Supplementary information

Prokineticin Receptor-1 Signaling Inhibits Dose- and Time-Dependent Anthracycline-Induced Cardiovascular Toxicity Via Myocardial and Vascular Protection

Short title: PKR1 inhibits doxorubicin-induced cardiotoxicity

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Methods

Cells culture and treatments

H9c2 cardiomyocytes derived from embryonic rat heart was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). Cardiomyocytes were isolated by the Percoll gradient technique as described previously (1). Cells were maintained in DMEM 4,5g/l, L-Glucose supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were seeded on laminin-coated glassware's (10 µg/ml mouse laminin, Invitrogen) for 4 days then spontaneously beating cells were expose to IS20 (synthesized by Laurent Désaubry, Illkirch) or prokineticin-2 (PeproTech, 100-46) for 24h, before DOX (15 µM, Sigma-Aldrich, # 44583) treatment for an additional 4h.

Human Epicardial Progenitors Derived Cells were isolated from human heart right atrium appendage tissues or Tcf21 (CliniScience, aa151–179, # LS-C271190–200, 1/50 dilution) positive cells by FACS analyses as previously described (2). All experiments with human tissue specimens were carried out according to the official guidelines of the University of Strasbourg Medical Center with ethical approval from Comate de protection des personas "est. IV" with CPP n°IDRCB:2009-A01116-51-PRI 2009 HUS N°4603). Briefly, tissue explant was cut in small pieces (<1mm^3) and, transferred in 1-2% gelatin pre-coated dish and cultured in a DMEM 4,5g/l; L-Glucose supplemented with 15% FCS/M199 1:1 and 1% penicillin/streptomycin for 3-4 days to induced cells outgrowths. When epicardial outgrowths were visible, the coverslips were removed. The cells were treated with DOX (0.1µM) in the presence of IS20 or vehicle for 24h and the outgrown cells were visualized by inverted microscopy and quantified.

Human aortic endothelial cells (HAEC) were obtained from ATCC. The cells were cultured in endothelial cell medium containing 10% FBS, and 1% endothelial cell growth factors and 1% penicillin and streptomycin. HAEC cells expressing PKR1 were seeded into 24 well culture plates coated with Matrigel (BD Bioscience, # 356231). Cells were pretreated with IS20 for 10 h, and then DOX (0.1 µM) for 14 h. The wells were imaged, and the tube numbers were quantified at 24h as previously described (3).

In some settings cells were pretreated with kinase inhibitors such as LY294002 (1µM, Sigma-Aldrich # 15447-36-6), PI3/Akt inhibitor, PD 98056 (1µM, Sigma-Aldrich # 167869-21-8), MAPK; or PKR1 agonist PC25 (30 nM, kindly provided by Laurent Désaubry) for 1h before IS20 treatments.

Breast cancer cell line, MDA-MB-231, MCF7, Kuramochi ovarian cancer cells (ATCC), was maintained in a 3:2 mixture of DMEM (Gibco) and F-12 (Gibco) supplemented with 10% FBS (Gibco) and 50 units/ml of P/S (Gibco). Cells were incubated at 37°C, under 5% CO_2.

siRNA transfections

siRNA for PKR1 utilized to down regulate PKR1 levels by 85% as previously described [18]. A universal siRNA non-targeted DNA sequence: sense: UUCUCCGAACGUGUCACGUdTdT; antisense:

ACGUGACACGUUCGGAGAAdTdT) was used as negative control.

Western Blot assays

After the treatments, cells or hearts were lysed in cold RIPA buffer (50mM TrisHCl pH7.4, 150mM NaCl, 2mM EDTA, 1% NP-40, 1,1% SDS) and centrifuged 10min at 14 000rpm 4°C to give protein extract. In some experinexperiments cytosolic or nuclear proteins were separated as previously described (4) . The concentration of protein was determined with BCA assay (Thermo Scientific) according manufacturer's instructions. About 10-30μg of protein per sample was load in SDS-PAGE electrophoresis and transferred to Whatman PVDF membrane. Membranes were probed with antibody against Phosphorylated-H2A.X (ser 139, Cell Signaling, #9718, 1/1000), phosphorylated-Akt (ser 473, #4058, 1/1000) and total-Akt (Cell Signaling, #9272, 1/1000), Nrf2 (Invitrogen, #PA5-27882, 1/1000), and GAPDH (Santa-Cruz, # sc-32233, 1/1000), vinculin (Cell Signaling, #4650, 1/5000). Membranes were treated with peroxidase-conjugated secondary antibodies (Santa-Cruz, 1/10000) and immunoreactivity was detected with an ECL Prime chemoluminescence detection kit (Amersham Pharmacia).

Cell viability

Cell viability was evaluated using CyQUANT-NF proliferation kit (Invitrogen)(5). H9c2, cells were seeded at 10,000/well, in 96 wells plate in their completed medium 24h before the experiment. Cells were pretreated with IS20 in 1% serum medium for 10h and 1μmol/L of DOX for additional 14h (10nmol/L or 1000nmol/L). Then cell viability was determined on the supplier's protocol. Relative cell viability was then assessed, using a 96-well microplate reader (Thermo fisher) with absorbance set at 490 nM.

