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*Brief Report*

# SR3677 IS HEPATOPROTECTIVE IN MURINE LIVER ISCHEMIA/REPERFUSION INJURY: POTENTIAL ROLE OF BNIP3L/NIX (BCL2/ADENOVIRUS E1B 19 KDA PROTEIN-INTERACTING PROTEIN 3-LIKE)

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ABSTRACT—SR3677, a highly selective rho-associated protein kinase 2 inhibitor, administered prior to liver ischemia/reperfusion injury, induced hepatoprotection in both wild-type and *Parkin2*$^{-/-}$ mice. Experiments in hepatocytes identified BNIP3L/NIX, as a potential mediator of the hepatoprotective effects of SR3677.

KEYWORDS—Mitochondria; liver ischemia/reperfusion; Parkin; BNIP3L/NIX; hepatocytes

Liver ischemia/reperfusion (I/R) is a pathophysiological process underlying the hepatocellular injury observed in many clinical scenarios, including hemorrhagic shock/resuscitation (HS/R) following trauma, blood loss during major elective liver surgery, and organ transplantation. Mitochondrial dysfunction is a hallmark—causative pathology—of I/R injury and is characterized by calcium overload-induced mitochondrial permeability transition, mitochondrial membrane depolarization, disruption of electron flow, depletion of cellular ATP, and generation of reactive free radical species. The degradation of dysfunctional mitochondria, through a cellular process called mitophagy, has recently gained attention as a potential target for therapeutic development in liver I/R, as it lessens elaboration of reactive oxygen species and allows replacement with new mitochondria *via* biogenesis. Two key mechanisms of mitophagy are well-studied: PINK1/Parkin/ubiquitin-dependent and receptor-mediated.

In 2020, two rodent studies implicated PINK1 (PTEN-induced kinase 1)/Parkin-dependent mitophagy as contributing to a hepatoprotection phenotype in liver I/R models (1,2). Gu *et al.* demonstrated that PINK1 is activated and translocated to mitochondria-associated membranes to mediate mitophagy *in vivo* and companion studies *in vitro* showed that knocking down Parkin contributed to hepatocellular injury following hypoxia/reoxygenation (2). Xu *et al.* showed significant hepatic inflammation and activation of the NLRP3 (nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3) inflammasome in mouse liver I/R and primary mouse Kupffer cells (KCs), accompanied by increased PINK1-mediated mitophagy (2). *In vivo* overexpression of PINK1 attenuated liver I/R injury, ROS production, NLRP3 activation, and inflammation (1). Additionally, *in vitro* anoxia/reoxygenation triggered NLRP3 activation in KCs along with increased mitophagy. Enhanced mitophagy mediated by PINK1 overexpression further inhibited NLRP3 activation and reversed KC-mediated inflammatory injury to hepatocytes. The protective effects of PINK1 were abolished by a kinase-dead mutant of PINK1. Pharmacological development to target PINK1/Parkin-dependent mitophagy would represent a novel strategy to treat liver I/R.

Systematically, Moskal *et al.* screened chemical libraries of ~3,000 small molecules, which identified several Parkin activators—recruitment of Parkin to mitochondria—with a common canonical target, namely, ROCK (3). ROCK2 is a serine/threonine kinase, which acts as an effector of RhoA (4). Among top-hits, SR3677 was investigated further because of its amenability for therapeutic development. SR3677 was found to be a potent activator of Parkin-dependent mitophagy and demonstrated neuroprotective effects in a *Drosophila melanogaster* model of paraquat-induced Parkinson's disease (3). Importantly, upon CCCP (carbonyl cyanide m-chlorophenyl hydrazone—inhibitor of oxidative phosphorylation)-induced (a form of chemical hypoxia) mitochondrial damage, SR3677 enhanced Parkin recruitment to mitochondria, and increased lysosomal degradation of mitochondria, in a PTEN/HK2 (phosphatase and tensin homolog/hexokinase 2)-mediated manner (3).

SR3677 is a potent, highly selective and highly efficient rho-associated protein kinase 2 (ROCK2) inhibitor, with $IC_{50}$ ~3 nM and an off-target hit rate of ~1% in a screen of >350 kinases (4). SR3677 inhibits both ROCK2 kinase activity and the ensuing phosphorylation of myosin light chain (MLC) (4). Compared to other ROCK inhibitors, SR3677 presents characteristics that

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may confer improved therapeutic advantages. In addition, literature suggests that classes of ROCK inhibitors result in the phenotype of mitophagy enhancement (3).

We have previously demonstrated that remote ischemic preconditioning to the hindlimb applied in advance of hemorrhagic shock/resuscitation in a murine model was hepatoprotective and did so by inducing Parkin-dependent mitophagy in the liver (5). We therefore tested the hypothesis that the ROCK2 inhibitor, SR3677, a Parkin-dependent mitophagy inducer, might exert hepatoprotective effects in a mouse liver I/R injury.

