

"BNIP3 peptides for treatment of reperfusion injury"

FIELD OF THE INVENTION

The present invention is directed to the treatment of reperfusion injury. IN particular, the present invention provides BNIP3-derived peptides which prevent cell damage and cell death by reducing the activity of BNIP3 and BAX at the mitochondria.

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BACKGROUND OF THE INVENTION

The occlusion of a vessel causes cessation of blood to part of the tissue, which leads in particular to insufficient oxygen supply, reduced availability of nutrients and inadequate removal of metabolic waste wreaking havoc on cells with subsequent deaths of cells. Whereas the acute occlusion is not predictable or avoidable, the restoration of vessel patency is feasible and essential for the outcome of the patients¹. A timely reperfusion regimen is the recommended therapy but the rapid restoration of blood and particularly O₂ supply imposes injury upon the tissue with no current treatment available. The early phase of reperfusion is characterized by a high level of oxygen leading to hyperoxic conditions, a burst of reactive oxygen species and an increased calcium level without acidosis. The pathology of reperfusion injury has been recognized in the heart, brain, liver, and kidney and is associated with serious clinical manifestations, including myocardial hibernation, acute heart failure, cerebral dysfunction, gastrointestinal dysfunction, renal dysfunction, systemic inflammatory response syndrome, and multiple organ dysfunction syndrome. Therefore, reperfusion injury is a critical medical condition

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that poses an important therapeutic challenge. Myocardial infarction (MI) is a sudden, temporally unpredictable event in which reperfusion is essential for survival, but determines up to 50% of the final infarct size². This fate also applies to transplanted organs. MI is the most common cause of heart failure cases, therefore therapeutic interventions to reduce reperfusion injury present an opportunity to salvage viable myocardium, limit MI size, preserve heart function and impact the incidence of heart failure³. In the reperfusion-induced infarct progression two forms of cell death, namely necrosis and apoptosis, play an essential role. A predomination of necrotic cardiomyocyte death could be observed in the initial infarct area. Necrosis induces downstream tissue responses such as inflammation, matrix remodeling, and later fibrosis⁴. Apoptosis occur in the infarct and the peri-infarct area and is a major component of early post-infarct remodeling⁵.

Cardiac damage is also a critical issue for cancer patients. Due to advances in screening and treatment strategies, the population of cancer survivors has steadily grown over the last three decades. Overall 5-year survival improved to 50-70% for ten year follow-ups. As a result, there is a growing prevalence of cancer therapy side effects, notably cardiovascular toxicity. Conventional chemotherapy (e.g. anthracyclines) are commonly used therapies for many cancer that have been widely recognized as contributors to asymptomatic and symptomatic declines in left ventricular ejection fraction (LVEF), cardiomyopathy, and heart failure (HF). Cancer-mediated cardiomyopathy is characterized by a dose-dependent decrease in LV systolic function mediated by reactive oxygen species (ROS) that is typically irreversible. Currently, neither approaches to prevent nor therapies exists to effectively reduce cardiotoxic side effects (for example reduction in heart function, cardiomyopathy etc.) in patients undergoing anthracycline chemotherapy or other cancer therapies. Several studies aimed to address this medical need but revealed only partial results/benefits. The CECCY trial failed to show a benefit of carvedilol in breast cancer patients, however, protection by reduction of troponin was shown⁶. The PRADA trial assessed candesartan and metoprolol and revealed a significant benefit for candesartan for the prevention of cardiomyopathy, despite the study being not sufficiently powered according to the current definition of cardiotoxicity⁷. The primary outcome of the MANTICORE trial was change in left ventricular diameter. Neither beta-blocker nor ACE-inhibitor had a significant impact on this outcome measure, but significantly prevented heart failure as secondary outcome⁸. Taken together, while existing literature points towards a potential benefit of heart failure therapy, currently no study has in depth evaluated the value of state-of-the-art heart failure therapy as defined by current guidelines in the prevention of cardiotoxicity in cancer patients.

Mitochondria are central to both necrotic and apoptotic signalling⁹. These include disruption of electron transport, oxidative phosphorylation, and ATP synthesis, DNA fragmentation, protein and lipid damage, and the excessive generation of ROS.

The defining event of necrosis at the mitochondria is the opening of a pore in the mitochondrial inner membrane (MIM), the so-called mitochondrial permeability

transition pore (mPTP). This creates an energetic collapse and rapid exchange of solutes with an influx of osmolytes into the mitochondria. The subsequent swelling of the matrix leads to rupture of the mitochondrial outer membrane, cellular swelling and cell disruption¹⁰. Necrotic stimuli, such as Ca^{2+} , are suggested to trigger opening of the mPTP and can be potentiated by ROS¹⁰. Despite extensive investigation, the components of the mPTP remain unknown, with transgenic animal studies excluding several putative components including the adenine nucleotide translocase¹¹, the voltage-dependent anion channel¹², the mitochondrial phosphate carrier¹³ (SLC25A3), and cyclophilin D¹⁴. Most recently, the c-subunit of the ATP synthase has been suggested to form the pore in the inner membrane^{15,16}. Preventing mPTP opening using pharmacological inhibitors such as cyclosporine A has been reported to reduce infarct size in pre-clinical models of I/R injury^{17,18}. In larger clinical trials, its effect was neutral¹⁹. The mitochondria-targeting peptide, elamipretide (formerly called Bendavia or MTP-131) as well as the mitochondrial targeting drug, TRO40303, have been demonstrated in animal studies to reduce infarct size by attenuating the production of mitochondrial-derived ROS when administered at the onset of reperfusion²⁰⁻²². However, in STEMI patient studies intravenous elamipretide²⁰ as well as TRO40303, both administered prior to PPCI, failed to reduce infarct size²³. Notably, more adverse events were reported in patients receiving TRO40303 when compared with the placebo arm, thereby limiting the clinical application of this therapeutic approach. Equally futile appeared Na^+/H^+ exchange inhibitors²⁴, anti-oxidants such as superoxide dismutase²⁵, and various anti-neutrophil antibodies^{26,27}.

Apoptotic cell death occurring in the infarct and peri-infarct area is initiated by mitochondrial outer membrane (MOM) permeabilization enabling the release of pro-apoptotic proteins, such as cytochrome c, apoptosis inducing factor, SMAC/DIABLO (Second Mitochondria-derived Activator of Caspases/Direct IAP Binding Protein with Low PI), and endonuclease G from the intermembrane space into the cytosol leading to the onset of cell death cascades via caspases and DNA fragmentation²⁸⁻³⁰.

The pro-death BCL-2 proteins BNIP3 (BCL-2 adenovirus E1B 19 kDa interacting protein-3) and BAX (BCL-2 associated X protein) induce MOM permeabilization and represent mediator and down-stream effector of mitochondrial apoptosis by translocation into the MOM and forming heterodimers³¹⁻³⁵. In addition, BNIP3 and BAX regulate perturbation of the MIM, thereby functioning as crucial activators of necrosis^{5,36}.

The present invention addresses the need for an optimal amelioration of both the acute injury in the central infarct zone and the subsequent cell death in immediately surrounding areas by providing an inhibitor of BNIP3 and BAX interaction activity, which interrupts the intra- and inter-pathway communication between BNIP3, BAX and mitochondria as individuals or triangle to treat reperfusion injury in heart, brain, liver and kidney and other indications in which the perturbation of mitochondria leads to cell damage and cell death, such as, for example heart failure, organ transplantation,

cardiac arrest or due to surgical and pharmacological intervention as well as stroke-, cancer- and cancer therapy-induced cardiac damage.

SUMMARY OF THE INVENTION

5 The present invention provides peptides which bind to BNIP3 and BAX as monomers as well as to their homo- and hetero-oligomers representing a broad-spectrum activity by circumventing individual activities and oligomer activities. The efficacy is not restricted to an organ or a species, as evidenced by protecting heart and brain tissue as well as human ventricular cardiomyocytes derived from human induced pluripotent stem cells against reperfusion injury. Myocardial infarct size was also markedly
10 reduced in swine.

The peptides are derived from the *N*-terminal portion of BNIP3 and an 8 amino acid stretch consisting of amino acids 13 to 20 of BNIP3 proved to be most active. It was highly surprising that such a short peptide was able to inhibit BNIP3 and BAX activities, block formation of homo- and hetero-oligomerization of these proteins, and induce
15 conformational changes in these homo- and hetero-oligomer within the cell. Certain mutations in the peptide sequence even enhanced its efficacy.

In view of these results, the present invention provides in a first aspect a peptide comprising

- (i) a cellular uptake signal; and
- 20 (ii) a BNIP3 fragment comprising positions 13 to 20 of BNIP3 or an amino acid sequence derived therefrom.

The peptide especially has a length of 50, in particular 40, amino acids or less.

In a second aspect, the present invention provides a pharmaceutical composition comprising the peptide according to the first aspect and its use in the treatment of
25 reperfusion-related and/or mitochondria-related disorders, as well as cancer therapy-induced cardiotoxicity and prevention of such.

In a third aspect, the invention provides a method for preventing cell damage or cell death, comprising contacting the cell with the peptide according to the first aspect.

The invention is further directed in a fourth aspect to a method of screening for a
30 compound suitable for prevention of reperfusion injury and/or mitochondria-related disorders and/or cancer therapy-induced cardiotoxicity, comprising

- (i) providing one or more candidate compounds;
- (ii) determining the ability of the candidate compounds to interfere with the binding of BNIP3 and BAX;

- (iii) selecting those candidate compounds which interfere with the binding of BNIP3 and BAX.