Appropriate 20,000 TNBC cells (MDA-MB 231) and MCF-7 breast cancer cells or Kuramochi ovarian cancer cells were seeded into 96-well plate 24h before the experiment. Cells were synchronized overnight in 1% serum medium and treated with various concentration of doxorubicin (DOX, Sigma) for 24h with or without IS20. Cell viability was determined using the Cell Counting Kit-8 (CCK-8, Sigma) according to the manufacturer's instructions. Relative cell numbers were then assessed, using a 96-well microplate reader (Thermo fisher) with absorbance set at 490 nM.

TUNEL and ROS assays

Apoptosis was detected by Terminal deoxynucleotidyl transferased UTP Nick End Labeling (TUNEL, Apoptag fluorescein) method (Millipore, #S7110). For that, the cryosectioned tissues sections were fixed with 3.7% formaldehyde and permeablized by ethanol/acetic acid 2:1 (v/v) solution in -20°C. Then the sample was incubated in enzymatic solution for 1h at 37°C. During this, the terminal deoxynucleotidyltransferase catalyzes the addition of dUTP-associated digoxygenin on 3'OH free of DNA strand damage. Anti-digoxygenin antibody conjugated-fluorescein labelled the apoptosis positive cells in green; DAPI stained all nuclei cells in blue. Cells percentages were calculated for TUNEL-positive nuclei per total DAPI x100 in 10 randomly selected fields of each condition (n=3) and expressed as a percentage of the total number of nuclei (6).

Intracellular ROS production was measured in the H9c2 cell lines or primary cardiomyocytes using 2', 7'-dicholorodihydrofluorescein diacetate (DCFDA) ROS

detection assay kit (Abcam, # ab113851) according to manufactures guidance. 1×10^4 cells were seeded in a clear bottom black 96-well plates and allowed for attachment overnight in medium containing 10% FBS at 37°C in a 5% CO2 incubator. Cells were then starved in the medium with 1%FBC for 10 h followed by IS20 treatment for additional 14h. Cells were washed and loaded with 25mM of DCFDA for 45 min at 37°C and exposed to DOX (1, 5, 10 or 15 μM) for 3h for the peak ROS accumulation. At the end of treatment period, fluorescence intensity was measured at Ex/Em, 485/535 nm using Fluoskan AscentFluorescan Reader (Thermo SCIENTIFIC). In some settings the cells were costained with mitoTracer and DCFDA for 45min and then ROS production in mitochondria was visualized using confocal microscopy.

Immunostaining analyses

Immunofluorescence assay was performed on cryosectioned hearts and cells (7). They were fixed with 3,7% formaldehyde and blocked with blocking buffer (from MOM kit ®VECTOR or 10% Donkey serum, 0,5% Triton 100X). The samples were probed with antibody, targeting α-SMA (Abcam, A7811, 1/500 dilution), calponin (Abcam, ab151427, 1/500 dilution), PECAM (Santa Cruz, sc-18916, 1/500 dilution), caspase-3 (Santa Cruz, sc-373730, 1/500), MF20-MHC (Hybridoma Bank, Iowa City, Iowa, 1/250 dilution), Nrf2 (Santa Cruz, # sc722, 1/500 dilution) and tcf21 (Abcam, (#ab329821, 1/250 dilution), phosphorylated-Akt (ser 473, Cell signaling #4075, 1/500). After, they were treated with second antibody conjugated with specific fluorochromes (e.g., Alexa 488, Alexa 555 or Fluorescein, Santa-Cruz, 1/500 dilution). Nuclei were stained with DAPI. The fluorescence signal was detected, and intensity was captured with Leica TCSNT fluorescent microscope. Signal intensity was quantified on digitalized-images and calculated as the product of averaged pixel intensity per high-power field (HPF) or per DAPI positive total nucleus number. The images were controlled by Image J software. The laser was chosen according to the sample. It was compared pixel intensity and pixel distribution after background intensity was subtracted.

cTnT levels were performed on serum, respectively as described (8,9).

Mice models of cardiotoxicity

All animal experiments were performed under using protocols approved by the Direction des Services Vétérinaires du Bas-Rhin, France (Authorization N° B67-274) and the French (APAFIS#4708) and European regulation-approved protocols from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes (EU0064). The animal experimentation and housing were conducted at the accredited animal experimentation and housing facility of the Institut de Recherche de l'Ecole de Biotechnologie de Strasbourg (Register number: C67-218-19).

For the chronic model, 10-week-old male C57BL/6J mice (total of at least 10 per group, Janvier Labs) were administrated serial DOX injections as previously described (5) with modifications. Mice were intra peritoneally (i.p.) injected with DOX (5 mg/kg; Sigma-Aldrich) once a week for 3 consecutive weeks (weeks 1–3). After one week of interval, they received again DOX for 4 weeks (weeks 5–8). Selection of IS20 dose and route of administration were based on a previous study (6). IS20 (1mg/kg, IP) was administrated daily (week 1) for 8 weeks. Vehicle-only treated animals were used as controls (0.1% DMSO in phosphate-buffered saline). Animals were randomly assigned.

Echocardiography was performed week 9 after the initial DOX injection by one operator blind for treatment. Anesthesia and analyzes were identical to those used in the acute model. Animals were euthanized after echocardiography, and hearts were excised, weighted and cryopreserved or included in cryomatrix for histology analysis. All animal experiments were carried out in accordance with current institutional guidelines for the care and use of experimental animals. All efforts were made to minimize suffering with respect to the regulation concerning genetically manipulation of organisms.

