A3 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 A3 adenosine receptor (A3AR) 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 A3AR.

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 is 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 A1, A2A, A2B and A3, 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 A2 receptor for adenosine (A2AR) has been identified to exert cardioprotection, neuroprotection and pulmonoprotection against IR injury (18-22).

Upon activation of the A2AR, specific signal transduction pathways are modulated, including the Wnt and NF-κB signaling pathways (23,24). The activation of A2AR 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), IkB kinase, IkB 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 A2AR resulted in increased differentiation of tumor cells compared with normal cells (28). CF102, a selective agonist to A2AR, 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 A2AR agonist, CF102, a compound also termed 2-chloro-N6-(3-isodobenzyl)-adenosine-5'-N-methyl-uronamide (C1-IB-MECA), was synthesized for Can-Fite BioPharma (Petal Tikva, Israel) by Albany Molecular Research, Inc. (Albany, NY, USA). A stock solution of 5 mg/ml was prepared in dimethysulphoxide 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).

**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), laparotomy was performed without IR procedure; ii) control (n=12) no treatment or surgery; and iii) CF102 (n=12) laparotomy 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). Laparotomy 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 (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 CO2 in a gas chamber. The livers were removed and fixed in 10% buffered formalin for pathological analysis and staining.

**Partial hepectomy.** 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 naïve 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). Laparotomy 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 CO2 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 peroxide 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 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 A3AR 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).

A3AR 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 treatment increased the regeneration rate of the remnant

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.
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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 A3AR 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 with 10 min of Pringle maneuver was used, in order to imitate the

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.

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.
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 A3AR agonist with
high receptor affinity, may be able to protect against liver IR
injury at low concentrations. Previous studies have extensively
reported that A3AR may be used as a target to mediate different
protective functions, including neuroprotection, chemoprotec-
tion and cardioprotection (21,33,34). Auchampach et al (33)
revealed that IB-MECA (CF102), administered at a concen-
tration 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 apop-
tosis in isolated rat cardiomyocytes (35). The PI3K-PKB/Akt
signal transduction pathway was identified to be responsible
for IB-MECA-induced glycogen synthase kinase-3β 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 A3AR 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 A3AR has been specifically identified
to contribute to the modulation of cell survival. Activation of
A3AR may induce cell protection or death, depending on the
degree of receptor activation (37,38).

Activation of the A3AR 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 A3AR and suppression of apoptosis occurred (21).

Apoptosis has been implicated in the pathophysiology of IR
liver injury (39); however, an association with A3AR 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<sub>1</sub>AR initiates a molecular mechanism that involves the deregulation of the PI3K-NFκB 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<sub>1</sub>AR 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.

References


