Supporting Information (SI)

WAIXENICIN A INHIBITS CELL PROLIFERATION THROUGH MAGNESIUM-DEPENDENT BLOCK OF TRPM7

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SUPPLEMENTAL PROCEDURES

Animal material

The soft coral *S. edmondsoni* was collected in waters adjacent to Lanikai Beach, on the island of Oahu. Samples were immediately frozen at –20 °C and then freeze-dried. The soft coral was identified as *Sarcothelia edmondsoni* (formerly known as *Anthelia edmondsoni*). The freeze-dried sample (325 mg dry wt) was extracted twice with methanol (11 ml), and solvent was removed from the combined extracts under vacuum to yield 117 mg of extract residue. Prior to assay, the extract was redissolved in methanol/ethyl acetate/*t*-butyl methyl ether (60:30:10) (MET) and diluted in Krebs-Ringer-HEPES (KRH) buffer (135 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 20 mM HEPES, and 5.6 mM glucose). The extract was tested for inhibitory effects on TRPM7-mediated cation flux in a fluorescence-based assay in duplicate on separate days at a concentration of 30 µg/ml (2% MET).

Fluorescence-based TRPM7 bioassay

The *S. edmondsoni* extract, its fractions, and isolated waixenicin-A (Fig. 1E) were assayed against TRPM7 using a previously reported (32) high-throughput bioassay system, that measures TRPM7-mediated Mn^{2+} influx via fluorescence quench of fura-2 calcium imaging dye in a TRPM7-overexpressing HEK293 cell line (2). In brief, HEK293-TRPM7 cells (50,000-60,000 cells/well) were plated in poly-L-lysine coated 96-well plates and TRPM7-expression was induced 2-3 hr post plating by the addition of 2 μg/ml tetracycline. The culture medium was completely removed at 18 hr post induction and replaced with fura-2 loading-buffer: 2 mM fura-2-acetoxymethyl ester (Invitrogen), 2 mM probenecid (Sigma), and 0.3% pluronic F-127 (Sigma) in KRH. Following incubation (45 min at 37 °C), the loading buffer was removed, and the cells were washed once with KRH before addition of fresh KRH as the assay buffer. The plates were then transferred to a pre-warmed (37 °C) FlexStation (Molecular Devices) fluorescence plate reader. Cells were initially incubated with test substances or vehicle for ~15 min. Vehicle-receiving, induced TRPM7-HEK293 cells served as positive control for the activation of a TRPM7-mediated Mn^{2+}-influx, while wells containing parental HEK293 cells (receiving vehicle) served as negative controls to define the background for the changes of cytosolic $[Mn^{2+}]$. The Ca^{2+}-independent fluorescence (excitation 360 nm; emission 510 nm) of fura-2 was monitored following the addition of 10 mM $MnCl_2$. The TRPM7-mediated increase in $[Mn^{2+}]_i$ was processed in SoftMax Pro (v 5.3, Molecular Devices) as the maximum slope of fluorescence change after $MnCl_2$ addition, and the data was background corrected and normalized by expressing sample-treated well maximum slope as a percent of vehicle-control. For dose-response determination, IC_{50} values were determined from dose-response curves fitted in Igor Pro (Wavemetrics) by constraining the top of the curve (no inhibition) to 100% vehicle control.

Bioassay-linked fractionation of the S. edmondsoni organic extract

The active extract was fractionated by semi-preparative reversed-phase HPLC (Agilent 1100 binary liquid chromatograph; Phenomenex Luna C18-2 column, 5 µm, 10 x 250 mm) using a linear gradient of

acetonitrile/aqueous 10 mM ammonium acetate (23-68% acetonitrile over 0-2 min, 68-95% acetonitrile over 2-25 min, 95% acetonitrile over 25-40 min; flow rate: 3.8 ml/min; UV detection: 220-240 nm). Extract components eluting between 5-40 min were collected as 30-sec fractions into a deep 96-well plate. Solvent and buffer was removed from the plate under vacuum in a centrifugal concentrator at room temperature and fractions were reconstituted with MET and apportioned into low-profile 96-well plates for bioassay and chemical analysis. Prior to assay, the fractions were reconstituted with MET and diluted with KRH. Fractions were tested at an average concentration of 15 mg/ml, and data was pooled from single replicates tested on separate days.

