase; SGPT, alanine aminotransferase.

experiments were performed. Data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

CF102 attenuates liver IR injury in rats. In the present study, rats were exposed to 30 min of ischemia. The serum levels of SGOT and SGPT, which may be used as a proxy of the extent of liver damage, were significantly reduced in the CF102 treatment group when compared with the control group 48 h after surgery (P<0.05;Fig. 1).

Pathological analyses were performed in order to obtain additional qualitative assessment of liver injury. Paraffin-embedded slides of liver tissues were stained by hematoxylin and eosin 48 h after liver reperfusion. The upper panel of Fig. 2 shows healthy hepatocytes and no signs of necrosis or inflammation in the naïve group, whereas large areas of necrosis surrounded by numerous inflammatory cells and pyknotic nuclei were observed in liver tissues from the control group (middle panel). By contrast, liver tissues from the CF102-treated group did not exhibit areas of necrosis, and only a few inflammatory cells were visible (lower panel). These findings confirm that the A_3AR agonist, CF102, allowed for substantial protection against IR injury from occurring (Fig. 2).

CF102 protects against IR injury in hepatectomized rats. Liver hepatectomy was performed on 70% of the rat's liver, whilst being exposed to 10 min of ischemia. Liver enzymic analysis revealed a marked increase in the levels of SGOT and SGPT 2 h after the hepatectomy, which peaked at 4 h and started to decrease after 48 h in the control group. CF102 treatment applied following reperfusion induced a reduction in the levels of SGOT and SGPT, which was maintained for 48 h (Fig. 3).

