Published in final edited form as:

Transplantation. 2011 February 27; 91(4): 406–412. doi:10.1097/TP.0b013e318204bdb2.

NIM811 Prevents Mitochondrial Dysfunction, Attenuates Liver Injury and Stimulates Liver Regeneration after Massive Hepatectomy^{§, #, &}

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Abstract

Background—Massive hepatectomy (MHX) leads to failure of remnant livers. Excessive metabolic burden in remnant livers may cause mitochondrial dysfunction. This study investigated whether blockade of the mitochondrial permeability transition (MPT) with N-methyl-4-isoleucine cyclosporin (NIM811) improves the outcome of MHX.

Methods—Mice were gavaged with NIM811 (10 mg/kg before surgery and 5 mg/kg daily afterwards) and underwent sham-operation or ~90%-partial hepatectomy.

Results—Serum alanine aminotransferase (ALT), necrosis and apoptosis increased, respectively, to ~1200 U/L, 6.1% and 7% after MHX. NIM811 decreased peak ALT release, necrosis and apoptosis by 70%, 100% and 42%. 5-Bromo-2'-deoxyuridine incorporation, proliferating cell nuclear antigen expression, and the remnant liver weights were all increased significantly by NIM811 treatment, indicating improved liver regeneration. NIM811 also blunted hyperbilirubinemia by 54%, increased serum albumin by 51% and improved survival from 6% to 40% after MHX. Hepatic mitochondrial depolarization, cell death and the MPT were detected by intravital confocal/multiphoton microscopy of rhodamine 123, propidium iodide and calcein. Mitochondrial depolarization occurred in many viable hepatocytes (13 cells/hpf), and non-viable hepatocytes increased slightly to ~1 cell/hpf at 3 h after MHX. Entry of calcein into mitochondria after MHX indicated MPT onset. Importantly, NIM811 decreased mitochondria depolarization by

[§]HR, JS, YS, ZZ, VKR, QL and RTC participated in the performance of the research; ZZ and JJJ participated in the research design and writing of the paper.

[#]Supported, in part, by Grants DK70844, DK084632, DK037034, DK073336 and 1P30 CA138313 from the National Institutes of Health.

[&]The authors have no conflict of interest to declare.

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>60%, blocked MPT onset, and prevented cell death. Decreases of hepatic ATP, mitochondrial cytochrome *c* release and caspase-3 activation after MHX were also partially blocked by NIM811.

Conclusions—NIM811 minimized liver injury and improved liver regeneration after MHX, at least in part, by preventing MPT onset and subsequent compromised energy supply and proapoptotic cytochrome *c* release.

Keywords

acute liver failure; major liver resection; massive hepatectomy; mitochondrial dysfunction; NIM811

INTRODUCTION

Major liver resection is performed in surgical treatment of liver malignancy and in living donor liver transplantation (1,2). Although liver can regenerate to a fully functional organ after resection, liver insufficiency and failure occur from time to time, leading to mortality (3-6). Clinically, the danger of liver failure increases when >50% of liver is removed (5,6). Patients exhibit progressive hyperbilirubinemia, coagulopathy, encephalopathy and eventually death. Histopathologically, liver failure following hepatectomy shows cholestatic changes or failure of regeneration with apoptosis of hepatocytes (7).

The mechanisms of liver failure after major hepatectomy are not well understood. Insufficient functional mass of remnant livers, aging, pre-existing liver disease, and post surgery complications such as sepsis can compromise outcomes after major liver resection (3,8-11). Procedures controlling blood loss (Pringle maneuver and total vascular exclusion) during surgery may cause IR injury (3,12), and decreased pro-mitotic cytokines and growth factors may also contribute to slower regeneration of remnant livers (13). Increased apoptosis is observed after major liver resection in some studies (7,14). Activation of the intrinsic rather than extrinsic apoptotic pathways and down-regulation of pro-regenerative factors might contribute to liver failure (3).