In vivo Tumorigenicity assay

10-week-old, athymic nude mice (BALB/cByJNarl) were purchased from the National Laboratory Animal Center, Taiwan. All mice were kept under specific pathogen-free conditions, using a laminar airflow rack, with free access to sterilized food and autoclaved water. All experiments were approved by the Animal Experimentation Ethics Committee of National Chung Cheng University, Taiwan (IACUC No. 1060501). $5x10^6$ of MDA-MB-231 breast cancer cells were resuspended in 0.15mL of 3:1 mixture of medium and Matrigel (BD Bioscience) and then injected subcutaneously into both side of the flank of each mouse. After tumor growth to a volume of around $20mm^3$, mice (n=12) for each group) were administered intra-peritoneal with DMSO (control), DOX (5mg/kg, twice per week for two weeks) or DOX + IS20 (1mg/kg, every day after DOX is given). Tumor size was measured daily with calipers in length (L) and width (W). Tumor volumes were calculated using the formula ($LxW^2/2$). At the end of experiment, all mice were sacrificed by cervical dislocation

Histological and electron microscopy analyses

For histology, hearts were removed from mice, dissected, and frozen for cryosectioning (5-10 μm), and the sections were stained with Mallory tetrachrome coloration (VWR Chemical)(6,10). The heart sections were analyzed under binocular LEICA MZ95 or microscope LEICA DME X10 magnifications connected with PROGRes C5 camera (JENOPTIK). For electron microscopy, hearts were fixed in 4% paraformaldehyde, 1.5% glutaraldehyde in 0.1mol/L Cacodylate buffer. The hearts were then sliced longitudinally into 2 pieces for continued immersion fixation in the same fixative and embedded in epoxy resin, using routine methods (11).

Echocardiography and MRI assessment

Echocardiography was performed on lightly anesthetized animals (≈1% Isoflurane in O2) with Vevo 2100 ultrasound machine (Visualsonics, Canada) equipped with a linear 22-55 MHz or 9-18 Mz transducer using a rail system to hold animal and probe. ECG and body temperature were monitored, and temperature maintained with a heating pad. LV imaging was obtained from a long-axis view for two-dimensional guided M-mode imaging and posterior wall tissue Doppler measurements and apical 4- to 5-chamber view for Doppler mitral and LV out flow assessment as well as tissue Doppler measurements at mitral annulus (10). End diastole was identified from ECG as the onset of QRS and end systole as the end of ECG T wave. Fractional shortening (FS) was computed from left ventricle diameters as: FS = ((LVEDD - LVESD)/LVEDD) *100. Where LVEDD is LV end-diastolic diameter and LVESD is LV end-systolic diameter. The mean velocity of circumferential fiber shortening (V_{cfc}) was corrected for heart rate as follows: $V_{cfc} = FS/ET_c$

(ejection time). Echocardiographic acquisitions at M Mode used to calculate cardiac output (CO) (ml/min) = ($LVEDD^3$ -$LVESD^3$) * heart rate) (Tournoux, et al, 2011).

MRI was performed Anesthesia was induced with a 2% mixture of isoflurane. The mouse forelimbs were fitted with pediatric ECG leads. ECG were monitored with an SAII Model 1025 monitoring and gating system (Small Animal Instruments, Inc., Stony Brook, NY). A black blood gradient echo sequence for multiphase cardiac MRI of the mouse heart was implemented on a Biospec 70/20 MR scanner (Bruker, Germany. A gradient echo pulse sequence was used to acquire data with TE/TR 1.72/61 ms, a matrix of 128 128 zero-filled to 256 256, a field of view of 3*3 cm, a slice thickness of 0.5 mm, flip angle 30°, and 8 frames. For black blood imaging, a nonselective/selective combination of 180o pulses (double inversion recovery (IR)) was used. A 50 ms square sinc pulse was used for selective second inversion. The %EF were calculated, utilizing the formula (DV-SV/DV*100).

Gene expression analysis

Total RNA from mice hearts or cells was isolated with TRIZOL Reagent (Life Technologies) and the concentrations were measured by nanoview. Minimums of 3 mice hearts or cell samples for each of groups were analyzed in each experiment. Retrotranscription and real time quantitative polymerase chain reaction (qPCR) was performed using GAPDH as an internal control and SYBR Green system with MyiQ cycler (Biorad). The primers used, and programs are shown in Table 1. All data of threshold Cycle (Ct) were normalized by Ct of house-keeping gene (GAPDH)(7).

***In silico* predictions of toxicity and degradation of IS20**

Median lethal dose (LD_{50}), Lowest Effect Level (LEL) for intra peritoneal toxicity and half-life of IS20 in mouse were assessed, utilizing a model based on RTECS (Registry of Toxic Effects dataset), described on On-line Chemical Database and Modeling Environment platform (OCHEM) http://ochem.eu. These models were developed using the default hyper-parameters of neural networks based on diverse sets of descriptors available at the OCHEM as previously described. The applicability domain of models was also estimated. The n-fold cross-validation results were used to identify the models with the highest prediction power.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM), except murine experimental data that are shown as mean ± standard deviation (SD). Statistical comparisons for TUNEL PECAM-1, Calponin, α-SMA, and flk-1 staining on 40 heart cryosections obtained from 4 mice were performed using a one-way analysis of variance (ANOVA) with alpha-correction for pairwise comparisons using Tukey's method. Kaplan-Meier survival analysis (log-rank) was performed for 2 groups comparisons. Statistical analysis for the murine experimental data were conducted by using GraphPad Prism software version 6.0 (GraphPad Software, La Jolla, California).

