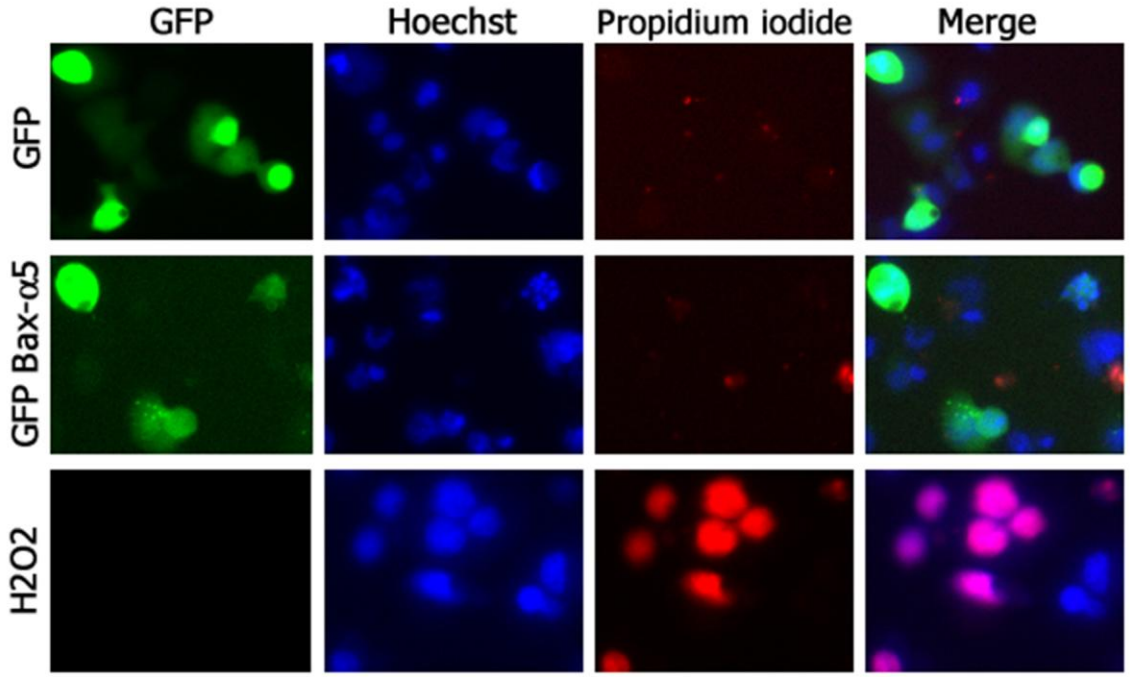
SUPPORTING INFORMATION

S1. Expression of GFP-Bax-α5/α6 induces caspase-dependent and Bax/Bak-independent cell death.

Top panel: Apoptosis/Necrosis test. In this assay, apoptotic nuclei are visualized using Hoechst 33342 (blue) and necrotic or late apoptotic cells are visualized with propidium iodide (red). GFP-positive cells exhibit green fluorescence.

Middle panel: Levels of cell death (apoptosis and necrosis) in HT1080 cells expressing GFP or different GFP-tagged, Bax-derived (poly)peptides. Cells were left untreated (-) or treated with 100 μM zVAD.fmk (+). Cell death was determined 24 hr post-transfection by analyzing GFP-positive cells (~300 cells in each experiment) under a fluorescence microscope. Data were compiled from 3 different fields (40 × magnification). The mode of cell death, necrosis *versus* apoptosis, was determined by the cellular permeability to propidium iodide (necrosis) and the morphology of the nuclei after staining with Hoechst 33342 (apoptosis). Propidium iodide-negative cells with condensed or fragmented nuclei were counted as apoptotic. Data are represented as mean values from three independent experiments ± SD. Experiments performed with SK-MEL-28 cells yielded similar results. GFP-$[KLAKLAK]_2$ transfection and staurosporine (STS) treatment were included for comparison.