Energy supply is critical for cell survival and proliferation. ATP is required not only for energy supply to maintain cell functions and survival but is also an essential molecule that controls regenerative signaling (15-17). Excessive metabolic burden in remnant livers after major hepatectomy may lead to mitochondrial dysfunction. Indeed, ATP decreases markedly after partial hepatectomy (15,18,19). Decreased ATP production in small-for-size liver grafts is associated with increased injury, suppressed regeneration and higher mortality (20,21).

Mitochondrial synthesis of ATP depends on an electrochemical gradient of H⁺ ions across the mitochondrial inner membrane. Mitochondrial membrane depolarization compromises energy production profoundly. After small-for-size liver transplantation, the mitochondria permeability transition (MPT) occurs (22,23). The MPT leads to mitochondrial depolarization, uncoupling of oxidative phosphorylation and large amplitude mitochondrial swelling, culminating in ATP depletion-dependent necrosis and/or cytochrome *c*-dependent apoptosis (24). Cyclosporin A (CsA) and its non-immunosuppressive derivative, *N*-Methyl-4-isoleucine cyclosporin (NIM811), inhibit the MPT in liver grafts after prolonged cold ischemic storage, cultured hepatocytes and isolated liver mitochondria (25,27). In particular, NIM811 prevents mitochondrial depolarization and improves survival of small-for-size liver grafts (22), indicating that the MPT plays an important role in small-for-size liver graft failure. However, two risk factors, namely cold preservation-reperfusion and small liver mass, co-exist under such conditions, and the MPT can be induced by ischemia/reperfusion (IR) *in vivo* (26,27). Thus, it remains unclear whether decreases in liver mass

alone cause the MPT in the absence of cold preservation-reperfusion injury. Accordingly, here we investigated whether MPT blockade with NIM811 alleviates injury and improves regeneration after massive hepatectomy.

RESULTS

NIM811 Decreases Liver Injury after Massive Hepatectomy

Liver injury after massive hepatectomy (MHX) was assessed from cell necrosis, apoptosis and ALT release. No pathological changes were observed in livers after sham-operation (**data not shown**). Small focal necrosis occurred in remnant livers after MHX (Fig. 1A). Necrotic areas increased gradually after MHX and reached 6.1% at 48 h (Fig. 1C). Necrosis was rarely present in remnant livers with NIM811 treatment (Fig. 1A and 1C).

Apoptosis in livers was detected by TUNEL. TUNEL-positive cells increased from the basal level of 0.13% to ~7% at 6 h after MHX (Fig. 1B and D) and then decreased to 2.9% at 48 h. NIM811 decreased TUNEL to 4.1 % at 6 h and to 1.2% at 48 h (Fig. 1B and D). Therefore, increased cell killing after MHX was partly due to necrosis and partly due to apoptosis, and both were blunted by NIM811.

ALT release signifies hepatocellular injury. Before MHX, serum ALT was about 60 U/L. After MHX, ALT increased to ~1200 U/L at 12 h and then gradually decreased to ~920 U/L at 48 h (Fig. 1E). Peak ALT was blunted by ~70% by NIM811 (Fig. 1E). These results indicated that NIM811 markedly reduces liver injury after MHX.

NIM811 Stimulates Liver Regeneration after Massive Hepatectomy

Liver regeneration was evaluated by BrdU incorporation and expression of PCNA (Fig. 2). BrdU-positive cells were barely detectable after sham-operation (Fig. 2A). After MHX, BrdU labeling remained barely detectable at 24 h (Fig. 2D) and was only ~0.8% at 48 h (Fig. 2B and D), indicating poor cell proliferation. By contrast, NIM811 treatment increased BrdU-positive cells to ~5% and ~13% in remnant livers at 24 and 48 h, respectively.