Other objects, features, advantages and aspects of the present invention will become apparent to those skilled in the art from the following description and appended claims. It should be understood, however, that the following description, appended claims, and specific examples, which indicate preferred embodiments of the application, are given by way of illustration only. Various changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Deletion of BNIP3 in mice reduces myocardial infarct size *in vivo*. **A** Schematic of the *in vivo* ischemia/reperfusion model. **B** Schematic of a heart section depicting non-ischemic area (remote), ischemic area (area at risk, AAR), infarct area (white, embedded in AAR). **C** Infarct sizes post 24 h of reperfusion in wild-type, BNIP3 deficient (*Bnip3*^{-/-}) and *Bnip3*^{-/-} mice treated with indicated TAT-BNIP3 doses (n = 3-7 mice). AAR – area at risk; Inf – infarct. Data are mean ± s.e.m. Statistical analyses are two-way analysis of variance (ANOVA) with Bonferroni's correction.

Figure 2. BNIP3 is a mediator of BAX activity in myocardial reperfusion injury. Mice were exposed to indicated reperfusion time durations *in vivo* after vessel occlusion. Increasing mitochondrial BNIP3 level (**A**) and mitochondrial BAX concentration (**B**) in the area at risk at baseline and after 10 min of reperfusion were observed (n = 5-7 mice). **C** Western blot monitoring revealed that BNIP3 is co-immunoprecipitated with BAX at baseline and after 10 and 30 min of reperfusion. **D** Quantification of mitochondrial BAX level in the area at risk of BNIP3 deficient (*Bnip3*^{-/-}) mice at baseline and post 10 min of reperfusion untreated and treated with BNIP3 (n = 3 mice). Translocation of BAX is dependent on the presence of BNIP3. Data are mean ± s.e.m. Statistical analyses are two-way analysis of variance (ANOVA) with Bonferroni's correction.

Figure 3. Interaction sites, secondary structure and *in silico* docking. **A** Schematic of the experimental set-up (JPT, Berlin, Germany, Berlin). **B** Heat map depiction of BNIP3 incubation with the BAX peptide library displaying the helices α5, α6 and α7+α8 as interaction sites. Colour coding ranges from white (0 or low intensity) over light gray (middle intensity) to dark gray (high intensity). **C** The three-dimensional structure model of BNIP3 obtained by homology modelling using Modeller 9.15. **D** Circular dichroism (CD) spectroscopic analysis of BNIP3. **E, F** Cartoon representations of BAX/BNIP3 interactions with indicated binding sites resulting from *in silico* docking experiments using HADDOCK. **G** Structure of TAT sequence. **H** Structure of BNIP3-20A structure. **I** Structure of BNIP3-20C structure. **J** Representative images of transmural distribution of TAT-BNIP3-20A (green) post 10 min of reperfusion *in vivo*. Scale bars, 1 mm. **K**

Western blot monitoring of TAT-BNIP3-20A co-immunoprecipitated with BNIP3 after 5 min of reperfusion post vessel occlusion *in vivo*.

Figure 4. TAT-BNIP3-20A reduces myocardial reperfusion injury *in vivo*. **A** Schematic of the *in vivo* myocardial infarction model. Mice were exposed to the indicated reperfusion time durations after vessel occlusion *in vivo*. The peptide was given into the left ventricle 5 min before reperfusion. **B** Infarct sizes post 24 h of reperfusion in wild-type mice treated with vehicle, the control peptide TAT-BNIP3-20C and TAT-BNIP3-20A (n = 7-10 mice). TAT-BNIP3-20A significantly reduces the infarct size. BNIP3-20C and vehicle are not effective to attenuate the infarction. TAT-BNIP3-20A inhibits BNIP3 interaction with mitochondria after 10 min of reperfusion (**C**) and caspase-3 activity after 4 h of reperfusion (**D**), whereas the control peptide TAT-BNIP3-20C does not (n = 6 mice). Data are mean $\pm$ s.e.m. Statistical analyses are two-way analysis of variance (ANOVA) with Bonferroni's correction.

Figure 5. **A** Schematic of the *in vitro* reoxygenation (study design in human ventricular cardiomyocytes derived from human induced pluripotent stem cells (humanCM). HumanCM were exposed to normoxia and 2 h of reoxygenation after hypoxia and were treated with the TAT-BNIP3-20A and the control peptide BNIP3-20C. **B** TAT-BNIP3-20A markedly inhibits the interaction of BNIP3 with mitochondria. **C** Representative images of apoptotic, necrotic and healthy humanCM. Scale bars, 1mm (left), 200 μ m (right). TAT-BNIP3-20A potently prevents humanCM death in reoxygenation. **D** Representative images of depolarized mitochondria, healthy mitochondria, nucleus. TAT-BNIP3-20A diminishes the loss of mitochondrial inner membrane potential induced by reoxygenation. Scale bars, 400 μ m (left), 100 μ m (right). Control peptide TAT-BNIP3-20C was not effective to inhibit BNIP3 interaction with mitochondria, loss of mitochondrial inner membrane potential and cell death. Data are mean $\pm$ s.e.m. Statistical analyses are two-way analysis of variance (ANOVA) with Bonferroni's correction.

Figure 6. **A** Structure of the BNIP3-8B sequence. **B** Structure of the BNIP3-8C sequence. **C** Circular dichroism (CD) spectroscopic analysis of the BNIP3-8B.

Figure 7. **A** Uptake of fluorescence labelled TAT-BNIP3-8B in different organs after 5 min of reperfusion post vessel occlusion. TAT-BNIP3-8B was given 5 min before starting reperfusion. **B** Survival of isolated adult cardiomyocytes treated with TAT-BNIP3-8B and TAT-BNIP3-8C control peptide after 24 h.

Figure 8. TAT-BNIP3-8B pharmacokinetics. Fluorescence labelled BNIP3-8B was incubated in human serum (**A**), plasma (**B**), and whole blood (**C**) at 37°C for indicated time durations and monitored by Western blot. Proteinase K treatment served as control.

Figure 9. TAT-BNIP3-20A reduces myocardial infarct size *in vivo*. **A** Schematic of the *in vivo* myocardial infarction model. Mice were exposed to 5 min and 24 h of reperfusion after vessel occlusion, respectively. The peptide was given into the left

ventricle 5 min before starting reperfusion. **B** Western blot monitoring of TAT-BNIP3-8B co-immunoprecipitated with BNIP3 and BAX 5 min after reperfusion. **C** Immunoblot for cytosolic BNIP3 and BAX in the area at risk following blue native-PAGE at baseline and 10 minutes reperfusion. Mice were treated with vehicle (NaCl) and TAT-BNIP3-8B. Sham operated mice served as controls. **D, E** Infarct sizes post 24 h of reperfusion in wild-type mice treated with vehicle, TAT- β -Gal, control peptide TAT-BNIP3-8C and indicated TAT-BNIP3-8B doses (n = 7-10 mice). TAT-BNIP3-8B significantly reduces the infarct size in a dose-dependent manner. Vehicle, TAT- β -Gal, and TAT-BNIP3-8C were not effective to attenuate the infarction.

Figure 10. BNIP3-8B diminishes myocardial reperfusion injury *in vivo*. Mice were exposed to the indicated reperfusion time durations after vessel occlusion *in vivo*. The peptide was given into the left ventricle 5 min before starting reperfusion. TAT-BNIP3-8B inhibits BNIP3 (**A**) and BAX (**B**) interaction with mitochondria after 10 min of reperfusion, mitochondrial swelling after 10 min of reperfusion (**C**), BAX activation after 30 min of reperfusion (**D**), cytochrome c release after 30 min of reperfusion (**E**), and caspase-3 activity after 4 h of reperfusion (**F**), whereas the control peptide TAT-BNIP3-8C does not (n = 5-12 mice). Sham-operated mice served as control (n = 5-8 mice). Data are mean $\pm$ s.e.m. Statistical analyses are two-way analysis of variance (ANOVA) with Bonferroni's correction.

Figure 11. A Schematic of the *in vitro* reoxygenation study design in human ventricular cardiomyocytes derived from human induced pluripotent stem cells (humanCM). HumanCM were exposed to normoxia and 2 h of reoxygenation after hypoxia and were treated with the TAT-BNIP3-8B and the control peptide TAT-BNIP3-8C. **B** TAT-BNIP3-8B potentially inhibits humanCM death in reoxygenation. Representative images of apoptotic, necrotic and healthy humanCM. **C** TAT-BNIP3-8B attenuates loss of mitochondrial inner membrane potential induced by reoxygenation. Representative images of depolarized mitochondria, healthy mitochondria, nucleus. Scale bars, 200 μ m. Control peptide BNIP3-8C was not effective to inhibit cell death and loss of mitochondrial inner membrane potential. Data are mean $\pm$ s.e.m. Statistical analyses are two-way analysis of variance (ANOVA) with Bonferroni's correction.

Figure 12. TAT-BNIP3-8B reduces brain infarct sizes after 24 h of reperfusion following a transient middle cerebral artery occlusion and in comparison to vehicle treatment. TAT-BNIP3-8B and vehicle were given immediately before reperfusion. Data are mean $\pm$ s.e.m. Unpaired Student's t-test was used, and statistical significance was set at the level of $P < 0.05$.

Figure 13. TAT-BNIP3-8B reduces myocardial infarct sizes after 4 h of reperfusion following left coronary artery occlusion and in comparison to vehicle treatment in swine. TAT-BNIP3-8B and vehicle were given 5 min before reperfusion. Data are mean $\pm$ s.e.m. Unpaired Student's t-test was used, and statistical significance was set at the level of $P < 0.05$.

Figure 14. TAT-BNIP3-8B protects against Doxorubicin-induced mitochondrial injury by preventing mitochondrial swelling. HL-1 cells were treated with 5 μ M Doxorubicin without or together with TAT-BNIP3-8B and swelling of the mitochondria was determined by optical density, wherein swollen mitochondria have a lower OD₅₄₀. Untreated cells were used as control.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides methods, compounds and compositions of treating a disease or condition in a subject in which it is desirable to inhibit the individual activity and inter-pathway communication of BCL-2 adenovirus E1B 19 kDa interacting protein-3 (BNIP3), BCL-2 associated X protein (BAX) and mitochondria to prevent cell damage and cell death.