Table 1. Primers for RT-qPCR

Gene	Primers	Sequence (5' -3')
Mouse ANF	Forward	CCTGTGTACAGTGCGGTGTC
	Reverse	CCTGCTTCCTCAGTCTGCTC
Mouse Col1A1	Forward	GCCAAGAAGACATCCCTGAAG
	Reverse	TGTGGCAGATACAGATCAAGC
Mouse BNP	Forward	AAGTCCTAGCCAGTCTCCAGA
	Reverse	GAGCTGTCTCTGGGCCATTTC
Mouse β-actin	Forward	GAGACCTTCAACACCCC
	Reverse	GTGGTGGTGAAGCTGTAGCC
Mouse TNF-α	Forward	TCTCATCAGTTCTATGGCCC
	Reverse	GGGAGTAGACAAGGTACAAC
Mouse SERCA2a	Forward	TGTGTAATGCCCTCAACAG
	Reverse	CCCCACGAGCCAGATATTCTC
Mouse RYR2	Forward	GGATGAATGTCTCACTGTCC
	Reverse	CTTAATGTGGCTTCCACTCC
Mouse PLB	Forward	CTGTGACGATCACCGAAGC
	Reverse	TGGTCAAGAGAAAGATAAAAAGTTGA
Rat Bcl2	Forward	ATCGCTCTGTGGATGACTGAGTAC
	Reverse	AGAGACAGCCAGGAGAAATCAAAC
Rat Bax	Forward	AGGGTGGCTGGGAAGGC
	Reverse	TGAGCGAGGCGGTGAGG
Mouse Bcl2	Forward	GACAGAAGATCATGCCGTCC
	Reverse	GGTACCAATGGCACTTCAAG
Mouse Bax	Forward	CTGAGCTGACCTTGGAGC
	Reverse	GACTCCAGCCACAAAGATG
Human HSP70	Forward	TGTTTGTCTTTGAGGTGGAC
	Reverse	AAGAATTCTAATGAACATATCGGTTG
Human β-actin	Forward	GCGAGAAGATGACCCAGAT
	Reverse	GAGGCGTACAGGGATAGC
Rat β-actin	Forward	ATCTGGCACCACACCTTC
	Reverse	AGCCAGGTCCAGACGCA
Human Bcl2	Forward	GATGTGATGCCTCTGCGAAG
	Reverse	CATGCTGATGTCTCTGGAATCT
Human Bax	Forward	CAGTGACCTGCGCTGTGGTA
	Reverse	CGGAGGAAGTCCAATGTC
Rat nrf2	Forward	TTTGTAGATGACCATGAGTCGC
	Reverse	GTCCTGCTGTATGCTGCTT
Rat GCLc	Forward	CCACTGTCCAAGGTTGACGA
	Reverse	CTTGCTACACCCATCCACCA
Rat NQO1	Forward	GGCCATCATTTGGGCAAGTC
	Reverse	TCCTTGTGGAACAAAGGCGA
Rat HO-1	Forward	TCTGCAGGGGAGAATCTTGC
	Reverse	TTGGTGAGGGAAATGTGCCA