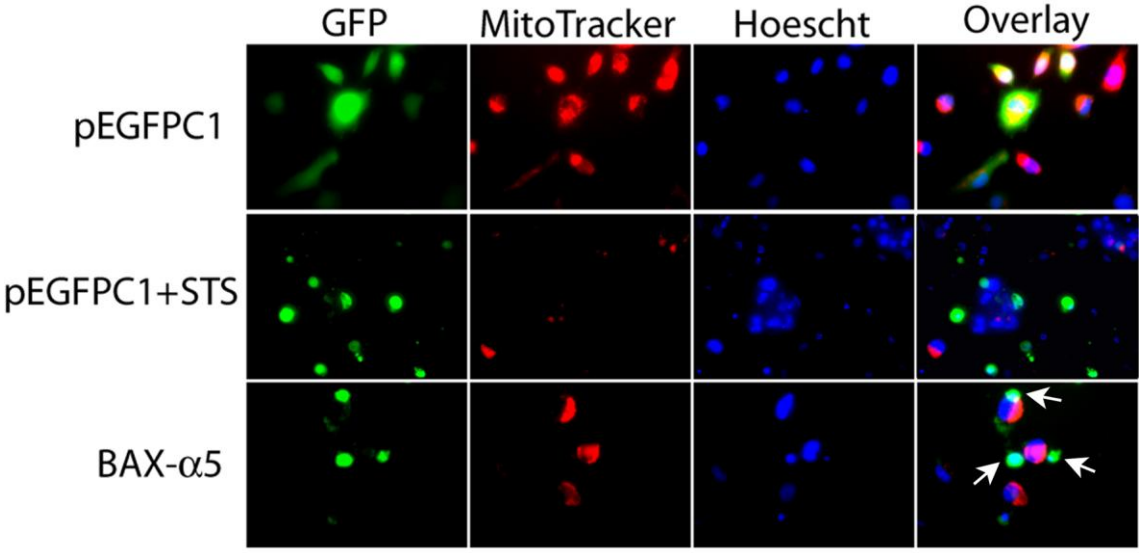
Bottom panel: Percentage of Annexin V staining in wild-type murine embryonic fibroblasts (MEF) or in Bax and Bak double knockout MEFs (DKO) transfected with control vector or with the GFP-Bax-α5 construct. The percentage of Annexin V-binding cells was determined by FACS analysis 6h, 24, 48 and 72h after transfection using an Annexin V-Cy3 apoptosis detection kit.



S2. GFP-tagged Bax-α5/α6 fragments induce loss of mitochondrial membrane potential.

(A) Representative microscopic fields showing fluorescence of cells transfected with constructs encoding GFP (in the presence or absence of 1 µM staurosporine) or GFP-Bax-α5. The arrows indicate GFP-Bax-α5-expressing cells that fail to exhibit MitoTracker Red staining.

(B) Flow-cytometric analysis of ΔΨm estimated by MitoTracker Red intensity. HT1080 cells were transfected with control vector or with the GFP-expressing constructs and MitoTracker Red fluorescence was analyzed by flow cytometry 24h later. Results are representative of three independent experiments (up). Data are represented as mean ± SD. A shift to the left indicates the loss of mitochondrial transmembrane potential (bottom).