Isolation of waixenicin-A

The scaled-up fractionation of the soft coral extract was guided by characteristic LC-MS data (electrospray ionization; ion trap analyzer; Thermo Finnigan LCQ Deca XP) obtained for the extract peak corresponding to the highest TRPM7 activity. Initially, the extract was subjected to vacuum liquid chromatography (stationary phase: silica; mobile phase: dichloromethane/methanol mixtures). The fraction eluting with dichloromethane/methanol (95:5) concentrated the targeted compound. Repeated semi-preparative reversed phase HPLC led to the isolation of waixenicin-A (Fig. 1E), which was identified by comparison of NMR data and optical rotation with literature values (34). The purity of our waixenicin-A sample was determined to be >95% based on LC-MS-evaporative light scattering detector (ELSD) analyses (Phenomenex Luna C18-2 column, 5 µm, 2 x 250 mm; mobile phase: linear gradient of acetonitrile/aqueous 10 mM ammonium acetate (50-100% acetonitrile over 0-50 min); Sedex 75 ELSD, 50 °C). For further physiological experiments, 25 µg of purified and lyophilized waixenicin-A was dissolved in 60 µl of methanol, diluted in the adequate buffer solution used for the subsequent experiments (100 µM stock solution) and stored at –20 °C.

Cell culture

A tetracycline-inducible HEK293 cell line overexpressing TRPM7 (2) was used for the fluorescence-based TRPM7 bioassay. For additional electrophysiological studies we used HEK293 cells transiently overexpressing TRPM7 (2) and TRPM7 mutants (6), TRPM2 (36) or TRPM4 (39), as well as rat basophilic leukemia (RBL1), RBL1-wt cells, and finally HEK293-wt cells transfected with a TRPM6 plasmid (kindly provided by Dr. Bindels). RBL1 cells and a human T-lymphocyte cell line (Jurkat) were used for proliferation, cell cycle and toxicity assays.

Electrophysiology

Patch-clamp experiments were performed in whole-cell configuration. High-resolution currents were acquired and recorded using EPC9 (HEKA) and PatchMaster (HEKA). All voltages were corrected for a liquid junction potential of 10 mV. Currents were elicited by a ramp protocol from –100 mV to +100 mV (TRPM7, I_{CRAC}) or –150 to +150 mV (TRPM2, TRPM4) over 50 ms acquired at 0.5 Hz and a holding potential of 0 mV. Inward current amplitudes were extracted at –80 mV, outward currents at +80 mV and plotted versus time of the experiment. Data were normalized to cell size as pA/pF. Standard extracellular solution contained (in mM): 140 NaCl, 1 $CaCl_2$, 2.8 KCl, 2 $MgCl_2$, 10 HEPES-NaOH, 11 Gluc (pH 7.2, 300 mOsm). Standard intracellular solution contained (in mM): 140 Cs-glutamate, 8 NaCl, 1 $MgCl_2$, 10 HEPES (pH 7.2, 300 mOsm). Mg-free intracellular solution contained (in mM): 120 Cs-glutamate, 8 NaCl, 10 HEPES, 10 Cs-BAPTA, 5 Na-EDTA (pH 7.2, 300 mOsm). For Mg^{2+} dose-response assessment intracellular solution contained (in mM): 140 Cs-glutamate, 8 NaCl, 10 Cs-BAPTA plus appropriate amounts of $MgCl_2$, as calculated with WebMaxC.

Proliferation and apoptosis assays

Cells were seeded at a density of 10^4 cells/ml in DMEM supplemented with 10% FBS and allowed to attach before waixenicin-A was added. Cell culture plates were incubated in a humidified incubator at 37 °C for two days.

ViCell Analysis: A Beckman Coulter ViCell Automated Cell Viability Analyzer (Beckman Coulter, Inc., Brea, CA) was used to stain RBL1 cells with trypan blue and count all live and dead cells of 100 digital

images. Total number of vital cells was normalized to the untreated control. Cells that were stained with trypan blue were considered as dead cells and normalized to the total amount of cells in the sample.