In one aspect the invention is a peptide inhibiting the BNIP3, BAX and mitochondria triangle which activates cell damage and cell death cascades. The peptide according to the invention comprises a cellular uptake signal; and a BNIP3 fragment comprising positions 13 to 20 of BNIP3 or an amino acid sequence derived therefrom and especially has a length of 50, in particular 40, amino acids or less.

The BNIP3 fragment

The BNIP3 fragment within the peptide according to the present invention in particular is capable of binding to BAX and/or BNIP3. Especially, the BNIP3 fragment is capable of binding to the BNIP3-binding region of BAX, such as the region of amino acids 108 to 164 of BAX. The BNIP3 fragment is in particular capable of interfering with or inhibiting the interaction of BNIP3 and BAX.

In certain embodiments, the BNIP3 fragment has a length of 20 amino acids or less. Especially, it has a length of 15 amino acids or less or even 10 amino acids or less. In particular embodiments, the BNIP3 fragment has a length of 8 amino acids.

In specific embodiments, the BNIP3 fragment has an amino acid sequence of the BNIP3 protein. The term "BNIP3" as used herein in particular refers to mouse BCL-2 adenovirus E1B 19 kDa interacting protein-3 having the amino acid sequence of SEQ ID NO: 1. The BNIP3 fragment hence may have an amino acid sequence which is identical to a consecutive part of the amino acid sequence of SEQ ID NO: 1 which comprises the sequence of amino acid positions 13 to 20. For example, the BNIP3 fragment may have the amino acid sequence of positions 1 to 20 of SEQ ID NO: 1. In certain embodiments, the BNIP3 fragment has an amino acid sequence selected from the group consisting of positions 4 to 20 of SEQ ID NO: 1, positions 11 to 20 of SEQ ID NO: 1, positions 12 to 20 of SEQ ID NO: 1, and positions 13 to 20 of SEQ ID NO: 1. In specific embodiments, the BNIP3 fragment consists of the amino acid sequence of positions 13 to 20 of SEQ ID NO: 1. In these embodiments, the BNIP3 fragment in particular does not comprise any further amino acid residues. The amino acid

sequence of positions 13 to 20 of mouse BNIP3 is identical to the amino acid sequence of positions 73 to 80 of human BNIP3 (SEQ ID NO: 2). Alternative to the amino acid sequences of mouse BNIP3 referred to herein, also the corresponding amino acid sequences of human BNIP3 can be used.

- 5 In further embodiments, the BNIP3 fragment has an amino acid sequence which is derived from BNIP3. A target amino acid sequence is "derived" from or "corresponds" to a reference amino acid sequence if the target amino acid sequence shares a homology or identity over its entire length with a corresponding part of the reference amino acid sequence of at least 60%, more preferably at least 70%, at least 80%, at
10 at least 90% or at least 95%. In particular embodiments, a target amino acid sequence which is "derived" from or "corresponds" to a reference amino acid sequence is 100% homologous, or in particular 100% identical, over its entire length with a corresponding part of the reference amino acid sequence. A "homology" or "identity" of an amino acid sequence or nucleotide sequence is preferably determined according to the invention
15 over the entire length of the reference sequence or over the entire length of the corresponding part of the reference sequence which corresponds to the sequence which homology or identity is defined. The BNIP3 fragment in particular may be derived from one of the BNIP3 fragments described above. For example, the BNIP3 fragment may have an amino acid sequence which is at least 60% identical, especially at least
20 70% identical, to an amino acid sequence selected from the group consisting of positions 1 to 20 of SEQ ID NO: 1, positions 4 to 20 of SEQ ID NO: 1, positions 11 to 20 of SEQ ID NO: 1, positions 12 to 20 of SEQ ID NO: 1, and positions 13 to 20 of SEQ ID NO: 1. In specific embodiments, the BNIP3 fragment comprises an amino acid sequence which is at least 60% identical to positions 13 to 20 of SEQ ID NO: 1.
- 25 In certain embodiments, the BNIP3 fragment comprises positions 13 to 20 of BNIP3, optionally comprising 1, 2 or 3 amino acid substitutions compared to positions 13 to 20 of BNIP3. BNIP3 especially has the amino acid sequence of SEQ ID NO: 1. In these embodiments, the 1, 2 or 3 amino acid substitutions are preferably present at one or more of the position corresponding to positions 13, 15, 17, 18, 19 and 20 of BNIP3, in
30 particular positions 15, 17 and 19 of BNIP3. In particular, the amino acid substitutions are selected from the group consisting of
- (i) substitution of glutamic acid at position 15 of BNIP3 to phenylalanine, isoleucine, leucine, valine, tyrosine, cysteine, histidine, arginine or threonine,
 - (ii) substitution of histidine at position 17 of BNIP3 to valine, and
 - 35 (iii) substitution of serine at position 19 of BNIP3 to tyrosine, cysteine, phenylalanine or histidine.

In particular embodiments, the BNIP3 fragment comprises positions 13 to 20 of BNIP3 comprising 1 or 2 amino acid substitutions compared to positions 13 to 20 of BNIP3, wherein the amino acid substitutions are selected from the group consisting of

- (i) substitution of glutamic acid at position 15 of BNIP3 to histidine, isoleucine, leucine, valine or tyrosine, and
- (ii) substitution of serine at position 19 of BNIP3 to tyrosine, cysteine or phenylalanine.

5 In certain embodiments, serine at position 19 of BNIP3 is substituted to tyrosine, cysteine or phenylalanine, in particular to phenylalanine.

10 In specific embodiments, the BNIP3 fragment comprises or consists of an amino acid sequence selected from the group consisting of SEQ ID NOs: 3 to 17. In special embodiments, the BNIP3 fragment consists of the amino acid sequence of SEQ ID NO: 7 or 8, especially 8.

15 The BNIP3 fragment may optionally comprise further amino acid residues derived from BNIP3. Especially, the entire BNIP3 fragment is derived from a consecutive amino acid sequence of BNIP3. In particular, the entire BNIP3 fragment is derived from amino acid positions 1 to 20 of BNIP3 or a part thereof which comprises at least positions 13 to 20 of BNIP3. The BNIP3 fragment may have an amino acid sequence identical to the corresponding part of BNIP3 or may have 1 to 8 amino acid substitutions, especially 1 to 6 amino acid substitutions. The BNIP3 fragment may for example comprise positions 12 to 20 of BNIP3, optionally comprising 1, 2, 3 or 4 amino acid substitutions compared to positions 12 to 20 of BNIP3, or it may comprise positions 4 to 20 of BNIP3, optionally comprising 1, 2, 3, 4, 5 or 6 amino acid substitutions compared to positions 4 to 20 of BNIP3, or it may comprise positions 1 to 20 of BNIP3, optionally comprising 1, 2, 3, 4, 5 or 6 amino acid substitutions compared to positions 1 to 20 of BNIP3. In particular, 1, 2 or 3 of these amino acid substitutions are in positions 13 to 20 and the remaining amino acid substitutions are in positions 1 to 12. In these embodiments, the amino acid substitutions are preferably present at one or more of the position corresponding to positions 4, 11, 12, 13, 15, 17, 18, 19 and 20 of BNIP3, in particular to positions 4, 11, 12, 15, 17 and 19 of BNIP3.

25 In specific embodiments, the BNIP3 fragment comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 18 to 30 and 70 to 75.

30 A substituted amino acid residue in particular is substituted for another naturally occurring amino acid residue. The phrase "substitution" as used herein also includes the use of a chemically derivatized residue in place of a non-derivatized residue provided that such polypeptide exerts the requisite activity. The term "derivative" as used herein refers to a peptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The

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imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5 hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine.

In some embodiments, the term "substitution" includes a linkage of two or more substituted amino acid residues. In particular, two or more amino acid residues may be substituted with crosslinkable moieties and/or linked and each optionally comprises an additional α -carbon substitution selected from substituted, optionally hetero- lower alkyl, particularly optionally substituted, optionally hetero- methyl, ethyl, propyl and butyl. Furthermore, two substituted amino acid residues may be substituted with homocysteines connected through a disulfide bridge to generate a ring and tail cyclic peptide. Alternatively, two or more substituted amino acid residues may be replaced by a linker. Suitable linkers in this respect include, for example, $-(CH_2)_nONHCOX(CH_2)_m-$, wherein X is CH_2 , NH or O, and m and n are integers 1-4, forming a lactam peptide; $-CH_2OCH_2CHCHCH_2OCH_2-$, forming an ether peptide; or $-(CH_2)_nCHCH(CH_2)_m-$, forming a stapled peptide.

In certain embodiments, the BNIP3 fragment comprises at least one of the substitutions E15Y, S19F and S19Y with respect to the sequence of BNIP3.

The cellular uptake signal

The cellular uptake signal within the peptide according to the invention is in particular capable of mediating the uptake of the peptide into a target cell. Especially, the cellular uptake signal is capable of mediating uptake into a mammalian cell, in particular a human cell.

In certain embodiments, the cellular uptake signal is a peptide. In particular, the cellular uptake signal is a cell penetrating peptide or protein transduction domain. It may have a length of 5 to 30 amino acids, in particular 8 to 20 amino acids or 10 to 16 amino acids.

The cellular uptake signal may for example be a hydrophilic peptide or an amphiphilic peptide. Examples of cellular uptake signals include the protein transduction domain of the TAT protein of HIV (in particular amino acid residues 48-59), penetratin, antennapedia PTD, SynB1, SynB3, PTD-4, PTD-5, FHV Coat-(35-49), BMV Gag-(7-25), HTLV-II Rex-(4-16), D-Tat, R9-Tat, Transportan, MAP, SBP, FBP, MPG, MPG^(ANLS), Pep-1, Pep-2, polyarginines and polylysines. In certain embodiments, the cellular uptake signal is a peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs: 31 to 50.