A mouse model of 70% warm liver I/R was established, as previously described (6). Briefly, wild-type C57BL/6J mice (JAX #000664) or *Parkin*$^{-/-}$ mice (JAX #006582) (26 ± 0.4 g, 9–12 weeks) were environmentally acclimated for 1 week before surgery. Thirty minutes prior to surgery, SR3677 (2 mg/kg) was intraperitoneally injected. Surgical plane of anesthesia was induced using isoflurane (2.5%–3.5% in 100 mL/min $O_2$). Mice were placed on a self-regulated warming pad with constant temperature monitoring (37 ± 1°C). Midline laparotomy, dissection, and clamping were achieved. Seventy percent left/median-lobe warm ischemia was timed for 1 h, followed by clamp removal and 2 h of liver reperfusion. Sham-operated (all surgical manipulations except liver ischemia) controls were conducted. Mice were sacrificed by exsanguination and liver tissue and blood were collected following 2 h of liver reperfusion.

In wild-type mice, liver I/R significantly induced release of liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), consistent with hepatocellular injury (Fig. 1, A and B). Administration of SR3677 prior to surgery significantly reduced the release of liver enzymes, ALT and AST, in mouse liver I/R (Fig. 1, A and B), indicating hepatoprotection. Treatment of sham-operated mice with SR3677 did not by itself cause a rise in liver enzymes, indicating that SR3677 is safe at a dose of 2 mg/kg (Fig. 1, A and B). Liver histology confirmed that SR3677 significantly reduced hepatocellular injury in liver I/R. As shown in Figure 1C, SR3677 lessened markers of injury including cytoplasmic vacuolization, parenchymal necrosis, and hepatic congestion (left panels *vs.* right panels). This effect was quantified using the Suzuki liver injury score (Fig. 1D). Together, these studies in wild-type mice, demonstrated that SR3677 is a hepatoprotective small molecule in a mouse model of liver I/R.

To determine whether the hepatoprotective effects of SR3677 were dependent on Parkin, we performed I/R studies in *Parkin2*$^{-/-}$ mice with and without SR3677. In *Parkin2*$^{-/-}$ mice, liver I/R significantly increased the release of liver enzymes, ALT and AST, compared to sham animals (Fig. 1, E and F). In *Parkin2*$^{-/-}$ animals, SR3677 significantly reduced the release of liver enzymes, ALT, in mouse liver I/R (Fig. 1, E and F), and also AST, albeit not to a level of significance. Together, these studies indicate that SR3677 was able to induce hepatoprotection, following liver I/R in a Parkin-independent manner.

To gain further mechanistic understanding of SR3677-induced hepatoprotection, we conducted *in vitro* experiments. Human hepatocytes (Huh7.5) were subjected to chemical hypoxia, using CCCP, a potent mitochondrial depolarizer which induces mitochondrial damage, similar to hypoxia/reoxygenation. The tetrazolium ring of salts like MTT (3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide) is cleaved by succinate dehydrogenase in active mitochondria. MTT assays measure mitochondrial metabolic rate and therefore indirectly reflect the percent of viable cells (i.e., metabolically active cells).

After 24 h of treatment with CCCP, hepatocytes experienced abundant cell death (Fig. 1G). Consistent with our *in vivo* studies, SR3677 (500 nM) significantly rescued hepatocytes from CCCP-induced hepatocellular death (Fig. 1G). Having shown that Parkin was not required for the protective effect of SR3677, we hypothesized that another mitophagy effector, BNIP3L/NIX (a hypoxia-inducible mitophagy receptor), might be involved. To test this, we knocked down BNIP3L/NIX using siRNA. As shown in Figure 1H, SR3677 failed to exert hepatocellular protection in BNIP3L/NIX-deficient cells (Fig. 1H). Neither BNIP3L/NIX siRNA nor SR3677 by themselves affected cell viability. Therefore, in hepatocytes, the protective phenotype induced by SR3677 appeared to be mediated by BNIP3L/NIX.

In summary, SR3677 was hepatoprotective in both wild-type and *Parkin2*$^{-/-}$ mice subjected to liver I/R. Moreover, *in vitro*, we found that SR3677 protects hepatocytes from CCCP-induced mitochondrial damage and the resultant hepatocellular death, in a BNIP3L/NIX-mediated manner. Together, these findings highlight the protective effects of SR3677 in the liver.