A_3AR agonist enhances liver regeneration following partial hepatectomy. Hepatic regeneration rates were higher in the CF102 treatment group compared with the saline-treated control group 48 h after the hepatectomy (Fig. 4). CF102
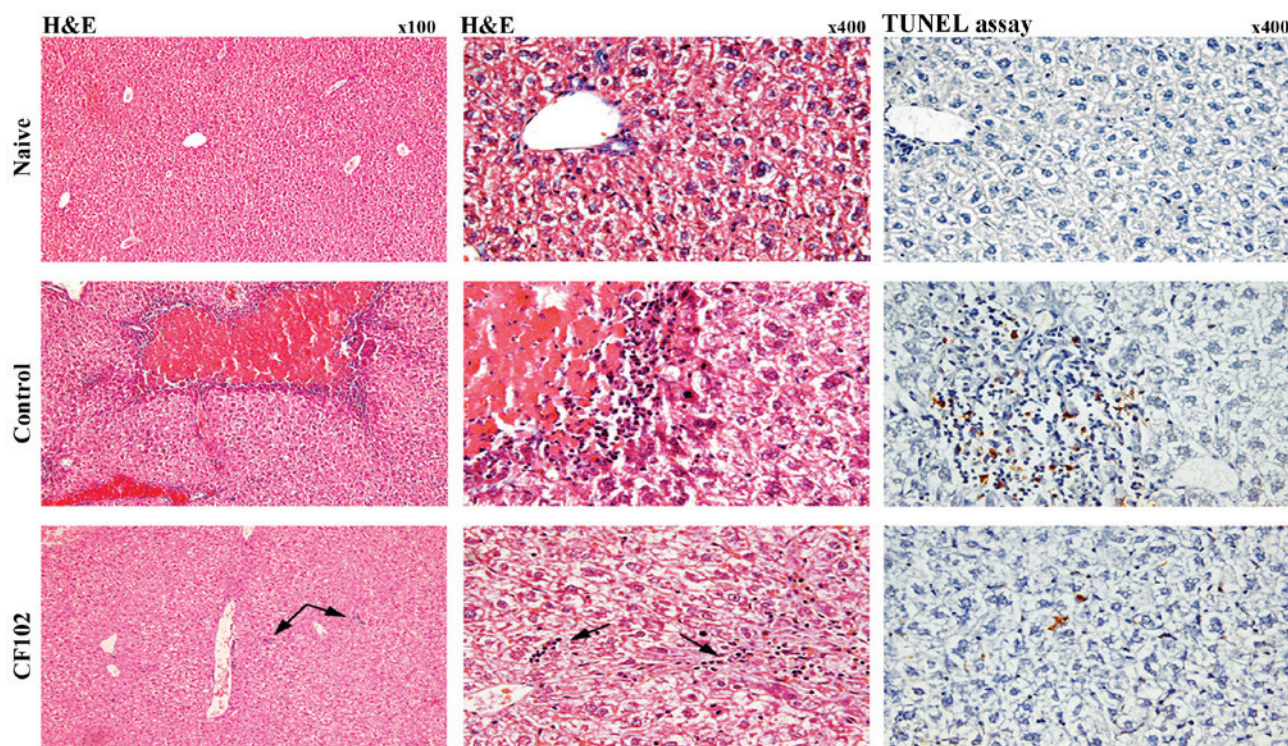
Figure 2. Histological evaluation of liver injury. No signs of necrosis or inflammation were observed in the naïve liver, and no sign of apoptosis was observed in the TUNEL assay. In the control group, large areas of necrosis and inflammatory cells were observed by H&E staining. The TUNEL assay revealed an abundance of cells undergoing apoptosis. In the CF102-treated group, no area of necrosis was observed, and only a few inflammatory cells appeared in the liver parenchyma (indicated by the arrows). Only a few cells were undergoing apoptosis in the TUNEL assay. H&E, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
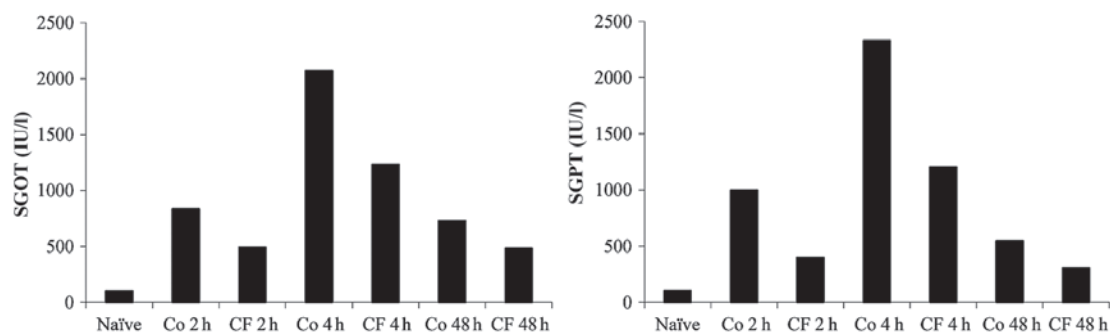

Figure 3. CF102 protects the liver following partial hepatectomy during ischemia. Liver ischemia for 10 min was performed during a 70% hepatectomy. CF102 (100 μg/kg) or saline were administered orally three times daily until the rats were sacrificed. CF102 treatment decreased the levels of the liver enzymes, SGOT and SGPT, at 2, 4 and 48 h after partial hepatectomy. Co, control; CF, CF102; SGOT, aspartate aminotransferase; SGPT, alanine aminotransferase.

treatment increased the regeneration rate of the remnant liver by 45% at 48 h following the 70% hepatectomy (Fig. 4). Additionally, in the CF102 treatment group, the PCNA labeling index (Fig. 5) and the MI (P=0.013; Fig. 6) were higher compared with the saline-treated control group.