MTT-assay: Part of the media was removed and 50 µl of 5 mg/ml thiazolyl blue tetrazolium bromide (MTT, Sigma) was added to the remaining 500 µl of media. Cells were incubated for 3 h at 37 °C until purple crystals formed. Finally, 500 µl of MTT solvent (0.1 N HCl in isopropanol) were added and after the crystals dissolved, the absorbance ratio A/R (with A = 540 nm and R = 630 nm) was read using a Benchmark Plus microplate spectrophotometer (Bio-Rad). Experiments were made in triplets.

Annexin-V–propidium iodide assay: A modified annexin-V-FITC Apoptosis Detection Kit (Sigma) was used to determine the percentage of apoptotic cells. Cells were collected in centrifuge tubes using Trypsin/EDTA and centrifuged at 1000 rpm for 7 min. The supernatant was removed and cells were resuspended in 300 µl of 1 x binding buffer (Sigma). Thereafter, 7 µl of propidium iodide (PI) and 10 µl of FITC-conjugated anti-Annexin-V antibody (Sigma) were added and incubated for 15 min. Finally, cells were analyzed using a fluorescence activated cell sorter (FACS Calibur, BD Biosciences).

Cell cycle analysis

For cell cycle analysis, a FITC BrdU Flow Kit (BD Pharmingen) was used according to manufacturer's instructions. In brief, cells were seeded at a density of 0.5 x 10^6 cells/ml and allowed to equilibrate for 10 min before waixenicin-A was added. Subsequently, cells were incubated for another 15 min before BrdU was added. After another 2-6 hours of incubation time, the cells were fixed and permealized. Thereafter, DNase was added to expose the BrdU epitopes, which were stained using a FITC conjugated anti-BrdU antibody while the DNA content was stained using 7-AAD. Cells were analyzed via flow cytometry (FACS, BD Biosciences) with excitation of 488 nm and FL-1 emission of 530 nm (bandpass filter) for FITC-conjugated anti-BrdU antibody detection and FL-3 emission of >670 nm (bandpass filter) for 7-AAD detection. The upper gate represents all cells that have incorporated BrdU, including cells in the synthesis (S) and mitosis (M) phase of the cell cycle. The lower left gate represents dead cells and apoptotic cell debris (A). The middle lower gate shows cells in G1/G0-phase of the cell cycle (G1) where they have a normal DNA pool (2 n), whereas the right lower gate represents cells that have already doubled their DNA content (4 n) and are in G2-phase of the cell cycle (G2).

Statistical analysis

Unless stated otherwise, data represent the mean of individual experiments ±standard error of mean (S.E.M.). An unpaired student's t-test was applied for significance analysis and $P<0.05$ was considered statistically significant.

SUPPLEMENTAL FIGURES AND LEGENDS

Fig. S1. Waixenicin-A affects outward and inward currents similarly
Fig. S2. Waixenicin-A and extracellular Mg^{2+} in TRPM7-wt and TRPM7-ΔK
Fig. S3. Waixenicin-A blocks native TRPM7 currents.
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Fig. S5. Toxicity of waixenicin-A is above 10 µM
Fig. S6. Waixenicin-A-treated cells exhibit green auto-fluorescence at 488 nm laser excitation