The cellular uptake signal may be composed of naturally occurring amino acid residues and may optionally be a peptide derivative comprising chemically derivatized amino

acid residue as described herein. Furthermore, it may be a peptide mimetic or comprise a D-retro-inverso sequence. In certain embodiments, the cellular uptake signal comprises a D-retro-inverso sequence of a cell penetrating peptide as disclosed herein.

5 **The peptide**

10 The peptide according to the invention comprises the cellular uptake signal and the BNIP3 fragment. The expression "comprise", as used herein, besides its literal meaning also includes and specifically refers to the expressions "consist essentially of" and "consist of". Thus, the expression "comprise" refers to embodiments wherein the subject-matter which "comprises" specifically listed elements does not comprise further elements as well as embodiments wherein the subject-matter which "comprises" specifically listed elements may and/or indeed does encompass further elements. Likewise, the expression "have" is to be understood as the expression "comprise", also including and specifically referring to the expressions "consist essentially of" and "consist of". The term "consist essentially of", where possible, in particular refers to 15 embodiments wherein the subject-matter comprises 20% or less, in particular 15% or less, 10% or less or especially 5% or less further elements in addition to the specifically listed elements of which the subject-matter consists essentially of.

20 In certain embodiments, the peptide according to the invention consists of the cellular uptake signal and the BNIP3 fragment.

25 In further embodiments, the peptide according to the invention comprises the cellular uptake signal, the BNIP3 fragment, and a linker between the cellular uptake signal and the BNIP3 fragment. Especially, the peptide according to the invention consisting of the cellular uptake signal, the BNIP3 fragment, and a linker between the cellular uptake signal and the BNIP3 fragment. The linker may be a peptide linker composed of amino acids, or a chemical linker. The linker in particular has a small size. For example, it is a peptide linker having 10 or less amino acids, such as 8 or less, 6 or less or 5 or less amino acids. A chemical linker for example has a molecular weight of 1,500 Da or less, such as 1,000 Da or less, 750 Da or less or 500 Da or less. In embodiments where the 30 cellular uptake signal is a peptide moiety, the linker preferably is a peptide linker.

35 In certain embodiments, the peptide according to the invention has a length of 40 amino acids or less. In specific embodiments, the peptide according to the invention has a length of 35 amino acids or less, especially 30 amino acids or less. The peptide according to the invention may for example have a length of 25 amino acids or less, such as about 20 amino acids. Shorter peptides are especially desired because they can more easily be produced, formulated and handled. Therefore, the peptide according to the invention preferably has a length of 35 amino acids or less, especially 25 amino acids or less. This in particular refers to embodiments where the cellular uptake signal is a peptide moiety. In specific embodiments, the peptide according to

the invention has a molecular weight of 10,000 Da or less, especially 5,000 Da or less, 4,000 Da or less or 3,000 Da or less.

In certain embodiments, the peptide according to the invention has an amino acid sequence selected from the group consisting of SEQ ID NOs: 51 to 67.

5 Generally, the peptide according to the invention is composed of naturally occurring L-amino acids. In certain embodiments, the peptide may also include artificial amino acids. For example, the peptide may comprise one or more D-amino acids, E- β -homo amino acids, and/or N-methylated amino acids; or it may be composed thereof. In certain embodiments, the cellular uptake signal and/or the BNIP3 fragment are the D-retro-inverso sequence, especially the D-retro-inverso sequence of an amino acid
10 sequence described herein. For example, the peptide has the D-retro-inverso sequence of QPRRRQRRKKRG-NSFHLEVWSGQLNEEGSQSM (SEQ ID NO: 68) or QPRRRQRRKKRG-NSFHLEVW (SEQ ID NO: 69).

15 In certain embodiments, the peptide is acetylated, acylated, formylated, amidated, phosphorylated, sulfated, nitrosated, glycosylated, suomyated, hydroxylated, alkylated and/or isomerized. For example, the peptide may comprise an N-terminal acetyl, formyl, myristol, palmitoyl, carboxyl or 2-furosyl group, and/or a C-terminal hydroxyl, amide, ester or thioester group. Furthermore, the peptide may be cyclized.

20 In specific embodiments, the peptide according to the invention is a peptide mimetic of any of the peptides described herein. The term "peptide mimetic" as used herein refers to structures which serve as substitutes for peptides in interactions between molecules. Peptide mimetics include synthetic structures which may or may not contain amino acids and/or peptide bonds but retain the structural and functional features of a BNIP3 peptide. Peptide mimetics also include molecules incorporating peptides into larger
25 molecules with other functional elements, peptoids, oligopeptoids, and peptide libraries containing peptides of a designed length representing all possible sequences of amino acids corresponding to a peptide of the invention. All of these peptides as well as molecules substantially homologous, complementary or otherwise functionally or structurally equivalent to these peptides may be used for purposes of the present
30 invention.

In certain embodiments, the peptide according to the invention is present in a composition further comprising nanoparticles. Especially, the peptide is present within a nanoparticle. The nanoparticles may be any nanoparticles suitable to encapsulate the peptide. Exemplary nanoparticles include liposomes, nanoemulsions, solid-liquid
35 nanoparticles, nanostructured lipid carriers, polymeric nanoparticles and dendrimers. In certain embodiments, the nanoparticle comprises targeting molecules on its outer surface such as peptides, ligands or antibodies which enable targeting the peptide of the invention to the desired cells or tissues.

40 In specific embodiments, the nanoparticles enable uptake of the peptide according to the invention into the target cells. In these embodiments, the nanoparticle may perform

the task of the cellular uptake signal and in particular be or replace the cellular uptake signal. Hence, the present invention also provides a nanoparticle comprising a BNIP3 fragment as defined herein.

Therapeutic uses of the peptide

5 The invention provides methods, compounds and compositions of treating a disease or condition in a subject in which it is desirable to inhibit the individual activity and inter-pathway communication of BNIP3, BAX and mitochondria comprising administering to the subject the compound in an amount effective to treat the disease or condition in a subject.

10 The invention also provides pharmaceutical compositions comprising the peptide according to the invention. In particular, the pharmaceutical compositions comprise the peptide according to the invention in unit dosage, administrable form. The invention further provides methods of inhibiting cell damage and cell death, comprising administering to a person in need thereof an effective amount of the peptide according to the invention. The present invention also provides the use of the peptide according to the invention or the pharmaceutical composition comprising the same in medicine, especially in the treatment of reperfusion-related and/or mitochondria-related disorders as well as cancer therapy-induced cardiotoxicity and prevention of such, respectively.

20 The invention includes all combinations of the recited particular embodiments as if each combination had been laboriously separately recited.

The invention also provides a method of inhibiting BNIP3 in a subject comprising contacting the BNIP3 with one or more of any of the peptides or pharmaceutical compositions disclosed herein in an amount effective to inhibit BNIP3. Preferably, the BNIP3 is in a subject, and the one or more peptides or compositions is administered to the subject.

25 The invention also provides a method of inhibiting BAX in a subject comprising contacting the BAX with one or more of any of the peptides or pharmaceutical compositions disclosed herein in an amount effective to inhibit BAX. Preferably, the BAX is in a subject, and the one or more peptides or compositions is administered to the subject.

30 The invention also provides a method of inhibiting BNIP3/BAX dimer and/or oligomer activity in a subject comprising contacting the BNIP3/BAX dimers and/or oligomers with one or more of any of the peptides or pharmaceutical compositions disclosed herein in an amount effective to inhibit BNIP3/BAX dimer/oligomer activity. Preferably, the BNIP3 and the BAX is in a subject, and the one or more peptides or compositions is administered to the subject.

35 The invention also provides a method of treatment of reperfusion-related and/or mitochondria-related disorders in a subject, comprising administering the peptide or

pharmaceutical composition according to the invention to the subject in a therapeutically effective amount.

The invention also provides a method of the treatment or prevention of tissue damage due to mitochondria-induced apoptosis or necrosis in a subject, comprising administering the peptide or pharmaceutical composition according to the invention to the subject in a therapeutically effective amount. The peptide may be administered to the subject before, during and/or after occurrence of cell death or damage.

The subject being administered the peptide or pharmaceutical composition, and being treated, may have, for example, a disease or condition selected from the group consisting of hypoxic and/or ischemic cells; cardiac, brain, liver, kidney, bowel, limb ischemia, limb vessel occlusion; cardiac, brain, liver and kidney, bowel, limb reperfusion injury; myocardial infarction and reperfusion injury; chemotherapy-, radiotherapy-, targeted therapy-, and immunotherapy-induced cardiotoxicity; atherosclerosis; heart failure; heart, liver, kidney transplantation; aneurism; chronic pulmonary disease; ischemic heart disease; hypertension; pulmonary hypertension; embolisms; thrombosis; cardiomyopathy; stroke; neurodegenerative disease or disorder; an immunological disorder; renal hypoxia; hepatitis; a liver disease; a kidney disease; cerebellar degeneration; organ transplantation rejection, and a disease or disorder involving cell death and/or tissue damage. In certain embodiments, the subject has an ischemia-, reperfusion- and/or mitochondria-related disorder, especially after vessel occlusion, especially myocardial infarction, ischemic stroke, acute kidney injury, trauma, circulatory arrest, and ischemia during organ transplantation. In further embodiments, the subject suffers from cancer therapy-induced cardiotoxicity, for example cardiotoxicity induced by chemotherapy, radiotherapy, immunotherapy and/or targeted therapy. Or the subject may undergo any kind of cancer therapy and the therapy may be applied for prevention of cardiotoxicity. Chemotherapy in particular refers to anthracycline-based chemotherapy such as therapy with doxorubicin. In a specific embodiment, the subject suffers from cardiotoxicity induced by anthracycline-based chemotherapy such as chemotherapy with doxorubicin.

A reperfusion-related disorder may generally refer to a disorder which involves reperfusion injury. Reperfusion injury is tissue damage caused when e.g. blood supply returns to tissue after a period of ischemia, in which the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function. The invention thus provides a method of treatment of reperfusion injury in a subject, comprising administering the peptide or pharmaceutical composition according to the invention to the subject in a therapeutically effective amount.