Expression of PCNA, a protein that acts as a processivity factor for DNA polymerase δ , was used as another indicator of cell proliferation (28). PCNA labeling increased slightly from the basal level of 0.05% to 1.6% and 2.5% at 24 h and 48 h after MHX, respectively (Fig. 2E). NIM811 treatment markedly increased PCNA expression after MHX to 6% and 10% at 24 h and 48 h, respectively (Fig. 2E). In addition, remnant liver weight increased from 0.32 g in vehicle-treated mice at 48 h after MHX to 0.41 g in NIM811-treated mice ($p < 0.05$) (Fig. 2F). These results indicate that NIM811 increases cell proliferation and improves liver regeneration after MHX.

NIM811 Improves Liver Function and Survival after Massive Hepatectomy

Total bilirubin was 0.17 mg/dL before massive hepatectomy (MHX), which increased to 3.8 mg/dL at 48 h after MHX, indicating poor liver function. NIM811 blunted hyperbilirubinemia by 54% (Fig. 3A). Serum albumin, another indicator of liver function, decreased from a basal level of 4.1 g/dL to 1.2 g/dL after MHX. NIM811 blunted the decrease of albumin after MHX by 51% (Fig. 3B). These data indicate that NIM811 significantly improves liver functions after MHX.

Since MHX leads to failure of remnant livers, we investigated whether NIM811 improves survival of remnant livers. Survival decreased to 6% after MHX and mortality mainly occurred in the first 3 days (Fig. 3C). Importantly, survival increased significantly to 40% after MHX in NIM811-treated mice ($p < 0.05$ vs. vehicle treatment).

NIM811 Prevents the MPT after Massive Hepatectomy

Energy supply is essential for survival of remnant livers. Therefore, we explored whether the MPT and mitochondrial depolarization occur after MHX in living mice using intravital multiphoton microscopy. Green Rh123 fluorescence was punctate in virtually all hepatocytes at 3 h after sham-operation, indicating mitochondrial polarization, and red propidium iodide (PI)-labeling of nuclei, an indicator of cell death, was rare (Fig. 4A). Three hours after MHX, mitochondria in many still viable hepatocytes (PI negative) did not accumulate Rh123, indicating mitochondrial depolarization (Fig. 4B). A few hepatocytes not accumulating Rh123 were also labeled with PI (Fig. 4B, ~1/hpf), indicating cell death. PI-labeled hepatocytes never contained polarized mitochondria, whereas many hepatocytes with depolarized mitochondria excluded PI (Fig. 4B). NIM811 decreased mitochondrial depolarization by 66% (Fig. 4C and G) and hepatocyte death to a level which was not statistically different from sham-operation (**data not shown**). Mitochondrial depolarization is difficult to detect in non-parenchymal cells due to their paucity of mitochondria. Non-parenchymal cell death after MHX (~0.3 cells/hpf) was not different with or without NIM811 treatment (**data not shown**). Together, these results showed that mitochondrial depolarization occurred after MHX in many hepatocytes and that mitochondrial depolarization preceded cell death. NIM811 significantly blunted mitochondrial depolarization, suggesting that mitochondrial depolarization was induced by MPT onset.

MPT onset after MHX was further confirmed by intravital multiphoton microscopy of calcein, a fluorophore that enters mitochondria when permeability transition (PT) pores open. In livers of sham-operated mice, cytosolic calcein outlined mitochondria as dark voids (Fig. 4D). These dark voids disappeared at 3 h after MHX (Fig. 4E), indicating MPT onset. NIM811 prevented entry of calcein into mitochondria after MHX (Fig. 4F).

Decreased ATP Production, Cytochrome *c* Release and Caspase-3 Activation after Massive Hepatectomy: Prevention by NIM811

The MPT collapses the mitochondrial membrane potential and causes large amplitude mitochondrial swelling with rupture of the outer membrane, leading to failure of ATP production and release of cytochrome *c* (29,30). Accordingly, ATP was detected in liver tissue after surgery. At 3 h after MHX, ATP decreased from the basal levels of 2.1 nmoles/g liver to 0.7 nmoles/g liver, indicating less ATP production (Fig. 5A). NIM811 blunted this decrease of ATP by ~60%.