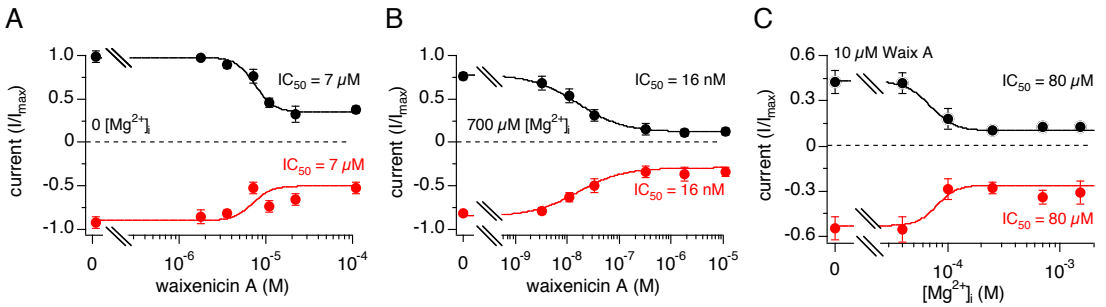

Fig. S1. Waixenicin-A affects outward and inward currents similarly. A. Same data set as in Fig. 2E. Different concentrations of waixenicin-A were applied as shown in Fig. 2A. Currents were extracted at +80 mV (black) and –80 mV (red) at 500 s, normalized to the maximal current amplitude before application (200 s), averaged, plotted against waixenicin-A concentration and approximated by a dose-response fit function (n = 8-10). *B.* Same data set as in Fig. 2F. Different concentrations of waixenicin-A were applied as shown in Fig. 2C. Currents were extracted at +80 mV (black) and –80 mV (red) at 600 s, normalized to the maximal current amplitude before application (300 s), averaged, plotted against waixenicin-A concentration and approximated by a dose-response fit function (n = 5-13). *C.* Same data set as in Fig. 3A for TRPM7-wt. Application solution contained 10 μM waixenicin-A. Currents were extracted at +80 mV (black) and –80 mV (red) at 600 s, normalized to the maximal current amplitude before application, averaged, plotted against $[Mg^{2+}]_i$ and approximated by a dose-response fit function (see Methods; n = 6-10). Note that inward and outward currents reveal the same IC_{50} and Hill coefficients for all conditions. Error bars represent S.E.M.

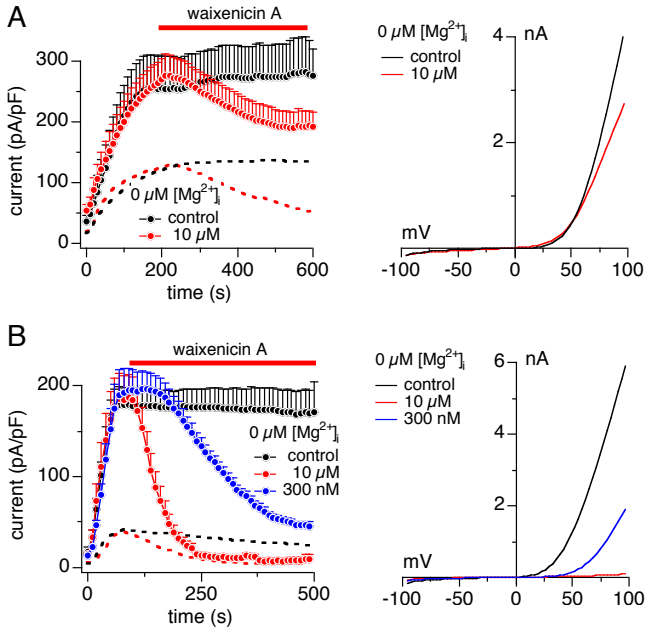

Fig. S2. Waixenicin-A and extracellular Mg2+ in TRPM7-wt and TRPM7-ΔK. A. Currents in HEK293 cells overexpressing TRPM7-wt were recorded in the absence of extracellular Mg^{2+} and normalized to cell size without (black, n = 12) and with 10 µM waixenicin-A application (red, n = 12). The dotted lines represent the same data set as in *Fig. 2A* in the presence of extracellular Mg^{2+}. Error bars represent S.E.M. Corresponding I/V relationships are representative currents in response to voltage ramps obtained at 500 s into the experiment. The external solution contained nominally zero Mg^{2+} (in mM: 140 NaCl, 1 $CaCl_2$, 2.8 KCl, 10 HEPES-NaOH, 11 Gluc). *B.* Time course of TRPM7-ΔK currents normalized to cell size without (black, n = 9) and with 10 µM (red, n = 10) or 300 nM (blue, n = 8) waixenicin-A application. Experimental conditions were the same as in *A*. The dotted lines represent the same data set as in *Fig. 3C* in the presence of extracellular Mg^{2+}. Error bars represent S.E.M. Corresponding I/V relationships are representative currents in response to voltage ramps obtained at 400 s into the experiment.