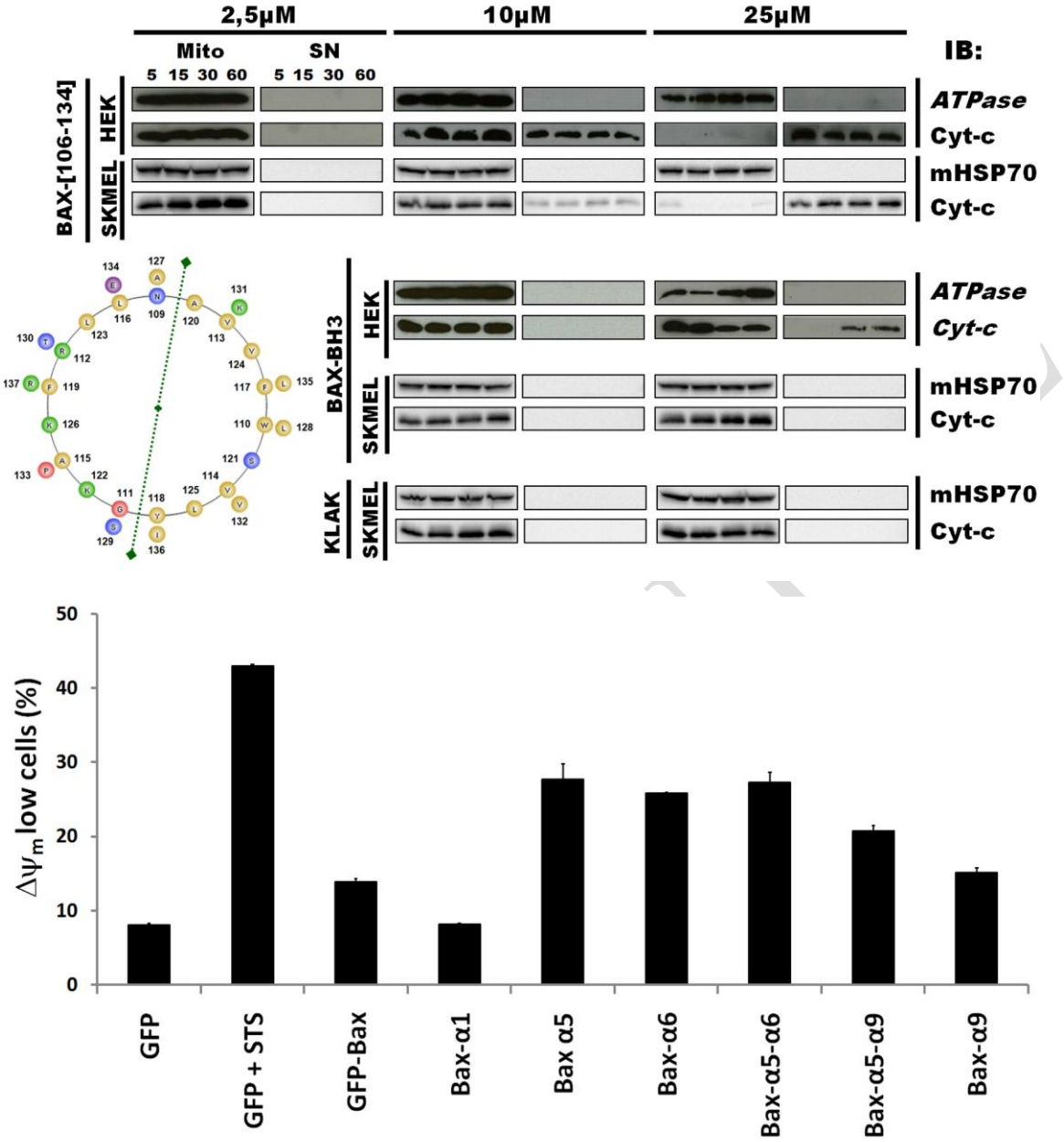
S3. Effect of synthetic Bax-derived peptides on isolated mitochondria.

Crude mitochondria were prepared from SK-MEL-28 and HEK 293T cells. In brief, cells were mechanically broken one time using a 2 ml glass/glass Dounce homogenizer (Kontes) (30 strokes). Homogenates were cleared at 1,500 g and mitochondria were spun down at 10,000 g. For cytochrome c release assays, 30 μg of crude mitochondria were resuspended at 1 mg/ml in KCl buffer supplemented with succinate (5 mM) and EGTA (0.5 mM). Peptides (2.5, 10 and 25 μM) were added to the samples and incubations were carried out at 30°C under agitation (300 rpm). At the indicated time points, samples were centrifuged (5 min, 10,000 g, 4°C); supernatants and pellets were recovered and analyzed by immunoblotting for cytochrome c and ATPase (subunit 6) or mitoHsp70.

(A) Cytochrome c release assays for the Bax[106-134] peptide using mitochondria isolated from SK-MEL-28 (upper panels) or HEK293T cells (lower panels). Results of the assays for the Bax BH3 and $(KLAKLAK)_2$ peptides are also shown. Peptides were incubated with isolated mitochondria for the indicated time periods (min) and the release of cytochrome c was monitored by immunoblotting (IB). MitoHSP70 or ATPase (subunit 6) was used as an equal-loading control for the pellet fraction. Control lanes indicate that in the preparation the MOM is intact and cytochrome c is retained within the intermembrane space. *Inset*: Helical wheel projection of the Bax[106-134] peptide. This amphipathic peptide includes the first helix (α5) of the pore-forming hairpin domain, the inter-helical residues previously implicated in the addressing and insertion of Bax into the mitochondrial membrane (Bellot et al., 2007), and the first amino acids of the second helix (α6) (Garcia-Saez et al., 2005; Garcia-Saez et al., 2006). Negatively charged residues are shown in purple, positively charged residues in green, polar residues in blue and hydrophobic amino acids in yellow. Numbers indicate amino acid positions in the native Bax protein.