FIGURES

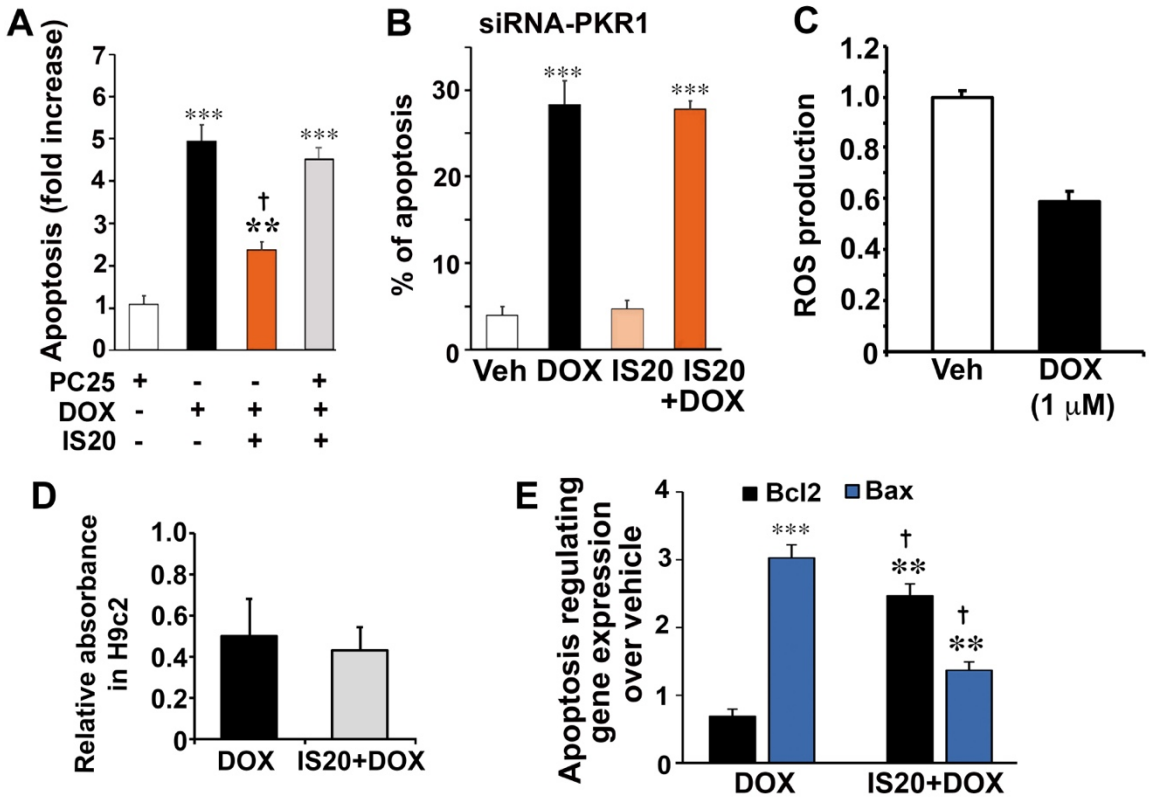

Figure S1. A) IS20 (10nM, 10h) inhibits DOX (1μM, 14h)-mediated apoptosis in H9c2 cells that was reversed by in the presence of PKR1 antagonist PC25 (30nM). **B)** IS20 (10nM, 10h) cannot inhibit DOX (1μM, 14h)-mediated apoptosis in H9c2 cells where PKR1 is downregulated by siRNA-PKR1. 250-300 cells were analyzed across three independent experiments. * $p<0.05$ vs. vehicle, ** $p<0.05$ vs DOX. **C)** H9c2 cell were treated as in A for detection of ROS production, using ROS detection kit according to manufactures protocol. **D)** Spectrofluorometric measurements (maximum excitation wavelength of 493 nm and a maximum emission wavelength of 592 nm) to detect the cellular uptake of DOX in the absence and presence of IS20. **E)** QPCR analyses of apoptotic gene expression (Bcl2/Bax) over vehicle. Data are presented as the mean ± SEM. * $p<0.05$ vs. vehicle, ** $p<0.05$ vs DOX n=4, ANOVA test).

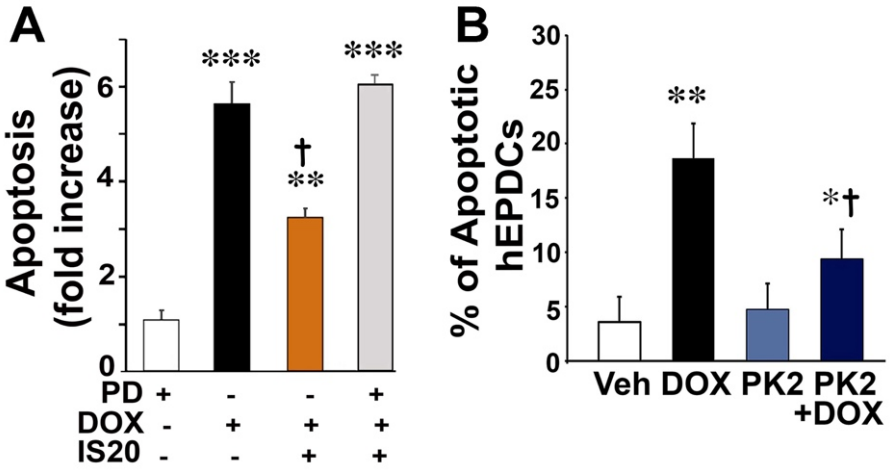

Figure S2. A) IS20 (10nM, 10h) was not able to inhibit apoptosis induced by DOX (1μM, 14h) in the presence of MAPK inhibitor PD 98056 (1μM, 1h before IS20) in HAECs. **B)** Prokineticin-2 was able to inhibit apoptosis induced by DOX in hEPDCs. 250-400 cells were analyzed across three independent experiments. Data are presented as mean± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. vehicle, t $p < 0.05$ vs. DOX,

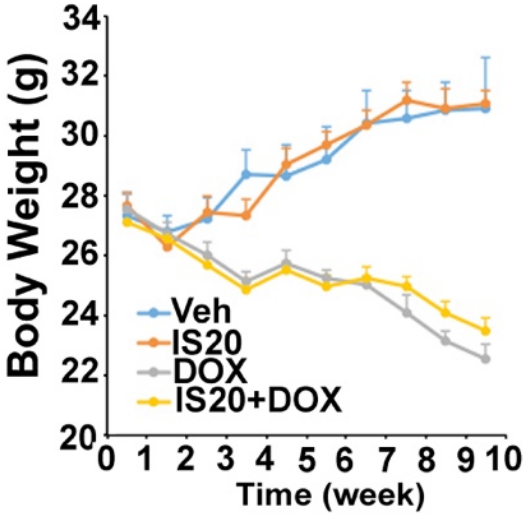

Figure S3. Body weight evaluation among the indicated group of mice.