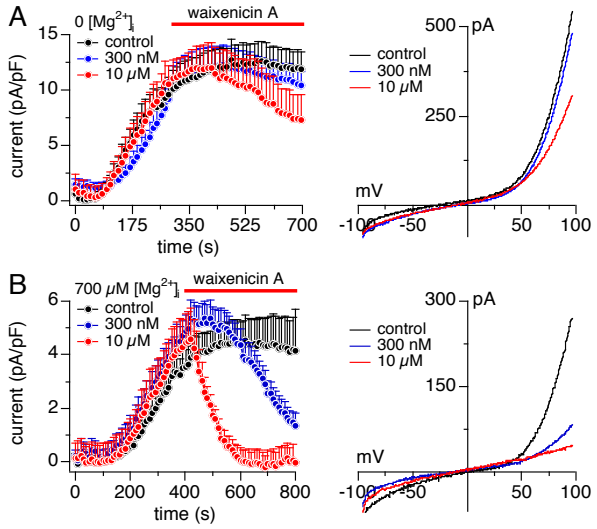

Fig. S3. Waixenicin-A blocks native TRPM7 currents. A. Normalized native TRPM7 currents at +80 mV in RBL cells without (black, n = 8) and with 300 nM (blue, n = 8) or 10 µM (red, n = 8) waixenicin-A application. TRPM7 currents were elicited by intracellular zero Mg^{2+}. Corresponding I/V relationships are average currents in response to voltage ramps obtained at 700 s in untreated controls (black, n = 5) and 300 nM (blue, n = 4) or 10 µM waixenicin-A (red, n = 5). *B.* Same experimental protocol as in *A*, except that intracellular free Mg^{2+} was clamped to 700 µM. Normalized native TRPM7 currents at +80 mV without (black circles, n = 7) and with 300 nM (blue, n = 9) or 10 µM waixenicin-A (red, n = 5). Corresponding I/V relationships are average currents in response to voltage ramps obtained at 800 s in untreated controls (black, n = 3) and 300 nM (blue, n = 3) or 10 µM waixenicin-A (red, n = 3). Error bars represent S.E.M.

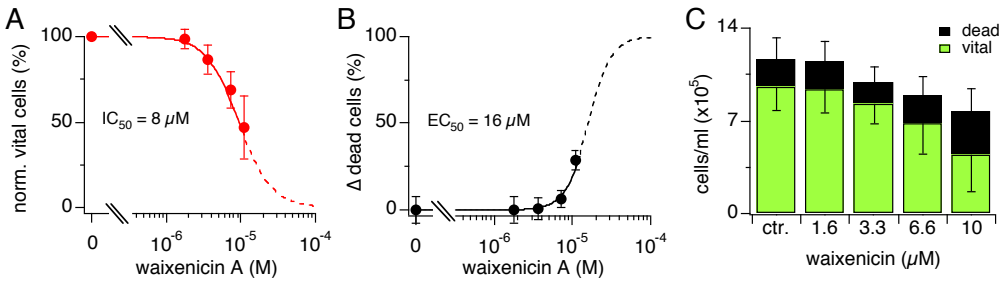

Fig. S4. Waixenicin-A dose-dependently inhibits proliferation without being toxic. A. Trypan blue staining of RBL1 cells treated with different concentrations of waixenicin-A for 2 days. Cells were analyzed via a VICell counter screening 100 individual images (n = 3). Relative numbers of unstained cells were averaged, normalized to the untreated control and plotted against waixenicin-A concentration. Error bars represent S.E.M. *B.* Trypan blue positive cells were analyzed from the same data set as in *A*. Relative numbers of dead cells were averaged and plotted against waixenicin-A concentration. Error bars represent S.E.M. *C.* Total cell count of trypan blue-stained cells per ml. Raw data are represented as mean of three independent experiments. Error bars indicate S.E.M. Note a reduction in total cell number with increasing compound concentrations.