(B) Bax[106-134]-induced mitochondrial swelling (left) and ΔΨm loss (right). Mitochondrial swelling and ΔΨm dissipation were measured using liver mitochondria as previously described (Jacotot et al., 2006). From that study, it appears that Bax[106-134] is a more potent inducer of mitochondrial swelling (SD50 = 3.98 ± 0.57 μM) and ΔΨm loss (DD50 = 1.68 ± 0.39 μM) than Bax-BH3 (SD50 > 200 μM; DD50 > 200 μM). NT = non-

treated.



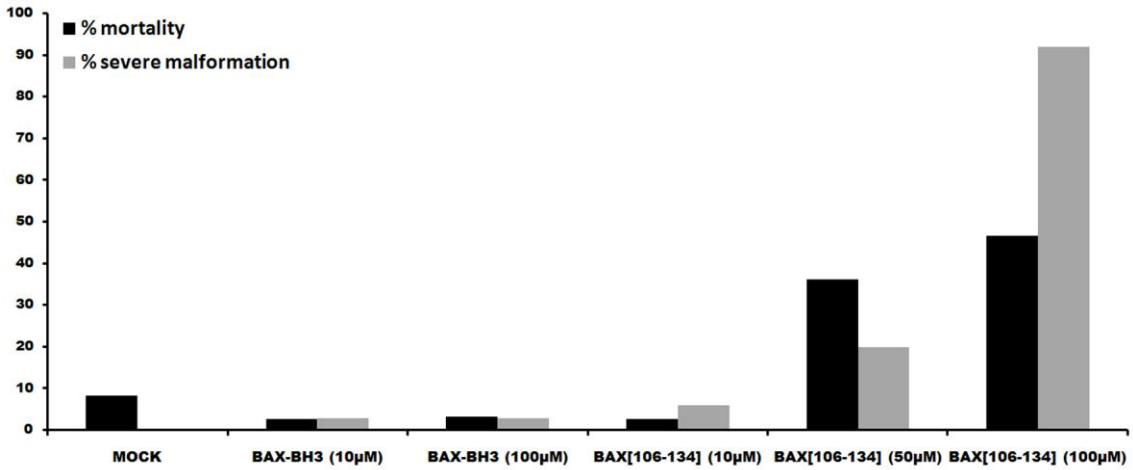
S4. Poropeptide-Bax[106-134] is toxic upon microinjection into zebrafish eggs.

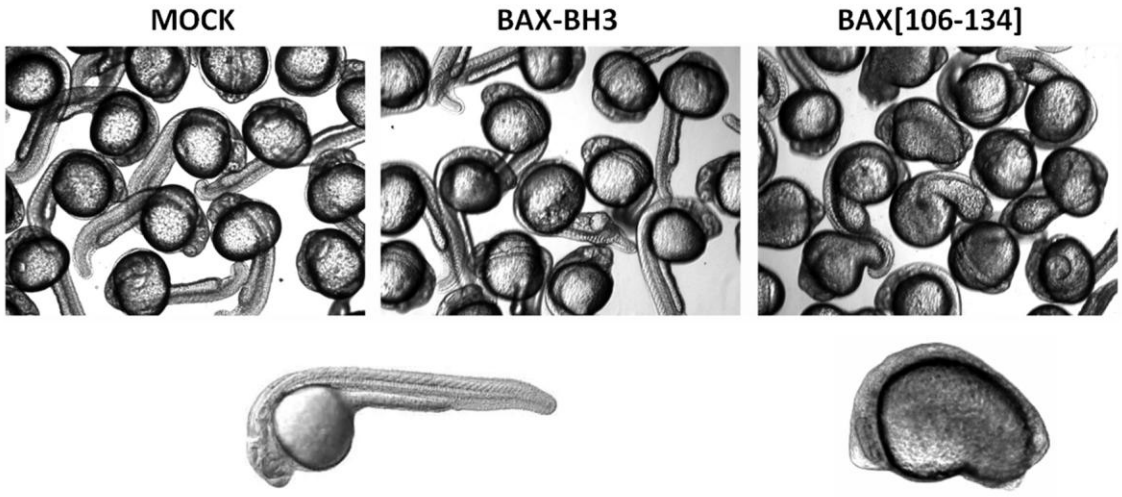
The zebrafish system is a useful cellular model because (i) its apoptotic machinery is similar to the one of mammals, (ii) it is easier in principle to inject peptides into zebrafish eggs than in mammalian cells and (iii) it allow testing *in ovo* toxicity and possible developmental defects. Peptides (Bax[106-134] and Bax-BH3) were injected (~10 nL) into 1–2 cell stage *Danio rerio* embryos at 10, 50 and 100 µM. Ultrapure water was injected as a negative control. About 80 embryos were injected per condition. Zebrafish embryos were maintained at 28°C in 30% Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM

Ca(NO3)2, 5 mM Hepes, pH 7.6). The egg morphology was observed 24h after the initial injection.

(A) Zebrafish eggs were microinjected at the 1-2 cell stage embryos with synthetic Bax[106-134] and Bax-BH3 peptides at 10, 50 and 100 µM (this later concentration corresponding to around $6x10^{12}$ molecules of peptide per egg) or ultrapure water ('mock'). Histograms represent the percentage of mortality at 24h post fertilization and the percentage of embryos with severe malformations among the surviving embryos. Data are from a representative experiment repeated twice with similar results. Embryos microinjected with 10, 50 or 100 µM (in the injection capillaries) of Bax[106-134] showed 2%, 36% and 47% mortality at 24h, respectively. On the other hand, at the doses assayed, Bax-BH3 was ineffective in triggering specific embryonic death after microinjection in zebrafish eggs.

(B) Embryo morphology 24h after injection. Severe morphological malformations are observed in the group injected with Bax[106-134] compared to the group injected with Bax-BH3. Peptide concentration in the microinjection capillaries was 100µM. Note that all surviving embryos exhibited major malformations.



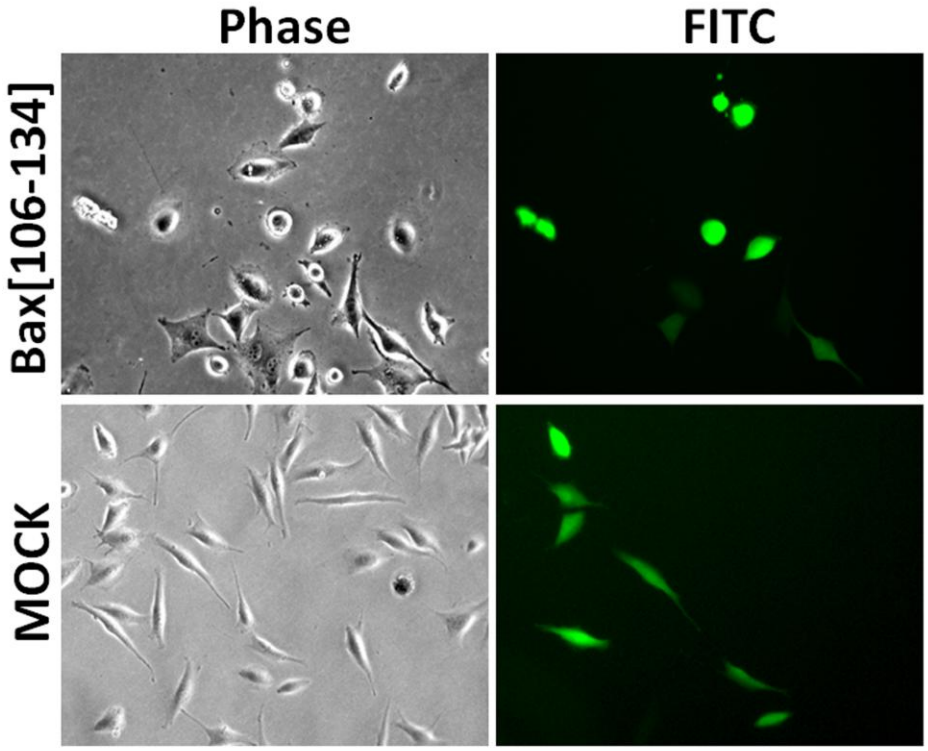


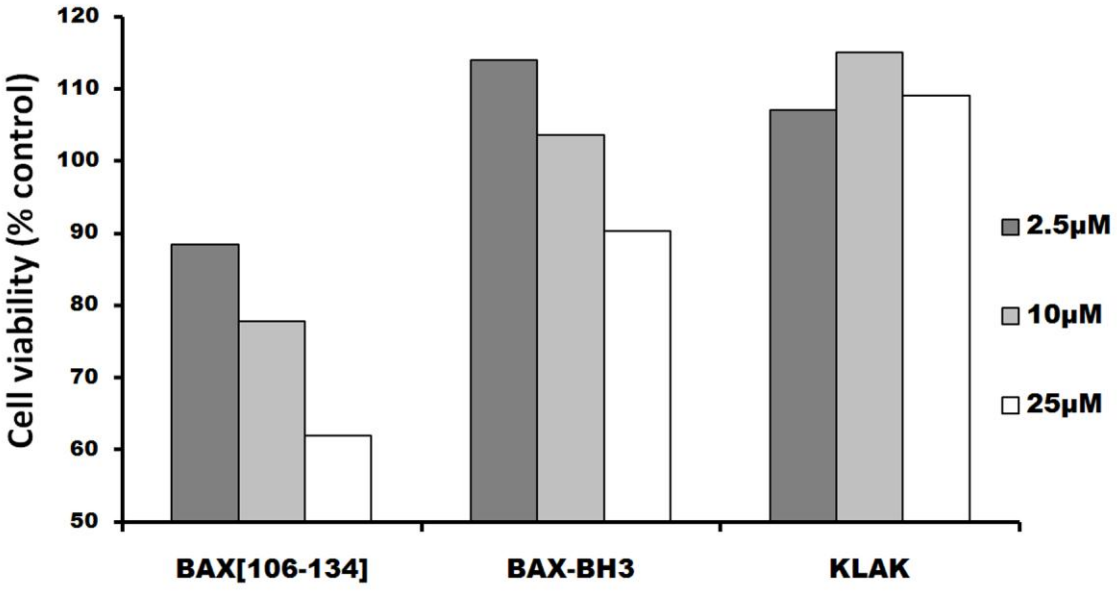
S5. Poropeptide-Bax[106-134] is toxic upon microinjection into human cancer cells.

Cytosolic microinjection was performed under a Nikon Eclipse TE200 inverted microscope using an Injectman NI2 (Eppendorf) and microinjector/micromanipulator Femtojet (Eppendorf). Cells were microinjected by using sterile microcapillaries (Femtotips, Eppendorf) loaded by sterile microloaders (Eppendorf). SK-MEL-28 