Cytochrome *c* immunostaining was punctate in hepatocytes of sham-operated mice, consistent with mitochondrial localization of cytochrome *c* (Fig. 5B). After MHX, cytochrome *c* staining became diffuse, indicating release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol (Fig. 5B). NIM811 largely prevented release of cytochrome *c* after MHX (Fig. 5B).

To determine if release of cytochrome *c* from mitochondria triggered caspase activation and apoptosis, caspase 3 activity was measured (29,30). Caspase-3 activity increased 6.3-fold at 6 h after MHX and remained at high levels afterwards (Fig. 5C). NIM811 treatment blunted caspase-3 activation by ~55% (Fig. 5C).

DISCUSSION

Prevention of Mitochondrial Dysfunction Improves Outcomes after Massive Hepatectomy

The mechanisms by which MHX leads to failure of remnant livers remain unclear. An important risk factor for liver failure is low remnant liver volume. A remnant liver mass of less than 250 ml/m² is associated with higher risk of liver failure (31,32). Engraftment of

small-for-size liver grafts with a graft volume-to-standard liver volume ratio less than 30-40% or a graft-to-recipient weight ratio of $<1\%$ decreases survival significantly (33,34). Small liver volume can also lead to a remarkable increase in hepatic vascular resistance in remnant livers (35). In addition, substantially decreased liver tissue mass causes excessive metabolic burden and mitochondrial dysfunction. Indeed, hepatic energy levels fall following major liver resection (18,19,36,37). After quarter-size liver transplantation, ATP decreases 67% in liver tissue, and compromised energy supply is associated with increased graft injury, poorer regeneration and higher mortality (20). After living donor liver transplantation, alteration of arterial ketone body ratios, which indicates poor hepatic mitochondrial function, is associated with partial graft dysfunction (38), suggesting the importance of mitochondrial function after liver resection and partial liver transplantation.

No doubt, proper mitochondrial function is essential for survival and regeneration of remnant livers. A deficiency of ATP both limits energy supply, which is needed to support increased metabolic demand, and biogenesis of new liver cells in remnant livers. In this study, we observed wide-spread mitochondrial depolarization after MHX (Fig. 4) which was associated with decreased ATP production (Fig. 5). This mitochondrial dysfunction occurred prior to cell death and was associated with subsequent increased liver injury (ALT release and necrotic and apoptotic cell death), inhibited liver regeneration (decreased BrdU incorporation, PCNA expression and remnant liver weight), poorer liver function (increased bilirubin and decreased albumin) and decreased survival (Figs. 1-3). These data suggest that despite increased energy requirements, a defect of energy production occurs in remnant livers, consistent with previous observations (18-20,36,37). Prevention of mitochondrial depolarization by NIM811 attenuated liver injury, stimulated regeneration and improved liver function and survival (Figs. 1-3). Therefore, mitochondrial dysfunction is likely responsible, at least in part, for liver failure after MHX. However, NIM811 can not completely prevent acute liver failure after MHX. It is possible that factors other than mitochondrial dysfunction (e.g. decreased growth factors and activation of extrinsic apoptotic pathway) also play a role in suppressed regeneration and cell death. As a result, in some animals, liver mass and function recovery is still not rapid enough to prevent encephalopathy and mortality.

The Role of MPT in Liver Failure after Massive Hepatectomy

MPT onset is important in failure of small-for-size liver grafts (22). It remains unknown whether the MPT also plays a role in liver failure after MHX. Similar to small-for-size liver grafts, remnant livers after MHX experience a substantially higher metabolic burden. Unlike small-for-size grafts, remnant livers after MHX are not exposed to cold-preservation/reperfusion. Therefore, we investigated whether the MPT could be caused by reduced liver mass alone. Interestingly, after MHX, mitochondrial depolarization occurred although the remnant liver tissue was never subjected to vascular occlusion (Fig. 4), indicating that substantially reduced liver mass alone can lead to mitochondrial dysfunction.