PINK1/Parkin-dependent mitophagy is a ubiquitin-mediated cellular process by which dysfunctional mitochondrial undergo macroautophagic degradation, whereas BNIP3L/NIX-dependent mitophagy is hypoxia-responsive, and initiates mitophagy through direct interaction with LC3 (microtubule-associated protein 1A/1B-light chain 3) autophagosomes. In fact, these two classes of mitophagy are distinct in their kinetics and cellular stress stimuli yet converge at macroautophagic degradation through LC3. The large majority of the literature has described PINK1/Parkin-dependent mitophagy as the canonical pathway; however, many of these studies are in model systems of rodent neurodegeneration. BNIP3L/NIX-dependent mitophagy has been previously demonstrated as an essential protective mechanism in ischemic brain injury (i.e., stroke, brain I/R) and in myocardial I/R (7,8). It is therefore reasonable to postulate that SR3677 functions differently in cerebral cells *versus* hepatic cells. In addition, there may also be compensatory crosstalk between receptor-mediated and ubiquitin-dependent mitophagy. For example, *Parkin2*$^{-/-}$ mice have increased protein expression of mitophagy receptors, BNIP3L/NIX and FUNDC1 (FUN14 domain containing 1), in liver tissue (5).

Prolonged hypoxia activates RhoA (9), and RhoA signaling increases mitophagy through PINK1 accumulation on mitochondria (10). In cardiomyocyte ischemic stress, active RhoA localizes to mitochondria and interacts with PINK1 (10). How ROCK2 inhibition is protective is still unknown and requires more study. SR3677 binds ROCK2, inhibits its kinase activity and lowers the phosphorylation of MLC. Crucial effectors of ROCK2 include MLC, PTEN, and HK2. ROCK was shown to activate PTEN, which acts as a *negative* regulator of Parkin-mediated mitophagy. PTEN dephosphorylates mitochondrial ubiquitin, which is the key signal promoting the feedforward loop of Parkin activity at the mitochondria. In addition, PTENα-deficient mice develop cardiac hypertrophy, dysfunctional mitochondria, and impairments in Parkin-mediated mitophagy (11). Downstream signaling from ROCK2 remains to be elucidated and how it is deleterious to mitochondrial function, for example,