cells were seeded on glass cellocate coverslips (Eppendorf) in 8 cm2 dishes 20h prior to microinjection. An average of 100 cells per dish were injected with peptide solutions at different concentrations (2.5 μM, 10 μM and 25 μM) in sterile buffer (Hepes 25 mM, $CaCl_2$ 8 mM, pH=7.4) and containing dextran conjugated Alexa Fluor 488 (10,000 MW, anionic, fixable, Molecular Probes). Control injections were done with dextran-conjugated Alexa Fluor 488 alone in buffer. All experiments were performed at an initial pressure of 85 Hpa for 0.2 seconds and a compensation pressure of 30 Hpa. The estimated injected volume into the cytoplasm is about 5 pl. Cells were moved into fresh medium immediately after microinjection and counted at various times (2, 8 and 24 h) using fluorescence microscopy.

(A) Microphotographs of SK-MEL-28 cells coinjected with FITC-dextran (mock) and Bax[106-134] and visualized by epifluorescence microscopy 12h after microinjection. These cells exhibited cytoplasmic blebbing and budding, which was not observed in cells microinjected with FITC-Dextran alone. Magnification, ×800.

(B) Cell viability after microinjection of Bax[106-134], Bax-BH3, $(KLAKLAK)_2$ or R8 synthetic peptides. All microinjections were visualized by coinjection of FITC-labeled dextran. Cell death of SK-MEL-28 cells was determined from morphological alterations (cell shrinkage and round-up) 12h after microinjection. Data are from a representative experiment repeated twice with similar results. The Bax[106-134] peptide induced substantial cell death. In contrast, Bax-BH3, $(KLAKLAK)_2$ or R8-treated cells showed no significant difference from control cells.