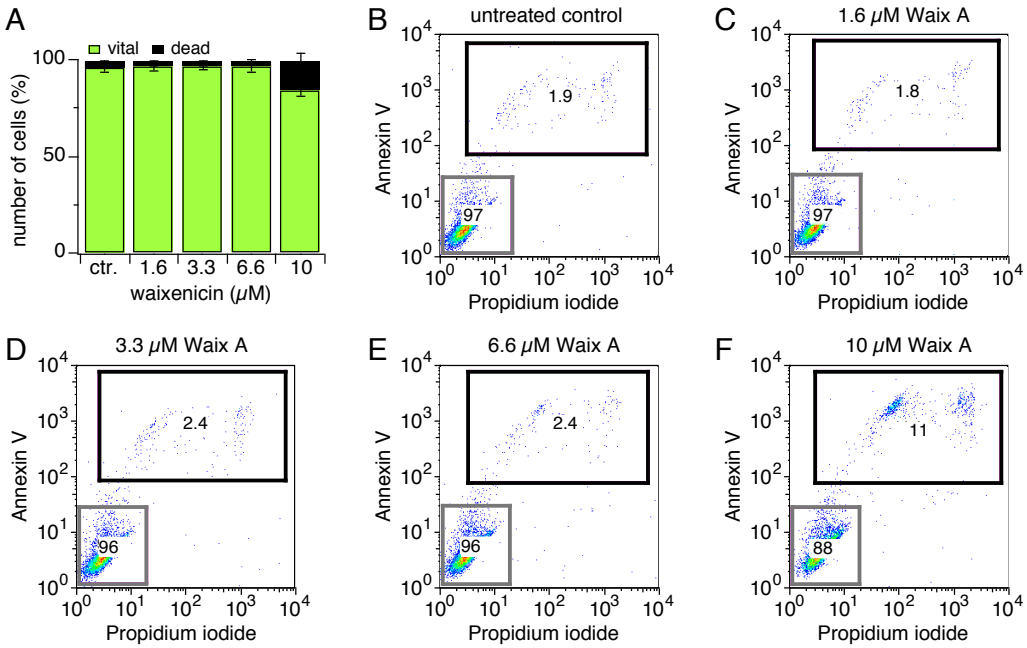

Fig. S5. Toxicity of waixenicin-A is above 10 μM. A. Same data set as in *Fig. 6B*. Averaged, normalized cell count of Annexin-V/PI-stained cells. Data are represented as mean of three independent experiments. Error bars indicate S.E.M. Note an increase in number of dead cells only with the highest compound concentration of 10 μM. *B – F.* Flow cytometric analysis of Annexin-V/PI-stained and waixenicin-A-treated RBL1 cells. Annexin-V-positive cells are plotted against propidium iodide staining. Cells in the lower left quadrant were considered healthy (grey gate), whereas cells in the upper two quadrants (black gate) were considered dead. Untreated control cells *B* versus cells treated with distinct waixenicin-A concentrations: *C*. 1.6 μM, *D*. 3.3 μM, *E*. 6.6 μM and *F*. 10 μM waixenicin-A.

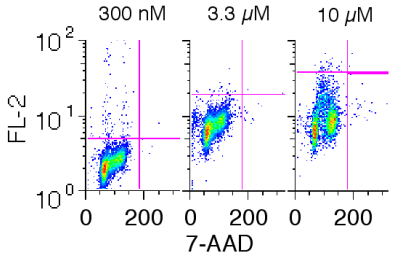

Fig. S6. Waixenicin-A-treated cells exhibit green auto-fluorescence upon excitation with a 488 nM laser. Cell-cycle analysis of Jurkat cells incubated in RPMI medium supplemented with 10% FBS and different waixenicin-A concentrations without BrdU for 2 h. Cells were stained with antiBrdU antibody and 7-AAD and analyzed via flow cytometry. Plots are representative examples for three independent experiments. Note that even without BrdU incorporation, waixenicin-A-treated cells show green fluorescence upon excitation with a 488 nM laser. Emission was collected with a bandpass filter of 530 nm.