Discussion

The present study determined that the selective A$_3$AR agonist, CF102, when administered during liver reperfusion, may attenuate cellular injury, apoptosis and the extent of necrosis in liver subjected to IR. Additionally, CF102 stimulated liver regeneration, which favored the survival of hepatocytes during IR injury. A rat model combining 70% partial hepatectomy
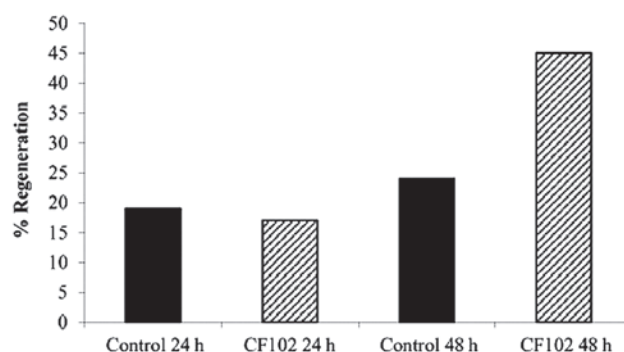

Figure 4. CF102 increases regeneration rate in hepatectomized livers. CF102 treatment accelerated liver regeneration after 48 h compared with the control-treated group. No changes were observed at 24 h.
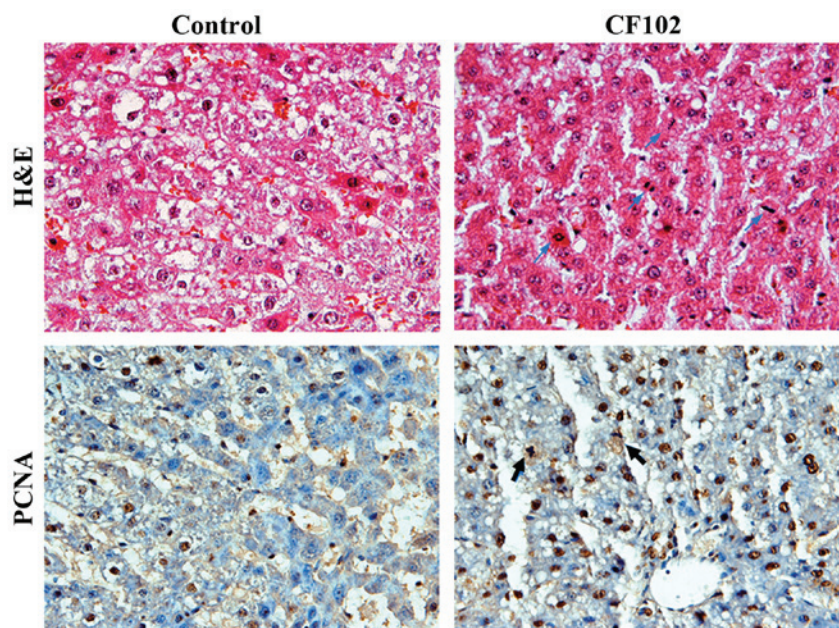
Figure 5. CF102 accelerates mitosis in hepatocytes following partial hepatectomy. Histological evaluation of the hepatectomized livers was performed. No sign of mitosis was noted in the control group. In the CF102-treated group, several mitotic figures were observed (denoted by the arrows). Magnifiction, x400. H&E, hematoxylin and eosin; PCNA, proliferation cell nuclear antigen.
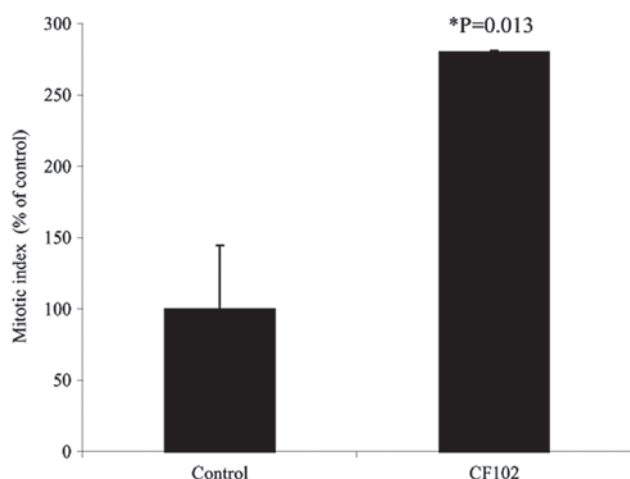