S6. Peritumoral administration of Cy5-labeled poropeptide-Bax[106-134] in mammary adenocarcinoma (TS/A-pc) tumor-bearing athymic nude mice. Fluorescence Reflectance Imaging.

Female NMRI nude mice (6-8 weeks old, Janvier, Le Genest-Saint-Isle, France) were injected subcutaneously with mouse TS/A-pc cells (10^6 cells per mouse). After tumor growth (10 days), anesthetized mice (isoflurane/oxygen 4% for induction and 1.5/2% thereafter, CSP, Cournon, France) were injected peritumorally with 100 µL of Cy5-Bax[106-134] suspension (300 nM of dye). Mice were illuminated by 633-nm light-emitting diodes equipped with

interference filters. Fluorescence images as well as black and white pictures were acquired by a back-thinned CCD camera (ORCAII-BT-512G, Hamamatsu, Massy, France) (Jin et al., 2006) fitted with a colored glass long-pass filter RG 665 (Melles Griot, Voisins Le Bretonneaux, France). At the end of the experiment, mice were euthanized to monitor peptide biodistribution in the different organs.

(A) Fluorescence reflectance imaging of athymic nude mice bearing subcutaneous TS/A-pc mammary adenocarcinoma tumor (right inferior limb) laid on the back, 1h (upper panel) and 24h (bottom panel) after peritumoral injection of Cy5-Bax[106-134] (representative example, N = 2).

(B) Corresponding peptide biodistribution data. Representative images of dissected organs (directly exposed under the camera) of mice sacrificed 24hrs p.i. The examined organs (arranged 4 by 4) are as follows: heart, lung, brain, skin, skeletal muscle, kidney, adrenal gland, urinary bladder, small intestine, spleen, pancreas, fat, stomach, uterus/ovary, liver, tumor. Upon peritumoral administration, the dye-conjugated peptide was mainly taken up by the tumor tissue, which exhibited strong Cy5 fluorescence intensity even 24h post-injection. These fluorescence data suggest that the Cy5-labeled 'poropeptide' has a sustained localization within the tumor micro-environment following peritumoral injection.

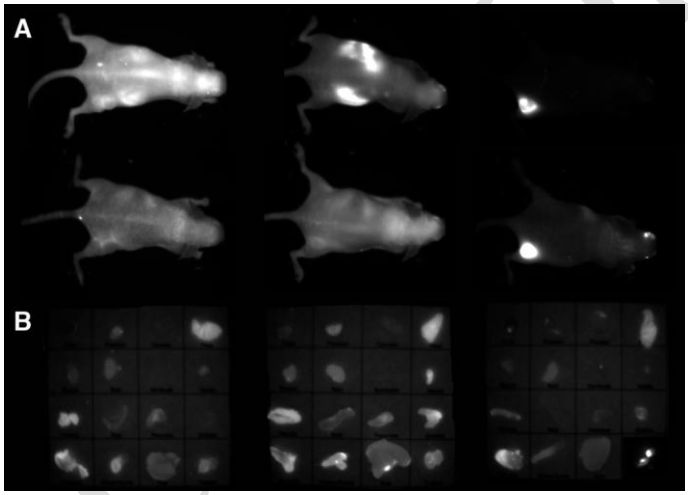


Table S1. Sequence of oligonucleotide primers used for this study.

Amino acid sequences of the peptides used in this study. MI = microinjection, CR = cytochrome c release, MSD = mitochondrial swelling and depolarization, CV = cell viability, ITA = antitumoral activity, IVB = in vivo biodistribution.

Peptide	Sequence	Length	Use
Bax[106-134]	NWGRVVALFYFASKLVLKALSTKVPELIR	29	CR, MI, MSD
Bax-BH3	VPQDASTKKLSECLKRIGDELDSNMELQR	29	CR, MI

KLAK	KLAKLAKKLAKLAK	14	CR, MI
R8-Bax[106-134]	FITC-RRRRRRRRRGNWGRVVALFYFASKLVLKALCTKVPELIR	38	CV, ITA
R8-Bax[Scr]	FITC-RRRRRRRRRGLWSVPLVELAFANTASYGIRLKFKLVRVK	38	CV, ITA
Cy5-Bax[106-134]	Cy5-NWGRVVALFYFASKLVLKALSTKVPELIR	29	IVB

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