Mitochondrial depolarization can be caused by a variety of factors. Inhibition of mitochondrial respiratory chain components and uncoupling of respiration from phosphorylation compromise ATP production (39). Alteration of activities of ion channels and transporters in mitochondria (e.g., PT pores, uncoupling proteins, mitochondrial ATP-sensitive potassium channel, adenine nucleotide transporter, and Ca^{2+} transporters) can also affect mitochondrial polarization and function (39,40). In this study, NIM811, a specific inhibitor of the MPT, blocked mitochondrial depolarization after MHX (Fig. 4), suggesting that mitochondrial depolarization is likely due to MPT onset. This hypothesis was further supported by the observation that calcein, a cytosolic fluorophore, gained entry into mitochondria in remnant livers after MHX (Fig. 4) which indicates opening of the PT pores to allow permeation of calcein.

Cyclophilin D (CypD), a peptidylprolyl isomerase within the mitochondrial matrix, plays an important role in onset of the MPT (40). Mice lacking the *Ppif* gene which encodes CypD are protected from IR-induced cell death, whereas CypD-overexpression caused mitochondrial swelling and spontaneous cell death in mice (41). Moreover, hepatocytes and fibroblasts from *Ppif*-null mice are protected from Ca^{2+} -overload and oxidative stress-induced cell death (41). CsA and NIM811 bind to CypD and prevent formation of PT pores (25). NIM811 is equipotent to CsA in inhibition of the MPT in cultured cells (25) and was shown recently to block the MPT in *in vivo* (22,27,42). Here we showed that entry of calcein into mitochondria and mitochondrial depolarization after MHX were also blocked by NIM811 (Fig. 4), consistent with the hypothesis that mitochondrial dysfunction after MHX is, at least in part, due to MPT onset. Collapse of the mitochondrial membrane potential caused by the MPT decreases ATP production (Fig. 5), leading to necrosis and suppression of liver regeneration (Figs. 1-2). In addition, mitochondrial swelling releases cytochrome *c* (Fig. 5) which triggers apoptosis (Fig. 1). Thus, the MPT most likely contributes to liver failure after MHX.

Taken together, previous studies showed that the MPT plays a critical role in IR injury (26,43), and this study further showed that decreased liver mass alone can induce onset of the MPT. Therefore, blockade of the MPT using MPT inhibitors such as NIM811 are beneficial not only after partial liver transplantation but also may prevent liver failure and enhance liver regeneration after major liver resection, such as liver donation and tumor resection. NIM811 is a CsA derivative without immunosuppressive activity but retaining full capacity for binding to cyclophilin. Previous studies show that NIM811 has good bioavailability after oral administration. Moreover, its nephrotoxicity is significantly lower than CsA (44). These features make it a promising candidate for use in clinical settings.

METHODS

Animals

Male C57BL/6 mice (8 - 12 weeks) were gavaged with *N*-methyl-4-isoleucine cyclosporin (NIM811, Novartis Pharma Ltd., Switzerland, 10 mg/kg) or an equal volume of vehicle containing 8.3% polyethoxylated castor oil (Sigma, St. Louis, MO) and 8.3% ethanol at 2 h before surgery. Mice underwent massive hepatectomy (MHX; ~87% of the liver mass) or sham-operation under ether anesthesia. Briefly, the left, medium, right lower and caudate lobes were ligated with 5-0 silk suture at the base and resected with only the right upper lobe (~13% of the total liver weight) remaining. For sham-operation, the ligaments around the liver were freed and the abdomen was closed 20 min later without MHX. NIM811 (5 mg/kg) or vehicle was gavaged daily post-operatively for 2 days. Mice were observed for 21 days for survival. All mice received humane care in compliance with institutional guidelines. Animal protocols were approved by the Institutional Animal Care and Use Committee.

Clinical Chemistry and Histology

Blood was collected from the inferior vena cava, and livers were harvested at times indicated in the figure legends. Liver necrotic areas were quantified by image analysis of liver sections stained with hematoxylin and eosin (42).

Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using an *In Situ* Cell Death Detection Kit (22). TUNEL-positive and negative cells were counted in a blinded manner in 10 randomly selected fields using a 20× objective lens. Serum alanine transaminase (ALT) and total bilirubin were measured using analytical kits from Pointe Scientific (Uncoln Park, MI) and serum albumin was measured using a Mouse Albumin ELISA kit from GenWay Biotechnology (San Diego, CA).

Measurement of Caspase-3 Activity

Caspase-3 activity in liver homogenates was detected using a kit from R&D Systems (Minneapolis, MN)(27). Protein was measured using a Protein Assay Kit from Bio-Rad Laboratory (Hercules, CA). Activities of caspase-3 were expressed as changes of optical density (OD) values per milligram of protein.

Immunohistochemistry

To detect cells synthesizing DNA, 5-bromo-2'-deoxyuridine (BrdU) was injected (10 mg/kg *i.p.*) 2 h prior to liver harvesting. BrdU incorporation, proliferating cell nuclear antigen (PCNA) and cytochrome *c* in liver sections were determined immunohistochemically (22,42). After BrdU and PCNA staining, positive and negative cells were counted in 10 randomly selected fields under the light microscope using a 40× and a 20× objective lens, respectively.

Intravital Confocal/Multiphoton Microscopy

At 3 h after MHX or sham-operation, hepatic mitochondrial polarization and cell death in living mice were detected by intravital confocal microscopy of green-fluorescing rhodamine123 (Rh123, Sigma, St. Louis, MO) which labels polarized mitochondria and red-fluorescing propidium iodide (PI) which labels the nuclei of non-viable cells, respectively (42). Therefore, viable cells with polarized mitochondria have cytoplasm with punctate green fluorescence and no red nuclear fluorescence, whereas viable cells with depolarized mitochondria have no red nuclear fluorescence and no or diffuse green cytoplasmic fluorescence. By contrast, non-viable cells show red nuclear fluorescence with depolarized mitochondria.

Mitochondrial inner membrane permeabilization at onset of the MPT was assessed by intravital multiphoton microscopy of calcein (42). Mice were loaded with calcein-AM which is cleaved by esterases in the cytosol, forming green-fluorescing calcein free acid. Mitochondria are normally impermeant to calcein, leaving mitochondria as nonfluorescent dark voids. These voids disappear after onset of the MPT when calcein, a fluorophore of 623 Da molecular weight, enters the mitochondrial matrix space through PT pores (45)

Detection of ATP

Livers were collected at 3 h after MHX or sham-operation by freeze-clamping using a tong chilled in liquid nitrogen and stored at -80°C. ATP in hepatic trichloroacetic acid extract was detected by luciferin-luciferase assay using an Enliten ATP Assay System (Promega Corp., Madison, WI) (20).

Statistical Analysis

Groups were compared using the Kaplan-Meier test and ANOVA plus Student-Newman-Keuls posthoc test as appropriate. Data shown are means ± S.E.M. Numbers of animals were 15 to 16 per group in the survival experiment and 4 per group for all other parameters. Differences were considered significant at $p < 0.05$.

Acknowledgments

We thank the Cell and Molecular Imaging Core of the Hollings Cancer Center at the Medical University of South Carolina for providing instrumentation and support for confocal/multiphoton microscopy.

ABBREVIATIONS

ALT	alanine aminotransferase
ATP	adenosine 5'-triphosphate
BrdU	5-bromo-2'-deoxyuridine
CDK	cyclin-dependent kinase
CsA	cyclosporin A
CyD1	cyclin D1
CypD	cyclophilin D
hpf	high power field
IR	ischemia/reperfusion
JNK	c-Jun- <i>N</i> -terminal kinase
MHX	massive hepatectomy
MKK	mitogen-activated protein kinase kinase
MPT	mitochondria permeability transition
NIM811	N-methyl-4-isoleucine cyclosporin
PCNA	proliferating cell nuclear antigen
PI	propidium iodide
PT	permeability transition
Rh123	rhodamine123
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
UW solution	University of Wisconsin cold storage solution