The invention also provides a method of treating an acute myocardial infarction, a myocardial reperfusion injury or heart failure in a subject comprising administering to the subject one or more of the peptides or pharmaceutical compositions disclosed herein in an amount effective to treat an acute myocardial infarction, a myocardial

reperfusion injury or heart failure in a subject in need thereof. Preferably, the one or more peptides or the pharmaceutical composition is administered in an amount effective to inhibit BNIP3, BAX or BNIP3/BAX dimer/oligomer activity, respectively in a subject.

5 In certain embodiments, the treatment includes alleviation and/or prevention of reperfusion and mitochondria-related injury. In further embodiments, the treatment includes alleviation and/or prevention of cancer therapy-induced cardiotoxicity.

The subject can be, for example, a mammal, and is preferably a human.

10 As used herein "treating" or to "treat" a disease or disorder means to alleviate or ameliorate or eliminate a sign or symptom of the disease or disorder that is being treated. When the peptides or composition is administered to a subject before or at the onset of the disease or disorder, the peptides or composition can prevent or reduce the severity of the disease or disorder. For example, administration of the peptides or composition to a subject can prevent or reduce the severity of cancer therapy-induced
15 cardiotoxicity, such as chemo-, radio-, targeted-, or immunotherapy-induced cardiotoxicity. In these embodiments, the peptide according to the invention can be administered before, during and/or after cancer therapy. Administration of the peptides can include preventive and/or therapeutic administration.

20 The peptides and compositions of the present invention can be administered to subjects using routes of administration known in the art. The administration can be systemic or localized to a specific site. Routes of administration include, but are not limited to, intravenous, intramuscular, intracardial, intrathecal or subcutaneous injection, oral or rectal administration, and injection into a specific site.

25 In specific embodiments, the peptide or pharmaceutical composition according to the invention is administered to the subject during or after occurrence of impaired blood supply, ischemia or vessel occlusion, especially prior to reperfusion of the tissue affected by vessel occlusion. In certain embodiments, the peptide or pharmaceutical composition is administered within 6 hours prior to reperfusion, in particular within 4 hours, within 2 hours or within 1 hour prior to reperfusion. In specific embodiments the
30 peptide or pharmaceutical composition is administered within 45 minutes, especially within 30 minutes prior to reperfusion.

All combinations of the various elements described herein are within the scope of the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

35 The method may include expressing in a cell an effective amount of the peptide according to the invention, wherein apoptosis, necrosis, or the combination thereof, is altered in the cell compared to a control cell. The expressing may include, for instance, introducing a polynucleotide encoding the peptide into the cell. The cell may be *ex vivo*

or *in vivo*, and may be a cardiac cell. Apoptosis, necrosis, or the combination thereof may be decreased in the cell.

5 The present invention provides a method that includes administering to a subject in need thereof an effective amount of a composition comprising a polynucleotide encoding the peptide according to the invention, wherein apoptosis, necrosis, or the combination thereof, is increased in the subject. The administering may include
10 delivery of the polynucleotide to cardiac tissue, brain tissue, liver tissue and kidney tissue. The subject may have signs of or is at risk of a disease chosen from acute infarction, hypoxia, ischemia, stroke, or vascular disease. The method may result in a reduction of a sign of disease.

As used herein, the term "polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides, and includes both double- and single-stranded RNA and DNA. A polynucleotide can be obtained directly from a natural source, or can be prepared with the aid of recombinant, enzymatic, or chemical
15 techniques. A polynucleotide can be linear or circular in topology. A polynucleotide may be, for example, a portion of a vector, such as an expression or cloning vector, or a fragment. A polynucleotide may include nucleotide sequences having different functions, including, for instance, coding regions, and non-coding regions such as regulatory regions.

20 As used herein, "gene" refers to a nucleotide sequence that encodes a mRNA. A gene has at its 5' end a transcription initiation site and a transcription terminator at its 3' end. As used herein, a "target gene" refers to a specific gene whose expression is inhibited by a polynucleotide as described herein. As used herein, a "target mRNA" is a mRNA encoded by a target gene. Unless noted otherwise, a target gene can result in multiple
25 mRNAs distinguished by the use of different combinations of exons. Such related mRNAs are referred to as splice variants or transcript variants of a gene.

As used herein, the terms "coding region" and "coding sequence" are used interchangeably and refer to a nucleotide sequence that encodes a polypeptide and, when placed under the control of appropriate regulatory sequences expresses the
30 encoded polypeptide. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. A "regulatory sequence" is a nucleotide sequence that regulates expression of a coding sequence to which it is operably linked. Non-limiting examples of regulatory sequences include promoters, enhancers, transcription initiation sites, translation start sites,
35 translation stop sites, and transcription terminators. The term "operably linked" refers to a juxtaposition of components such that they are in a relationship permitting them to function in their intended manner. A regulatory sequence is "operably linked" to a coding region when it is joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory sequence.

5 A polynucleotide that includes a coding region may include heterologous nucleotides that flank one or both sides of the coding region. As used herein, "heterologous nucleotides" refer to nucleotides that are not normally present flanking a coding region that is present in a wild-type cell. For instance, a coding region present in a wild-type
10 microbe and encoding a polypeptide is flanked by homologous sequences, and any other nucleotide sequence flanking the coding region is considered to be heterologous. Examples of heterologous nucleotides include, but are not limited to regulatory sequences. Typically, heterologous nucleotides are present in a polynucleotide of the present invention through the use of standard genetic and/or recombinant
15 methodologies well known to one skilled in the art. A polynucleotide of the present invention may be included in a suitable vector. The presence of heterologous nucleotides flanking one or both sides of a polynucleotide described herein result from human manipulation.

15 The terms "complement" and "complementary" as used herein, refer to the ability of two single stranded polynucleotides to base pair with each other, where an adenine on one strand of a polynucleotide will base pair to a thymine or uracil on a strand of a second polynucleotide and a cytosine on one strand of a polynucleotide will base pair to a guanine on a strand of a second polynucleotide. Two polynucleotides are complementary to each other when a nucleotide sequence in one polynucleotide can
20 base pair with a nucleotide sequence in a second polynucleotide. For instance, 5'-ATGC and 5'-GCAT are complementary. The term "substantial complement" and cognates thereof as used herein, refer to a polynucleotide that is capable of selectively hybridizing to a specified polynucleotide under stringent hybridization conditions. Stringent hybridization can take place under a number of pH, salt and temperature
25 conditions. The pH can vary from 6 to 9, preferably 6.8 to 8.5. The salt concentration can vary from 0.15 M sodium to 0.9 M sodium, and other cations can be used as long as the ionic strength is equivalent to that specified for sodium. The temperature of the hybridization reaction can vary from 30°C to 80°C, preferably from 45°C to 70°C. Additionally, other compounds can be added to a hybridization reaction to promote
30 specific hybridization at lower temperatures, such as at or approaching room temperature. Among the compounds contemplated for lowering the temperature requirements is formamide. Thus, a polynucleotide is typically substantially complementary to a second polynucleotide if hybridization occurs between the polynucleotide and the second polynucleotide. As used herein, "specific hybridization"
35 refers to hybridization between two polynucleotides under stringent hybridization conditions.

40 A polynucleotide that includes a coding region may include heterologous nucleotides that flank one or both sides of the coding region. As used herein, "heterologous nucleotides" refer to nucleotides that are not normally present flanking a coding region that is present in a wild-type cell. For instance, a coding region present in a wild-type microbe and encoding a polypeptide is flanked by homologous sequences, and any other nucleotide sequence flanking the coding region is considered to be heterologous. Examples of heterologous nucleotides include, but are not limited to regulatory

sequences. Typically, heterologous nucleotides are present in a polynucleotide of the present invention through the use of standard genetic and/or recombinant methodologies well known to one skilled in the art. A polynucleotide of the present invention may be included in a suitable vector. The presence of heterologous nucleotides flanking one or both sides of a polynucleotide described herein result from human manipulation.

The present invention further provides a method for preventing cell damage or cell death, comprising contacting the cell with the peptide according to the invention. The method may be performed *in vitro* or *in vivo*, and in particular is performed *ex vivo*.

The present invention further provides a method of screening for a compound suitable for prevention of reperfusion injury and/or mitochondria-related disorders and/or cancer therapy-induced cardiotoxicity, comprising

- (i) providing one or more candidate compounds;
- (ii) determining the ability of the candidate compounds to interfere with the binding of BNIP3 and BAX;
- (iii) selecting those candidate compounds which interfere with the binding of BNIP3 and BAX.

The candidate compounds may be any suitable compounds, especially including peptides and small molecule compounds.

EXAMPLES

Example 1: BNIP3 represents a therapeutic target for I/R injury

A combination of necrotic and apoptotic cardiomyocyte death is a hallmark of the early phase of reperfusion injury. Mitochondria play major roles in both processes. The BH-only BCL2-family member BNIP3 is a potential activator of mitochondrial-driven necrotic and apoptotic cell death cascades in cell culture and isolated rat hearts^{31,32,36-38}. BNIP3 has been previously implicated in left ventricular remodeling post-acute myocardial infarction and in heart failure with preserved ejection fraction^{33,39,40}. To investigate the involvement of BNIP3 in I/R injury and whether BNIP3 provides an attractive target for therapeutic intervention, wild-type and BNIP3 deficient mice were subjected to 24 h of reperfusion after occlusion of the left anterior descending coronary artery in a clinical relevant *in vivo* model⁴¹⁻⁴⁴ (Fig. 1A). For delineation of the affected area from the non-affected zone, Evans blue dye was injected into the aorta and coronary arteries. For demarcation of the non-viable myocardium, the infarcted area within the risk zone, heart sections were stained by triphenyl tetrazolium chloride (Fig. 1B). Consistently with a previous study in isolated rat hearts using a well-established dominant negative inhibitor of BNIP3³¹, genetic ablation of BNIP3 generated a significantly reduced infarct size by 46% in comparison to the wild-type mice (Fig. 1C).