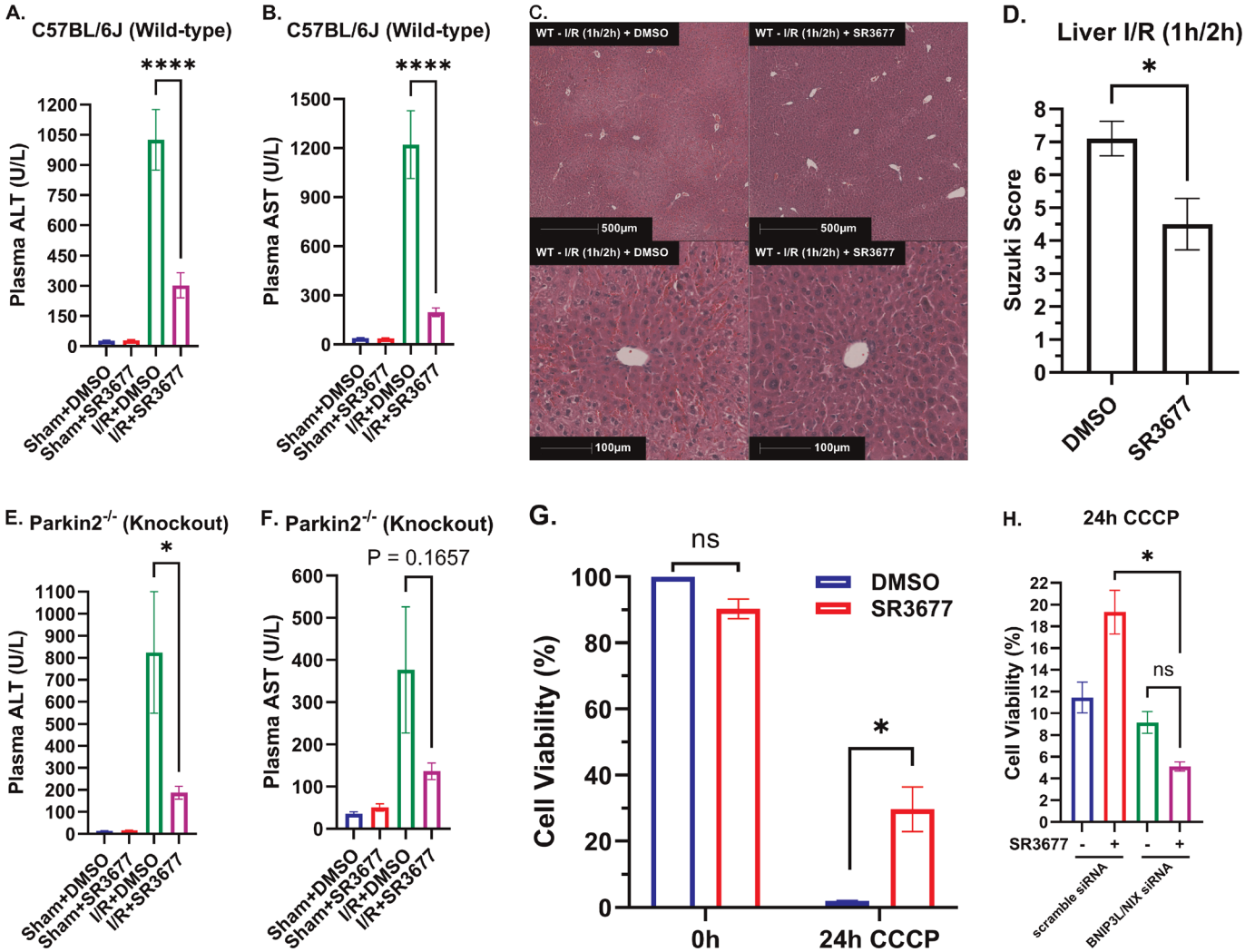


FIG. 1. **SR3677 is hepatoprotective in liver ischemia/reperfusion injury: potential role of BNIP3L/NIX**. A and B, SR3677 significantly reduced plasma ALT, alanine aminotransferase (1,026 ± 151 U/L in I/R + DMSO *vs.* 303 ± 62 U/L in I/R + SR3677) and AST, aspartate aminotransferase (1,221 ± 208 U/L in I/R + DMSO *vs.* 197 ± 26 U/L in I/R + SR3677) in wild-type C57BL/6 J mice subjected to liver I/R (1-h ischemia/2-h reperfusion). C and D, Representative liver hematoxylin and eosin histology images. Blinded quantification using the Suzuki liver injury score showed that SR3677 lowered hepatocellular injury in wild-type C57BL/6 J mice subjected to liver I/R (7.1 ± 0.5 in I/R + DMSO *vs.* 4.5 ± 0.8 in I/R + SR3677). E and F, SR3677 significantly reduced plasma ALT (824 ± 276 U/L in I/R + DMSO *vs.* 188 ± 29 U/L in I/R + SR3677) in *Parkin2*$^{-/-}$ mice subjected to liver I/R. SR3677 reduced plasma AST in *Parkin2*$^{-/-}$ mice subjected to liver I/R (377 ± 150 U/L in I/R + DMSO *vs.* 137 ± 20 U/L in I/R + SR3677), but this was nonsignificant in *post hoc* testing. G, SR3677 significantly rescued human hepatocytes (Huh7.5) from CCCP-induced cell death (5 µM) (2%-bar 3 *vs.* 30%-bar 4). Cell viability was measured by MTT assay and data normalized to control (ab211091). H, After 24 h of CCCP (2 µM) treatment, SR3677 does not protect in BNIP3L/NIX-deficient hepatocytes (19%-bar 2 *vs.* 5%-bar 4). BNIP3L/NIX siRNA transfection was done by jetPRIME (Polyplus #114-07). One-way ANOVA and *t* test were used for statistics. Asterisks denote significance by the Tukey's *post hoc* test or *t* test: ****$P < 0.0001$, *$P < 0.05$. n.s. = not significant. Mean ± SEM is reported. N = 4–6 mice per group for mouse studies and n = 3 independent experiments for cell culture experiments.

by sequential and systematic knockdowns of both Parkin and BNIP3L/NIX then measuring phosphorylation/activation and localization of MLC, PTEN, and HK2, with or without SR3677.

Although our studies in a hepatocyte cell line strongly implicated BNIP3L/NIX in the protective effect of ROCK2 inhibition, one of the limitations of this study is that we did not investigate the role of BNIP3L/NIX *in vivo*, in mouse liver I/R. A potential future direction of this study could be to generate hepatocyte-specific BNIP3L/NIX knockout mice and then induce liver I/R with or without SR3677. Such a study may elucidate a cell- and organ-specific mechanism for the action of SR3677, *in vivo*. Furthermore, ROCK2 could participate in injury by its other functions, such as increasing postischemic blood flow (12), inhibiting hepatic stellate cell activation (13) or regulating/mediating apoptosis (14). Thus, ROCK2 may not exclusively participate in tissue injury through mitochondrial damage.

In conclusion, we showed that SR3677 is hepatoprotective in both wild-type and *Parkin2*$^{-/-}$ mice subjected to liver I/R, suggesting the potential involvement of other mitophagy receptors. We found that SR3677 was protective in cultured hepatocytes, and this protective effect required BNIP3L/NIX. This suggests that ROCK2 involves a role for BNIP3L/NIX to promote protection in liver I/R. Here, we identify BNIP3L/NIX as a potential downstream molecular target for SR3677, which ultimately results in hepatoprotection. These findings are applicable in clinical scenarios where liver I/R contributes to patient morbidity and mortality.

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