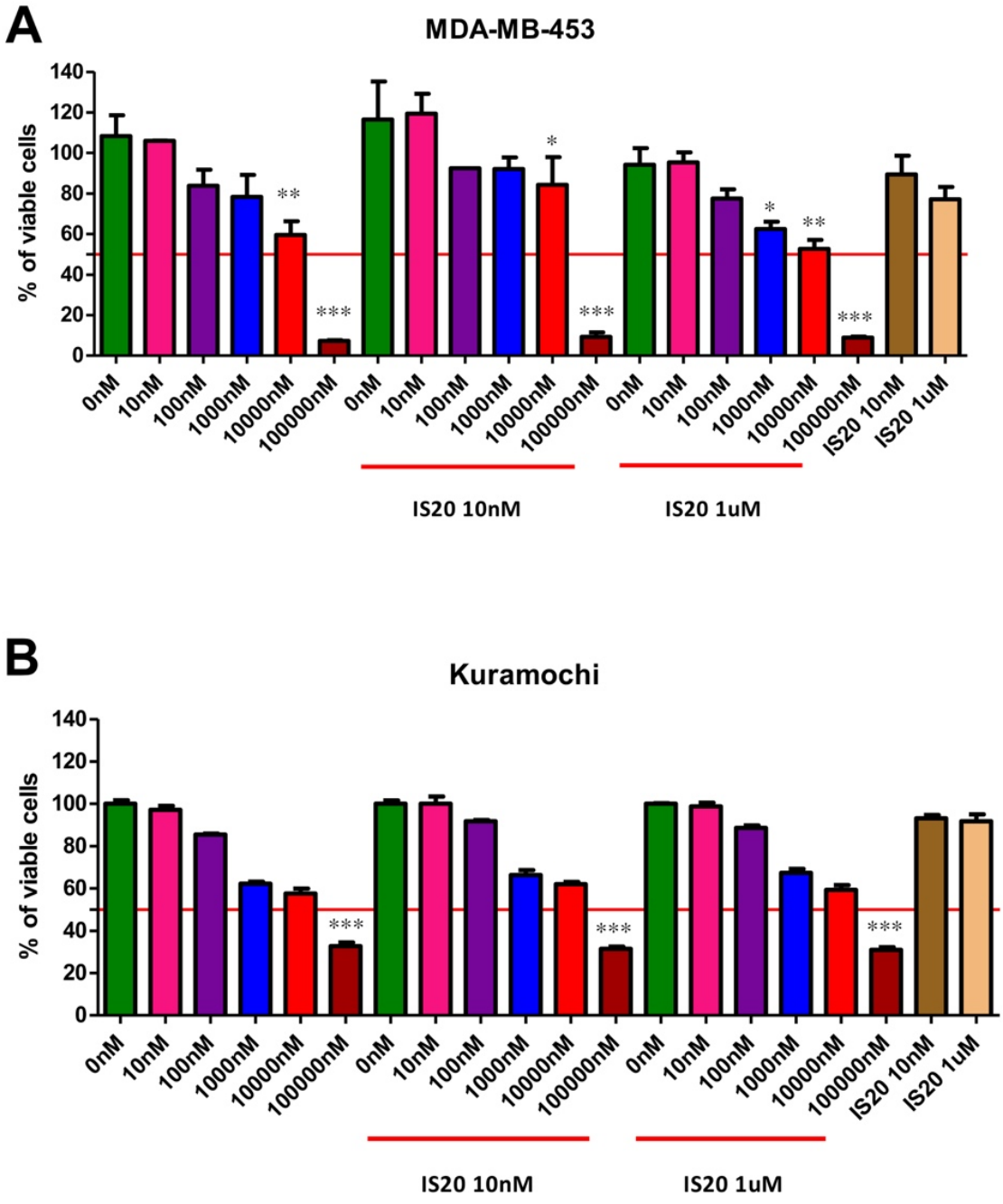

Figure S4. Cell viability assay on **A)** MDA-MB 453 breast cancer and **B)** Kuramochi ovarian cancer cells treated with different concentration of DOX (10nM-100μM) in the presence or absence of IS20 (10nM or 1μM). Data are presented as mean± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. vehicle.