Figure 6. Mitotic index analysis. The mitotic index of the CF102 group was significantly higher compared with that of the control group. Data are presented as the mean ± standard deviation.

with 10 min of Pringle maneuver was used, in order to imitate the surgical procedure performed in humans. In this model, 30% of the liver remained, which was exposed to IR injury. Following partial hepatectomy and liver reperfusion, serum levels of SGOT and SGPT, which may be used as a proxy of liver damage, were reduced in the CF102 treatment group when compared with the control group at 2, 4, and 48 h after the hepatectomy. Reduced liver necrosis and cell apoptosis were observed in the remaining liver obtained from the CF102-treated rats 48 h after the operation when compared with the control group. It is of note that the remnant liver in the CF102-treated rats exhibited a 45% increase in the hepatic regeneration rate when compared with the untreated control group rats. This was confirmed, as the MI and the

PCNA labeling index were higher in the CF102 treatment group compared with the control (Figs. 5 and 6). Therefore, CF102, a highly selective A_3AR agonist with high receptor affinity, may be able to protect against liver IR injury at low concentrations. Previous studies have extensively reported that A_3AR may be used as a target to mediate different protective functions, including neuroprotection, chemoprotection and cardioprotection (21,33,34). Auchampach et al (33) revealed that IB-MECA (CF102), administered at a concentration of 100 μg/kg intravenously 5 min prior to the onset of reperfusion, reduced the size of myocardial infarct in dogs (33). Similarly, 2-CL-IB-MECA administered during reperfusion reduced infarct size in isolated rat hearts and prevented apoptosis in isolated rat cardiomyocytes (35). The PI3K-PKB/Akt signal transduction pathway was identified to be responsible for IB-MECA-induced glycogen synthase kinase-3b inactivation, which is considered to be involved in cardioprotection (15).

Inhibition of the apoptotic response to IR injury in the lungs by the activation of A_3AR has also been previously described (36). Rivo et al (36) have established a spontaneously breathing cat model where the administration of IB-MECA (300 μg/kg) during reperfusion markedly attenuated indices of injury and apoptosis (36). Adenosine has been established as a regulator of cell viability (37), and A_3AR has been specifically identified to contribute to the modulation of cell survival. Activation of A_3AR may induce cell protection or death, depending on the degree of receptor activation (37,38).

Activation of the A_3AR has also been described to trigger neuroprotection. Chen et al (21) have determined that, when A3R knockout mice are treated with a high dose of CL-IB-MECA (0.2 mg/kg), reduced cerebral infarction through the activation of A_3AR and suppression of apoptosis occurred (21).

Apoptosis has been implicated in the pathophysiology of IR liver injury (39); however, an association with A_3AR activation remains to be described.

The present study determined that treatment with CF102 accelerated liver regeneration following a 70% hepatectomy and IR injury. Zhang *et al* (40) have determined that, in a regenerative state, the liver was protective against carbon tetrachloride-induced hepatotoxicity via a mechanism involving increased mitochondrial respiratory activity and plasma membrane fluidity (40).

Our previous study determined that activation of A_3AR initiates a molecular mechanism that involves the deregulation of the PI3K-NF-κB signaling pathway, which results in an effective anti-inflammatory effect (26). Therefore, this may be the possible mechanism through which CF102 exerts its hepatoprotection against IR injury, as the late phase of IR liver injury involved an inflammatory process.

In conclusion, the ability of CF102, an A_3AR agonist, to protect against hepatic IR injury, may include an anti-inflammatory and an anti-apoptotic effect, combined with an increased rate of liver regeneration. However, further studies should be conducted in order to elucidate the molecular basis for these clinically important effects of CF102.

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