Correspondingly, restitution of BNIP3 to the BNIP3 deficient mice caused infarct sizes comparable to wild-type mice in a dose-dependent manner and excluded possible side-effects of the genetic deletion of BNIP3 (Fig 1C). These results indicate that BNIP3 plays an important role in the evolution of infarction *in vivo*.

5 **Example 2: BNIP3 is a mediator of BAX-induced cell death in I/R injury**

The pro-death BCL-2 family member BAX seems to be placed at the intersection of mitochondrial-dependent necrosis and apoptosis as an effector protein⁵. For this, the translocation of BAX from the cytosol to mitochondria is the critical step^{37,45,46}. We recently demonstrated a basal interaction of BAX with BNIP3 taking place in the MOM in cardiomyocytes *in vivo*³⁵. To determine whether the translocation of BAX to mitochondria as most relevant step in cell death occurs in I/R and is dependent on BNIP3, first changes in mitochondrial BAX and BNIP3 concentration were investigated. After 10 min of reperfusion following vessel occlusion the level of BAX in the MOM increases markedly together with augmented mitochondrial concentration of BNIP3 (Fig. 2A and B). Co-immunoprecipitation revealed that BNIP3 and BAX form heterodimers in the MOM (Fig. 2C). By taking advantage of the BNIP3 deficient mice, next we examined whether BNIP3 is the upstream mediator of BAX for this translocation during reperfusion injury. No increase of mitochondrial BAX was observed within the early phase of reperfusion under genetic ablation of BNIP3 demonstrating the direct impact of BNIP3 (Fig. 2D). To verify that this effect really be achieved by BNIP3, we administered BNIP3 to BNIP3 deficient mouse hearts 5 min before starting the myocardial infarction procedure. The addition of BNIP3 restored the BAX translocation in BNIP3 deficient mouse hearts in the early phase of reperfusion and excluded possible side-effects of the genetic deletion of BNIP3 (Fig. 2D).

25 **Example 3: Identification of the critical BNIP3 sequence required for the BNIP3 inhibition in I/R injury**

As the BNIP3 interaction with BAX was recognized as a substantial activity in reperfusion injury *in vivo* evaluated by co-immunoprecipitation, we identified the helices α5, α6, α7 and α8 in BAX as potential binding sites by assessing a peptide microarray with a library of 13 BAX-peptides synthesized (Fig. 3A and B). For this study, we predicted the 3D structure of BNIP3 *in silico* by homology modelling using Modeller 9.15⁴⁷ (Fig. 3C). The created model was energy minimized using NAMD2.9 and the CHARMM36 force field. The BNIP3 model depicts 9 α-helices of variable length representing 64%, followed by random coils 19% and unidentified structures 17%, which was confirmed by circular dichroism spectroscopy (Fig. 3D). Computational docking simulations suggested the BAX helices α5, α6, α7 and α8 and the BNIP3 sequence MSQSGEENLQGSWVELHFSN (amino acids 1-20; SEQ ID NO: 20) as interaction sites (Fig. 3E), while the first 10 amino acids alone failed to bind to BAX (Fig. 3F). We hypothesized that amino acids 1-20 of BNIP3 may be sufficient to antagonize the activity of BNIP3, so we designed a cell-permeable peptide, TAT-BNIP3-20A peptide, composed of the HIV-1 TAT protein transduction domain (PTD,

GRKKRRQRRRPQ (SEQ ID NO: 31), Fig. 3G)^{48,49} attached via a covalent bond to 20 amino acids derived from amino acids 1-20 of BNIP3 (Fig. 3H). The amino acids 42-61 of BNIP3 were used to generate the control peptide, TAT-BNIP3-20C (Fig. 3I). The peptide fragments were capped at the N-terminus with an acetyl group and at the C-terminus with an amide group. Treatment of wild-type mice with the TAT-BNIP3-20A demonstrated that the peptide is taken up by the myocardium (Fig. 3J) and interacts there with endogenous BNIP3.

To examine whether the TAT-BNIP3-20A, given 5 min before reperfusion - a time point relevant for clinical practice, has the ability to antagonize BNIP3 activity and to reduce reperfusion injury *in vivo*, we used the given myocardial infarction model in mice (Fig. 4A). Treatment with TAT-BNIP3-20A, but neither vehicle nor TAT-BNIP3-20C, resulted in reduction of infarct size by 37% (Fig. 4B). This was due to the prevention of BNIP3 translocation to mitochondria by the TAT-BNIP3-20A peptide fragment (Fig. 4C) leading to a markedly inhibited caspase-3 activity, which is a key event in I/R after mitochondrial membrane perturbation (Fig 4D).

Example 4: TAT-BNIP3-20A peptide fragment inhibits apoptotic and necrotic human cardiomyocyte death

To meet the translational need, reoxygenation experiments in human ventricular cardiomyocytes derived from human induced pluripotent stem cells (humanCM) were conducted (Fig. 5A). The humanCM were exposed to 2h of reoxygenation after hypoxia. Notably, even in humanCM TAT-BNIP3-20A exhibits its protective properties. BNIP3-20A is capable of prevention the translocation of human BNIP3 to mitochondria (Fig. 5B) resulting in a considerable protection of mitochondria evidenced by a low mitochondrial inner membrane depolarization (Fig. 5C) and fewer apoptotic and necrotic cells (Fig. 5D).

Example 5: Identification and design of a peptide fragment inhibitor of BNIP3/BAX activity

N-terminal truncations of the BNIP3-20A peptide sequence followed by exchanging single residues revealed that a peptide containing the amino acids 13-20 in combination with a Ser-to-Phe-substitution at position 19 displayed a substantially higher BNIP3 binding in peptide microarrays (see Table 1).

Table 1: Truncated BNIP3-20A fragments showing the highest binding capacities to BNIP3

Sequence (SEQ ID NO)	Fluorescence intensity
MSQSGEENLQGSWVELHFSN (21)	1040.33
SQEENLQGSWVELHFSN (25)	2656.33
SWVELHFSN (29)	4889.33
WVELHFSN (7)	14410.00

Based on these results, we designed the BNIP3-8B peptide, composed of the PTD attached via a covalent bond to 8 amino acids derived from amino acids 13-20 of BNIP3 with the Ser-19 to Phe-19 substitution (WVELHFFN (SEQ ID NO: 8); Fig. 6A). To demonstrate the need for the residue phenylalanine in the peptide sequence, we substituted Phe-18 to Ala-18, additionally His-17 to Ala-17, and generated the BNIP3-8C peptide (Fig. 6B). Circular dichroism spectrum of BNIP3-8B showed that the peptide exhibits a random coil conformation (Fig. 6C).

Furthermore, BNIP3/BNIP3 interaction studies were performed with the BNIP3-20A peptide in which single residues of the wild-type sequence of BNIP3 1-20 were exchanged for 18 neutral amino acids. BNIP3 peptides with specific amino acid substitutions showed an increased binding capacity to BNIP3 (exemplary data shown in Table 2).

Table 2: Substituted BNIP3-20A peptides showing the highest binding capacities to BNIP3

Sequence (SEQ ID NO)	Amino acid substitution	Fluorescence intensity
MSQSGEENLQGSWVELHFSN (21)	-	1040.33
MSQSGEENLQGSWVELCFNS (70)	H17 -> C	6787.00
MSQSGEENLQGCWVELHFSN (71)	S12 -> C	7038.00
MSQSGEENLQGSWVCLHFSN (72)	E15 -> C	7149.00
MSQSGEENLQGSWVELYFSN (73)	H17 -> Y	7988.00
MSQSGYENLQGSWVELHFSN (74)	E6 -> Y	13122.50
MSQSGEENLQYSWVELHFSN (75)	G11 -> Y	10230.33
MSQSGEENLQGSWVELHFFN (22)	S19 -> F	15000.00

Example 6: *In vitro* and *in vivo* effects of TAT-BNIP3-8B peptide fragment uptake, toxicity and stability

First, we assessed the distribution and presence of BNIP3-8B on intracardiac injection at the time of desired effect *in vivo*. We next evaluated the pharmacokinetic profile of TAT-BNIP3-8B in human serum, plasma and whole blood and evaluated the toxicity in isolated adult cardiomyocytes. The TAT-BNIP3-8B is taken up by the heart, spleen and liver and is present in the plasma (Fig. 7A). Thus, TAT-BNIP3-8B is present in the heart 10 min after reperfusion, the time point when the BNIP3-BAX-mitochondria triangle cell death cascade occurs; cardiomyocytes showed no signs of overt toxicity (Fig. 7B), and the half-life of TAT-BNIP3-8B in human serum, plasma and whole blood enables its *in vivo* inhibitory capacity (Fig. 8A-C). Incubation of TAT-BNIP3-8B with proteinase K served as control.

Example 7: TAT-BNIP3-8B peptide mechanism of action

It was hypothesized that TAT-BNIP3-8B binds to BNIP3 and BAX monomers and homodimers and to BNIP3/BAX heterodimers and heterooligomers to interrupt their activity. To assess the interaction behavior of TAT-BNIP3-8B, BNIP3/TAT-BNIP3-8B as well as BAX/TAT-BNIP3-8B-overlay assays and docking simulations were performed. Both results suggested that TAT-BNIP3-8B binds to BNIP3 and to BAX (Data not shown).

Notably, in the myocardial infarction model (Fig. 9A), 5 min post reperfusion endogenous BNIP3 and BAX mono, homo- and heterodimers co-immunoprecipitated with the fluorescence-labelled TAT-BNIP3-8B (Fig. 9B). Furthermore, reperfusion drove BNIP3 and BAX to oligomerize evidenced by large-scale formation of oligomers consisting of BNIP3 and 3 BAX (Fig. 9C). SDS-PAGE and co-immunoprecipitation experiments unmask BNIP3/BAX hetero-interactions in higher order oligomeric complexes, and this oligomerization was strongly inhibited by treatment with TAT-BNIP3-8B (Figure 9C).