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Fig. 1. NIM811 Protects against Cell Death and Alanine Aminotransferase Release after Massive Hepatectomy

Sham-operation (**Sham**) and massive hepatectomy (**MHX**) were performed as described in **METHODS**. Livers were harvested at 48 h after surgery and stained with hematoxylin and eosin (**A**, bar is 100 μ m) to assess necrosis or harvested at 6 h and stained with TUNEL staining (**B**, bar is 50 μ m) to assess apoptosis. Representative images are shown. Ten random fields per slide were captured in a blinded manner using a 10 $\times$ objective lens. Necrotic areas were quantified by image analysis (**C**). TUNEL-positive and negative cells were counted in a blinded manner in 10 randomly selected fields using a 10 $\times$ objective lens (**D**). Blood samples were collected at 0, 24 and 48 h after surgery for serum alanine transaminase (ALT) measurement (**E**). *, $p < 0.05$ vs. MHX + NIM811 ($n = 4$ per group).

Fig. 2. NIM811 Improves Regeneration of Remnant Livers

Sham-operation (**Sham**) and massive hepatectomy (**MHX**) were performed. Livers were harvested at 24 h and 48 h after surgery. 5-bromo-2'-deoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA) were detected immunohistochemically. Representative images of BrdU staining are shown in **A-C**. Bar is 50 μ m. Quantification of BrdU- and PCNA-positive cells in 10 randomly selected fields was shown in **D** and **E**, respectively. The weight of remnant livers was measured at 48 h after surgery and standardized to 25 g body weight (**F**). *, $p < 0.05$ vs. MHX + vehicle treatment ($n = 4$ per group).

Fig. 3. NIM811 Improves Liver Functions and Survival after Massive Hepatectomy

Sham-operation and massive hepatectomy (MHX) were performed. Blood was collected at 48 h after surgery to determine total bilirubin (**A**) and albumin (**B**). *, $p < 0.05$ vs. MHX + vehicle treatment ($n = 4$ per group). Mice were observed 3 week after surgery for survival (**C**). Survival of the mice treated with NIM811 ($n = 15$) was significantly greater than untreated mice after MHX ($n = 16$; $p = 0.037$ by the Kaplan-Meier test).

Fig. 4. Mitochondrial Depolarization and Onset of the Mitochondrial Permeability Transition Occur after Massive Hepatectomy: Reversal by NIM811

Sham-operation (**Sham**) and massive hepatectomy (**MHX**) were performed. At 3 h after surgery, Rh123 and PI were infused, and intravital confocal microscopy was performed to detect mitochondrial polarization and cell death (**A - C**). Calcein-AM was infused and intravital multiphoton microscopy as performed to detect onset of the MPT (**D - F**). Representative images are shown. Rows are: **upper**, sham-operation; **middle**, MHX; **lower**, MHX plus NIM811 treatment. Bars are 10 μ m. Viable hepatocytes with depolarized mitochondria (**G**) were counted in 10 randomly selected fields after intravital confocal

microscopy. Group sizes are 5 livers in the MHX group and 4 livers per group in the sham-operation and MHX + NIM811 groups. **a**, $p < 0.05$ vs. sham-operation; **b**, $p < 0.05$ vs. MHX.

Fig. 5. NIM811 Prevents Decreases of ATP Production, Cytochrome C Release and Caspase-3 Activation after Massive Hepatectomy

Sham-operation (**Sham**) and massive hepatectomy (**MHX**) were performed. Livers were harvested at 3 h for ATP measurement (**A**). **a**, $p < 0.05$ vs sham-operation; **b**, $p < 0.05$ vs MHX ($n = 4$ per group). Cytochrome *c* was detected immunohistochemically (**B**). Bar is 10 μm . Caspase-3 activity in liver tissue was detected using a kit from R&D Systems (**C**). *, $p < 0.05$ vs. MHX + NIM811 ($n = 4$ per group).