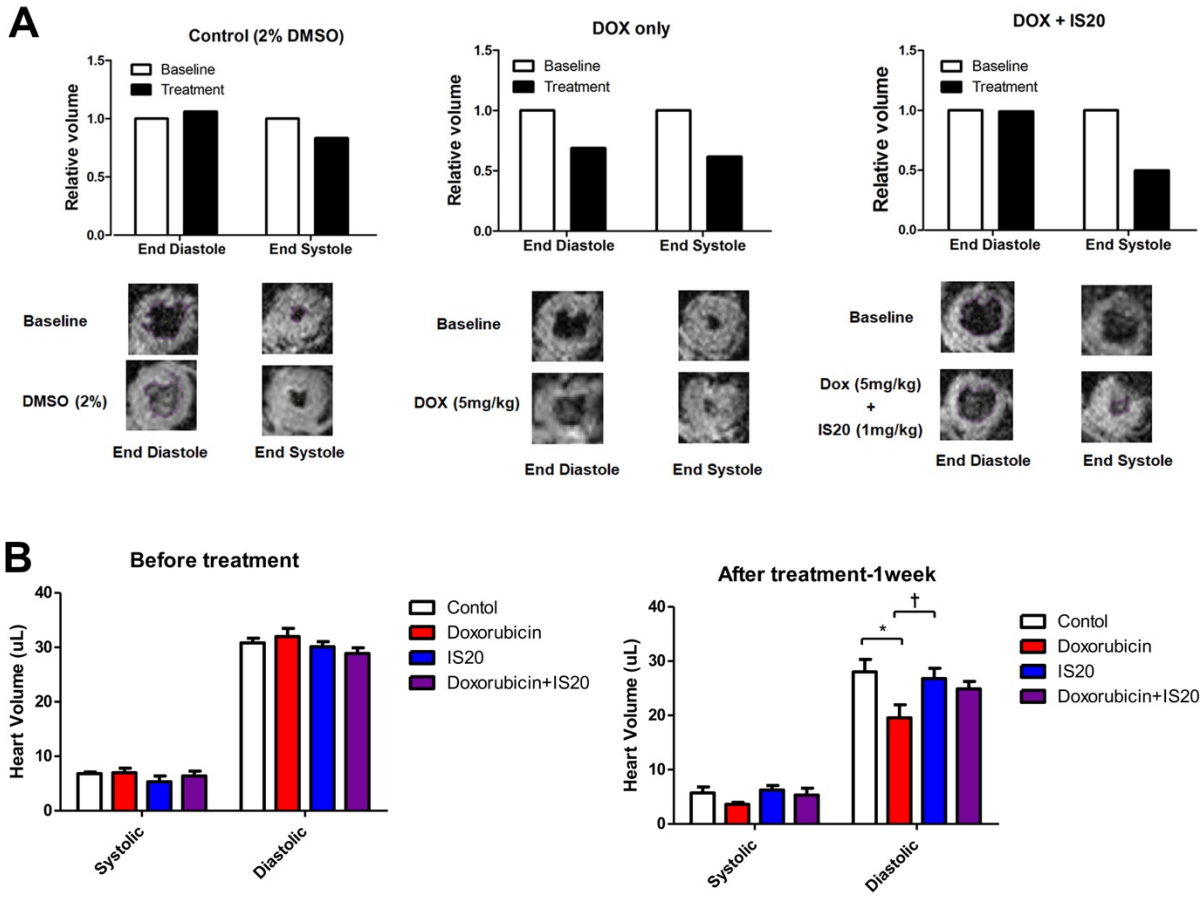

Figure S5. A) MRI analyses of the hearts of the mice treated with vehicle (left), DOX-only middle) and IS20 in the presence of DOX (right). **B)** Quantitative MRI analyses on heart volume during systole and diastole before treatments and 1 week after treatments. n = 6, multivariate ANOVA test. IS20, PRK1 agonist; PRK1, prokineticin receptor 1; DOX, doxorubicin. Data are expressed as mean ± SD. * $p < 0.05$ vs. vehicle, † $p < 0.05$ vs. DOX,

References

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Annex

Source of Data

Figure 1D. H2AX Phosphorylation by DOX

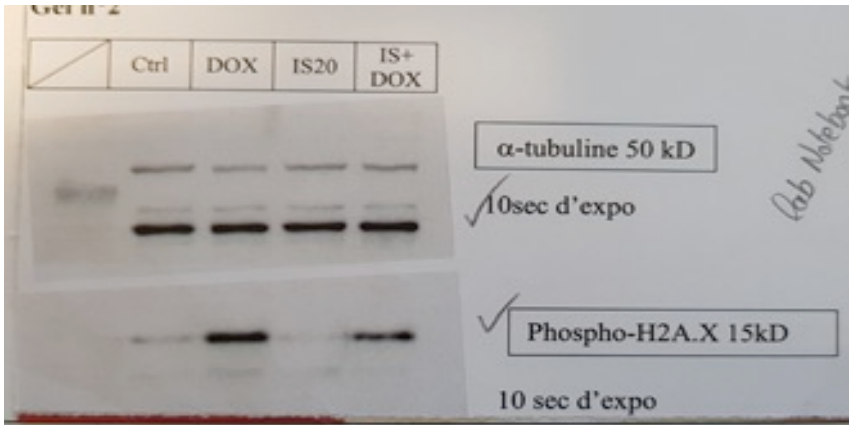

Fig1G. Activation of Akt (56kDa) by different concentration (nM) of IS20 in cardiomyocytes.