Example 8: TAT-BNIP3-8B reduces myocardial infarct size

Then we studied the efficacy and the effect of TAT-BNIP3-8B treatment given 5 min before starting reperfusion in the appropriate myocardial infarction model (Fig. 9A). Notably, TAT-BNIP3-8B reduces the infarct size in a dose-dependent manner up to 40% in comparison to vehicle, and TAT- β -Gal treatment (Fig. 9D and E). TAT-BNIP3-8C treatment does not appreciably affect the infarct size relative to TAT-BNIP3-8B pointing to the importance of the phenylalanine residue (Fig. 9D).

Example 9: TAT-BNIP3-8B reduces mitochondrial perturbation and cell-death cascade

Mitochondrial damage can occur by perturbation of the mitochondrial inner membrane (MIM) and the MOM. The critical mitochondrial event in necrosis is the early opening of the mitochondrial permeability transition pore (mPTP) in the MIM⁵⁰ causing a time-dependent dissipation of electrical potential difference across the MIM followed by mitochondrial swelling and cell disruption. The key mitochondrial event in apoptosis is BAX activation inducing its transmembrane domain exposure and MOM permeabilization allowing apoptogen release, e.g., cytochrome c and subsequent caspase activation⁵¹. TAT-BNIP3-8B injected into the left ventricle 5 min before reperfusion in the given I/R model in vivo prevents BNIP3 and BAX translocation to the mitochondria resulting in the inhibition of the down-stream cell death machinery including mitochondrial swelling, BAX activation, cytochrome c release and caspase-3 activity.

Example 10: TAT-BNIP3-8B inhibits apoptotic and necrotic cardiomyocyte death

It was tested if TAT-BNIP3-8B can inhibit cell death in humanCM. The humanCM were exposed to 2 h of reoxygenation after hypoxia (Fig. 11A). BNIP3-8B markedly inhibits necrotic and apoptotic cell death (Fig. 11B) and loss of mitochondrial inner membrane potential (Fig. 11C).

Example 11: TAT-BNIP3-8B reduces cerebral infarct size

Since BNIP3 is suggested to play a critical role in cerebral ischemia³⁸, we also evaluated the effects of the BNIP3-8B peptide on clinical outcome in a mouse model of focal cerebral reperfusion. We subjected mice to 24 h of reperfusion after a 30-min transient middle cerebral artery occlusion (tMCAO). BNIP3-8B treatment immediately post tMCAO markedly reduces the infarct size by 52% (Fig. 12).

Example 12: TAT-BNIP3-8B reduces myocardial infarct size in swine

Then we studied the efficacy and the effect of TAT-BNIP3-8B treatment given 5 min before starting reperfusion in a myocardial infarction model in swine. Pigs were subjected to a 60 min occlusion of the left anterior descending coronary artery followed by 4 h of reperfusion. Notably, TAT-BNIP3-8B markedly reduces the infarct size by 56% in comparison to vehicle (Fig. 13).

Example 13: TAT-BNIP3-8B protects against Doxorubicin-induced mitochondrial injury.

We evaluated the effects of the BNIP3-8B peptide on Doxorubicin-induced mitochondrial injury in HL-1 cells. To monitor mitochondrial swelling, HL-1 cells were treated with 5 μ M Doxorubicin, reproducing the plasma peak concentration reached by standard infusion in patients. Swelling of mitochondria was measured by optical density at 540 nm, where increased mitochondrial volume due to swelling results in reduced optical density. TAT-BNIP3-8B given simultaneously with Doxorubicin prevents mitochondrial swelling, as indicated by increased OD₅₄₀ (Fig. 14). These data indicate that TAT-BNIP3-8B can protect mitochondria against damage mediated by chemotherapies such as anthracyclines.

Example 14: Methods

Chemicals were obtained from Sigma Aldrich. Antibodies against BNIP3, tubulin, cytochrome c, and BAX were obtained from Abcam and activated BAX from Enzo.

Animals. Male mice with similar age (12 $\pm$ 3 weeks) and an average body weight of 30 g were used. C57BL/6 wild-type mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and kept for one week in the local animal house for acclimatization.

C57BL/6J-TgH (*Bnip3*^{-/-}) mice were obtained from Prof. Gerald W. Dorn, Center for Molecular Cardiovascular Research and Department of Pediatrics, University of

Cincinnati, Cincinnati, Ohio, USA. The mice were generated by replacing exons 2 and 3 with a neomycin resistance cassette³³. The mice were bred and held in the local animal house of the University Hospital Essen. All experiments were approved by the local ethics committee in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (Directive 2010/63/EU).

In vivo myocardial infarction model in mice. Wild-type and Bnip3 deficient (*Bnip3*^{-/-}) mice were anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and intubated. Mechanical ventilation parameters were set to a tidal volume of 2.1 to 2.5 ml and a respiratory rate of 140 breaths per min using a mouse mini-ventilator. Deep anaesthesia was maintained by adding 2 vol% isoflurane to the ventilation gas. The chest was opened through a lateral thoracotomy (1 cm left lateral incision between the 3rd and 4th ribs). A 6-0 prolene suture was placed around the left coronary artery (LCA) and a piece of soft silicon tubing placed over the artery. Coronary occlusion was achieved by tightening and tying the suture. After 30 min of occlusion, the silicon tubing was removed and the suture left in place. For longer reperfusion times the chest were closed using 4-0 prolene. 2, 6 and 9 nmol (in 50 µl 0.9% sodium chloride) BNIP3 were injected into the left ventricular cavity 5 min before vessel occlusion. Peptides with 20 amino acids (2 nmol/50 µl) and peptides with 8 amino acids (8 nmol/50 µl) in NaCl were injected 5 min before reperfusion. Sodium chloride injection (50 µl) served as control treatment.

In vivo myocardial infarction model in pigs. Following induction of anesthesia, a small incision was made over the femoral artery and vein; the vessels were isolated. A small opening was made in the artery and a sheath was introduced. In addition, a sheath was placed in the femoral vein or other appropriate vein to allow for emergency drug administration if necessary. A blood sample was taken prior to administration of heparin and was used to establish a baseline Activated Clotting Time (ACT) which was recorded. Subsequently, as directed by the surgeon, heparin (250 to 350 IU/kg) was administered as needed to achieve and maintain an ACT that is two times the level of the baseline ACT. Following the initial bolus of heparin ACT was recorded again and monitored at least every 60 minutes thereafter, until completion of surgery.

An appropriate guide catheter was advanced into the ostium of the left anterior descending artery (LAD) using visual guidance provided by fluoroscopy. Non-ionic contrast was used for all procedures. A balloon catheter was introduced by advancing it through the guide catheter to the left anterior descending (LAD) coronary artery. The balloon was advanced into the coronary arteries through a guide catheter to a suitable place above the 1st diagonal branch of the LAD. The balloon was then be inflated to a pressure sufficient to ensure complete occlusion of the artery. Occlusion of the artery was verified using fluoroscopy. After verification of occlusion, the balloon was left inflated in the artery for 60 minutes. Incidence supportive drugs and defibrillation were recorded in the study record. The peptide and the vehicle, respectively was administered via IV injection 5 minutes prior to reperfusion. At the end of the vessel

occlusion period, the balloon was deflated and the ischemic area was allowed to
reperfuse. Complete balloon deflation was verified with fluoroscopy. At the end of the
procedure all catheters were removed and the artery and vein was ligated and the
incision was closed in a standard fashion. 4 hours after reperfusion, animals were
euthanized.

Infarct size measurement. For analysis of infarct size, mice were euthanized after 24
hours of reperfusion; hearts were excised and perfused with PBS over 5 min. After
perfusion, the LCA was re-ligated in the same location as explicated before. Evans
blue dye (1 ml of a 1% solution) was injected into the aorta and coronary arteries for
delineation of the ischemic AAR from the non-ischemic zone. The tissue was wrapped
in a clear food wrap and stored for one hour in a -20°C freezer. The heart was then
serially sectioned perpendicularly to the long axis in 1 mm slices, and each slice was
weighed. The sections were incubated in 1 % TTC for 5 min at 37°C for demarcation of
the viable and non-viable myocardium within the risk zone. Infarction, AAR, and non-
ischemic left ventricle were assessed with computer-assisted planimetry by an
observer blinded to sample identity. The size of the myocardial infarction was
expressed as a percentage of the AAR.

Immunoblotting. Tissue and human CM cells were lysed in a RIPS buffer (50 mM
Tris-HCl, 150 mM NaCl, 0.5 mM EDTA, 1% NP-40 and protease- and phosphatase
inhibitor, pH 7.4). Isolated mitochondria were lysed in a Mito-lyse buffer (200 mM
sucrose, 10 mM HEPES, 1 mM EGTA, 1% Triton-X100 and protease- and
phosphatase inhibitor, pH 7.4). The lysates were cleared by centrifugation (20.000 xg,
15 min, 4°C). Protein concentrations in supernatant were measured using DC Protein
Assay (Bio-Rad). Samples were diluted into 4 x LDS sample buffer and 10 x Reducing
Agent (Invitrogen) and were prepared for SDS-PAGE by heating to 95°C for 5 min.
Equivalent amounts of protein were separated using 4-12% Bis-Tris Gels (Invitrogen),
transferred on to nitrocellulose and immunoblotted with primary antibodies. The used
secondary antibodies were horseradish peroxidase conjugated goat anti-mouse or anti-
rabbit IgG (Invitrogen). Immunoblotting was detected by ECL (Thermo Scientific) and
imaged on an Imager 600 (Amersham).