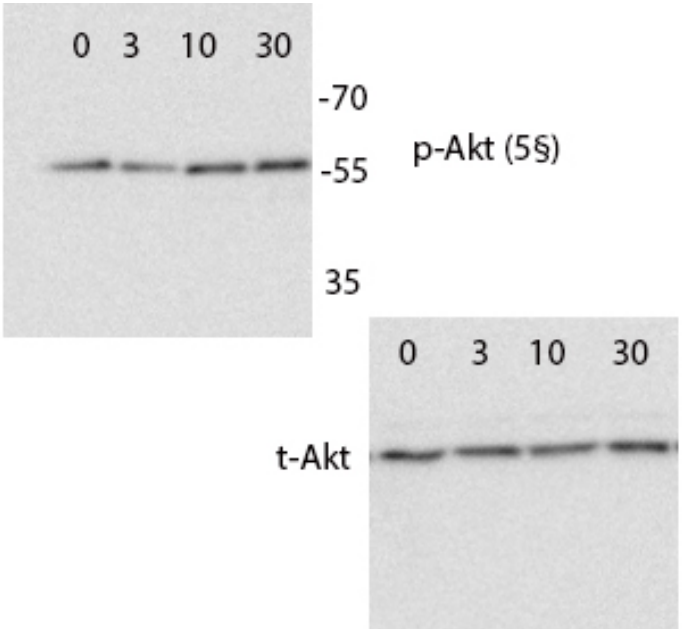

Fig2C: WB for Nrf2 (61 kDa) on proteins derived from Nuclear extracts

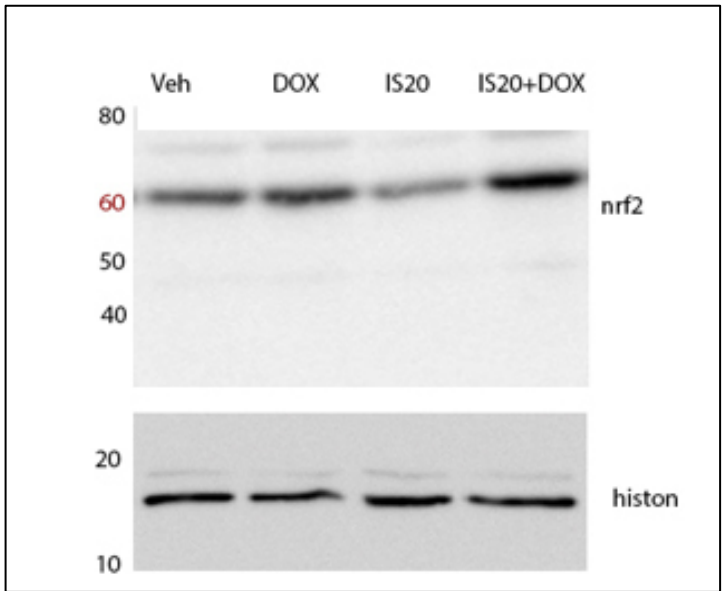

Fig2C WB for Nrf2 (60kDa) on protein derived from cytoplasmic extract

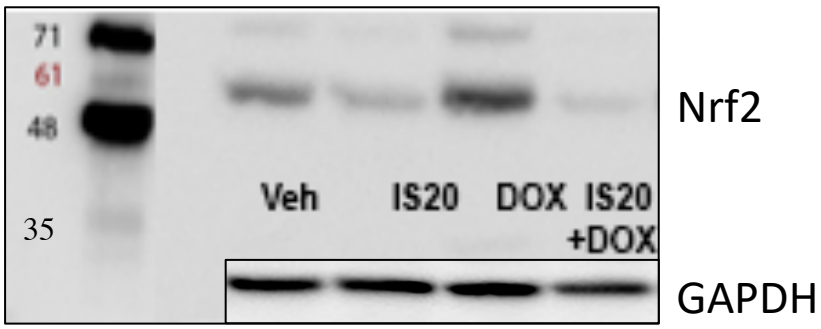

Fig 3B Western Blot analyses: Different concentration of IS20 (0,3,56, 10, 30 nM) mediated phosphorylation of ERK1 (42 kDa) and ERK2 (44kDa) (left), normalized by total ERK (right).

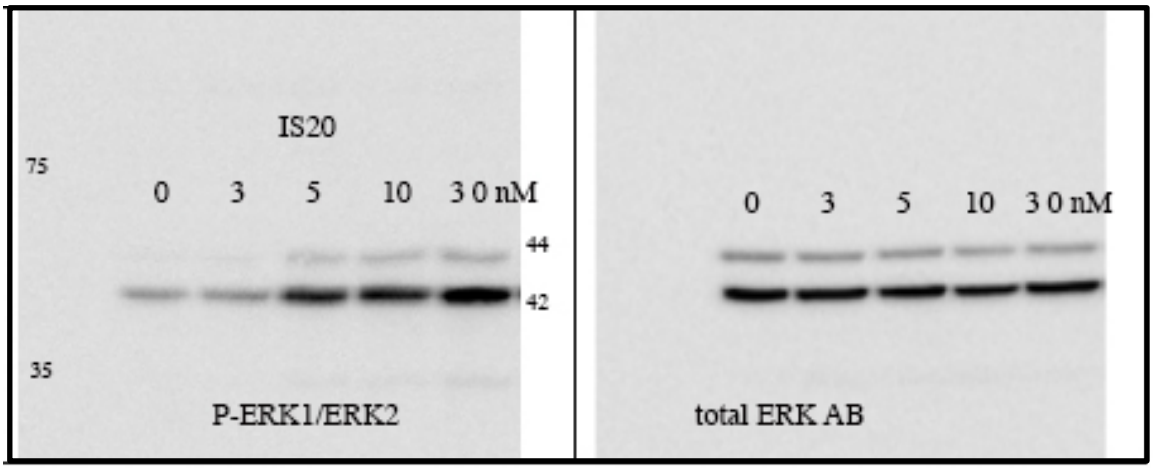

Fig 4A. Akt phosphorylation in the mice hearts

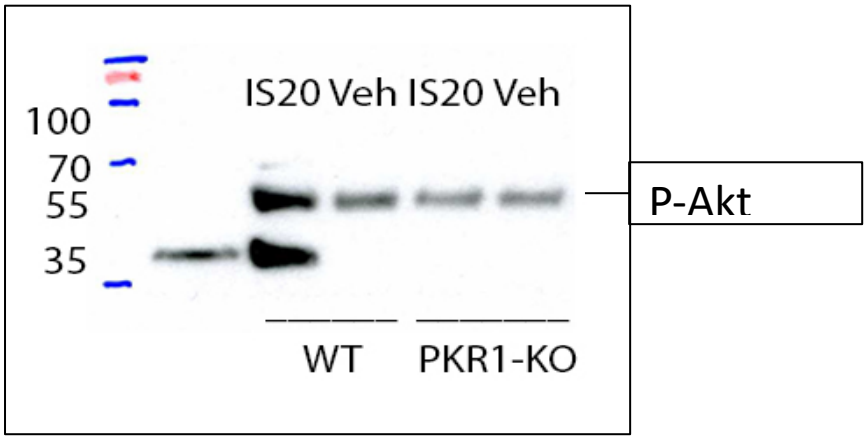

Fig 5A. H2AX phosphorylation in the mice hearts