Interaction studies. For protein-peptide interaction studies, peptide libraries were
synthesised and immobilised on microarray slides. Recombinant BNIP3 was used at
concentrations of 1 µg/ml. Peptides were synthesised and immobilised for: (i) the
BNIP3/BAX interaction study; (ii) the BNIP3/BNIP3 interaction study, in which C-, N- or
C/N-terminal truncations of the wild-type sequence of BNIP3 1-20 were performed; and
(iii) the BNIP3/BNIP3 interaction study, in which single residues of the wild-type
sequence of BNIP3 1-20 were exchanged for 18 neutral amino acids.

Microarray. For protein-peptide binding studies recombinant BNIP3 (cusabio) and BAX
(MyBioSource) were used in a concentration of 10 µg/ml resp. 1 µm/ml. As labelling kit
a DyLight Microscale Antibody Labeling Kit (Thermo) with label Dylight 650 was used.
The assay was performed using the automated TECAN HS4800 microarray processing

station. Microarrays were incubated with customer provided samples diluted in blocking buffer for 2 h at 30 °C. Before each step, microarrays were washed with washing buffer. Microarrays were scanned using a high-resolution fluorescence scanner. Laser settings and applied resolution were identical for all performed measurements. The resulting images were analyzed und quantified using spot-recognition software GenePix (Molecular Devices). For each spot, the mean signal intensity was extracted (between 0 and 65535 arbitrary units). For further data evaluation, the so called MMC2 values were determined. The MMC2 equals the mean value of all three instances on the microarray except when the coefficient of variation (CV) – standard-deviation divided by the mean value – is larger 0.5. In this case the mean of the two closest values (MC2) is assigned to MMC2. All steps were performed by JPT Peptide Technologies (Berlin, Germany).

Three dimensional (3D) structure modelling of BNIP3 in silico. The predicted in silico 3D structure of BNIP3 was obtained by homology modelling using Modeller9.15 and the created model was energy minimized using NAMD2.9 and the CHARMM36 force field.

Circular dichroism (CD) spectroscopy. CD spectra of the BNIP3 protein and BNIP3-8B peptide were recorded on a Jasco J-715 spectropolarimeter at 37°C, pH=7,4, 1x PBS.

Docking Simulation. Experiments were performed using Autodock Vina⁵² and HADDOCK⁵³. The structure of BAX (pdb-ID: 4S0O) was taken from the Protein Data Bank, whereas the structure of BNIP3 and peptide BNIP3-8B were modelled using Modeller9.15⁴⁷. The template structures correspond to PDB codes 2k7w and 2ka1. The created model was energy minimized using NAMD2.9⁵⁴ and the CHARMM36 force field.

Immunoprecipitation. Immunoprecipitation were performed using protein G-coupled Dynabeads (Invitrogen). 500 µg lysed proteins were incubated with 2 µg antibody over night at 4°C with shaking in PBS buffer containing 1 mM DTT, 0.005% Brij35 and protease-phosphatase inhibitors. Next day 20 µl Dynabeads were added and the solution was incubated again for 1h. The precipitated immune complex was washed 2 times and then resuspended in elution buffer containing LDS-Sample Buffer (1:4) und Reducing Agent (1:10) (Invitrogen) in PBS and heated for 5 min at 95°C. After removal of the Dynabeads the Eluate were analysed via immunoblotting.

Caspase-3 activity was measured using the Caspase 3 Assay Kit from Abcam (#ab39401). Murine hearts were harvested after 30 min ischemia and 4 h reperfusion, area at risk was isolated, lysed in containing buffer and the assay was performed according to manufacturer's instructions.

Cell culture. Human iPSC-derived ventricular cardiomyocytes (humanCM) were obtained from (axol) and were cultivated according to the manufacture's specification.

To simulate vessel occlusion, cells were incubated in buffer (113 mM NaCl, 4.7 mM KCl, 12 mM HEPES, 1.2 mM MgSO₄, 30 mM taurine, 1.3 mM CaCl₂, pH 7.4) under 1 % O₂, 37°C. Reoxygenation was performed in buffer supplemented with 5.5 mM glucose under 21% O₂, 37°C.

5 HL-1 cells were cultured in Claycomb medium as per manufacturer's protocol. The cells were treated with 5 µM Doxorubicin and 2 nmol TAT-BNIP3-8B for 30 min.

10 **JC-1 Assay.** To analyze mitochondrial inner membrane potential in humanCM cells were stained with 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanin Iodide (JC-1). Cells were incubated with 6 µM JC-1 in media at 37°C for 30 min. After washing cells with PBS buffer at 37°C they were fixed with 4% PFA for 15 min at room temperature. DAPI staining was performed and cells were analysed using EVOS FL (Life Technologies).

15 **Swelling Assay.** Mitochondrial swelling was measured by light scattering at 540 nm in a microplate absorbance reader FLUOstar Omega (BMG Labtech) at RT. The final assay volume was 200 µl, containing mitochondria at 0.5 mg/ml in buffer containing 250 mM sucrose, 10 mM HEPES, 1 mM EGTA, pH 7.4.

20 **Peptides.** The peptides were generated by the resin synthesis procedure (JPT International, Berlin, Germany). They were capped at the N-terminus with an acetyl group and at the C-terminus with an amide. For delivery, the peptides were attached via a covalent bond to the TAT-sequence GRKKRRQRRRPQ (SEQ ID NO: 31). For uptake and binding studies, peptides were labelled with a fluorophore. A complete peptide list is attached as appendix 1.

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CLAIMS

1. A peptide comprising
 - (i) a cellular uptake signal; and
 - 5 (ii) a BNIP3 fragment comprising positions 13 to 20 of BNIP3 or an amino acid sequence derived therefrom;wherein the peptide has a length of 50 amino acids or less.
2. The peptide according to claim 1, wherein the BNIP3 fragment has a length of 12 amino acids or less, especially 10 amino acids or less, in particular 8 amino acids.
- 10 3. The peptide according to claim 1 or 2, wherein the BNIP3 fragment comprises positions 13 to 20 of BNIP3, optionally comprising 1, 2 or 3 amino acid substitutions compared to positions 13 to 20 of BNIP3.
4. The peptide according to claim 3, wherein the amino acid substitutions are present at one or more of the position corresponding to position 15, 17 and 19 of BNIP3.
- 15 5. The peptide according to claim 3 or 4, wherein the amino acid substitutions are selected from
 - (i) glutamic acid at position 15 of BNIP3 to phenylalanine, isoleucine, leucine, valine, tyrosine, cysteine, histidine, arginine or threonine, and
 - (ii) histidine at position 17 of BNIP3 to valine, and
 - 20 (iii) serine at position 19 of BNIP3 to tyrosine, cysteine, phenylalanine or histidine.
6. The peptide according to any one of claims 1 to 5, wherein the BNIP3 fragment comprises a phenylalanine residue at the position corresponding to position 19 of BNIP3.
7. The peptide according to any one of claims 1 to 6, wherein the BNIP3 fragment
 - 25 (i) comprises positions 12 to 20 of BNIP3, optionally comprising 1, 2, 3 or 4 amino acid substitutions compared to positions 12 to 20 of BNIP3;
 - (ii) comprises positions 4 to 20 of BNIP3, optionally comprising 1, 2, 3, 4, 5 or 6 amino acid substitutions compared to positions 4 to 20 of BNIP3; or
 - (iii) comprises positions 1 to 20 of BNIP3, optionally comprising 1, 2, 3, 4, 5 or 6
 - 30 amino acid substitutions compared to positions 1 to 20 of BNIP3; or
 - (iv) consists of an amino acid sequence selected from the group consisting of SEQ ID NOs: 3 to 30.

8. The peptide according to claim 1, wherein the BNIP3 fragment comprises a D-retro-inverso sequence of positions 13 to 20 of BNIP3 or of any of the amino acid sequences defined in claims 2 to 7.
- 5 9. The peptide according to any one of claims 1 to 8, wherein the cellular uptake signal
- (i) is a peptide having a length of 5 to 30 amino acids, in particular 8 to 20 amino acids or 10 to 16 amino acids, especially 12 amino acids;
- (ii) is the protein transduction domain of the TAT protein of HIV; and/or
- 10 (iii) comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 31 to 50.
10. The peptide according to any one of claims 1 to 8, wherein the cellular uptake signal comprises a D-retro-inverso sequence of a cell penetrating peptide, in particular a cell penetrating peptide as defined in claim 9.
- 15 11. The peptide according to any one of claims 1 to 10, consisting of the cellular uptake signal and the BNIP3 fragment, optionally comprising a linker between the cellular uptake signal and the BNIP3 fragment.
12. The peptide according to any one of claims 1 to 11, having a length of 40 amino acids or less, in particular 35 amino acids or less, especially 30 amino acids or less.
- 20 13. A pharmaceutical composition comprising the peptide according to any one of claims 1 to 12.
14. The peptide according to any one of claims 1 to 12 for use in the treatment of reperfusion-related and/or mitochondria-related disorders as well as cancer therapy-induced cardiotoxicity.
- 25 15. The peptide for use according to claim 14, wherein the reperfusion-related and/or mitochondria-related disorder is selected from the group consisting of myocardial infarction, stroke, acute kidney injury, trauma, circulatory arrest, and cessation of blood flow during organ transplantation.
- 30 16. The peptide for use according to claim 14 or 15, wherein the peptide is administered to the patient during or after occurrence of impaired blood supply, especially prior to reperfusion of the tissue affected by the ischemia.
17. The peptide for use according to claim 16, wherein the peptide is administered to the patient within 2 hours, in particular within 30 minutes prior to reperfusion.

18. The peptide according to any one of claims 1 to 12 for use in the treatment or prevention of tissue damage due to mitochondria-induced apoptosis or necrosis.
19. A method of screening for a compound suitable for prevention of reperfusion injury and/or mitochondria-induced disorders and/or cancer therapy-induced cardiotoxicity, comprising
- 5
- (i) providing one or more candidate compounds;
 - (ii) determining the ability of the candidate compounds to interfere with the binding of BNIP3 and BAX;
 - (iii) selecting those candidate compounds which interfere with the binding of
- 10 BNIP3 and BAX.