Supporting Information

NAMI-A is highly cytotoxic toward leukemia cell lines: evidence of inhibition of KCa3.1 channels.

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Tables

Cell line	Ru ng/10^5 cells	Ru uptake%[a]
K562	**81.9**	**7.3**
IGROV-1	**61.6**	**5.5**

Table S1. Comparative ruthenium uptake, measured by ICP-AES, in a leukaemia cell line, K562, and in a solid tumor cell line, IGROV-1, after 72 h incubation. Data are referred to the same number of cells ; the percentage of uptake refers to the total amount of NAMI-A in the supernatant.

Instrument	Varian 720-ES
R. F. Power	1.25 KW
Plasma argon flow rate	16.5 L min^{-1}
Auxiliary argon flow rate	1.5 L min^{-1}
Nebulizer argon flow rate	0.75 L min^{-1}
Replicate Read Time	20 s
Instr Stabilization Delay	25 s
Sample introduction settings:	
Sample Uptake	20 s
Flow Rate	1 mL min^{-1}
Rinse Time	180 s
Fast Pump (samp delay/rinse)	Active
Smart rinse	Active
General settings:	
Replicates	3

Table S2. ICP-AES operating conditions for the quantification of ruthenium content in K562 and IGROV-1 cells.

Figures

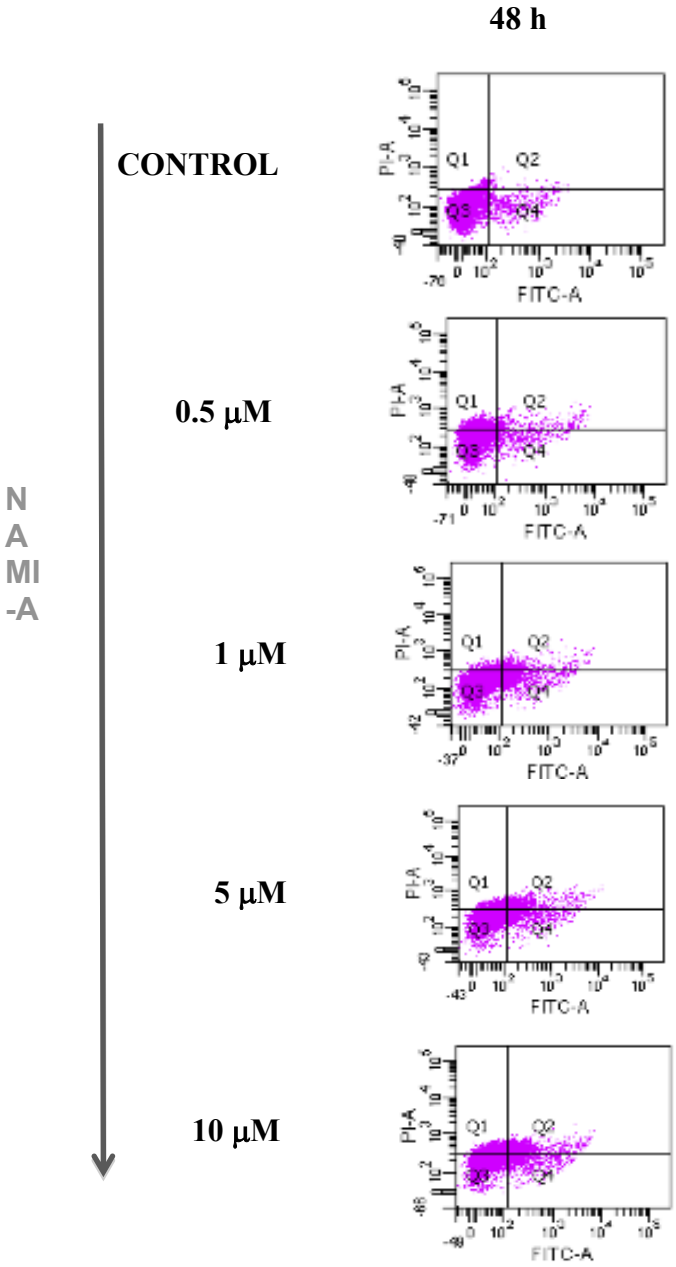

Figure S1. NAMI-A induces apoptosis in leukaemia cells. Leukaemia cells (K562 cell line) were exposed to increasing concentrations of NAMI-A (0-10 μM range) for 48 hours. The percentage of Annexin V+/Propidium Iodide- cells was measured. Values are means± SEM of two independent experiments. Representative dot plots of Flow cytometric analysis are shown.

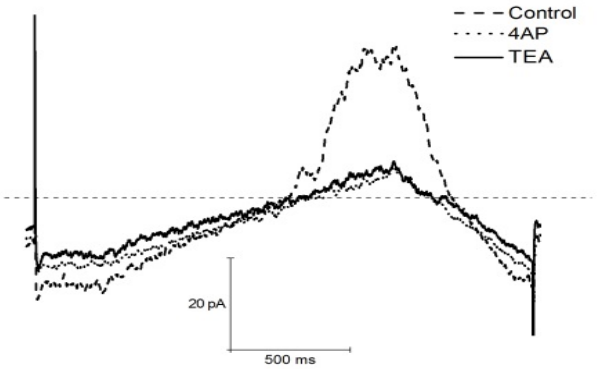

Figure S2. NAMI-A affects plasma membrane ion channels. Ramp protocol obtained in FLG 29.1 cells in the absence (dashed line) and in presence of 5mM TEA (continuous line) or 200 μM 4-Aminopiridine (dotted line).

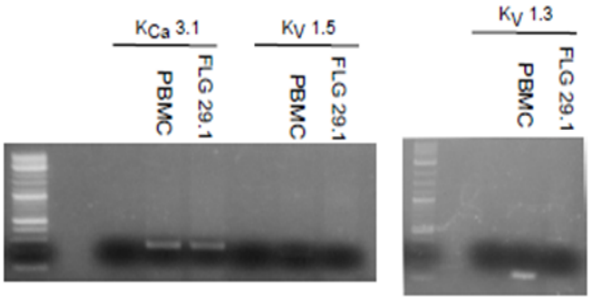

Figure S3. FLG 29.1 express KCa 3.1 channels. RT-PCR relative to KCa 3.1, KV 1.3 and KV 1.5 expression in FLG 29.1 and Peripheral Blood Mononuclear Cells (PBMC). The first lane of each gel shows the negative control of the PCR reaction.

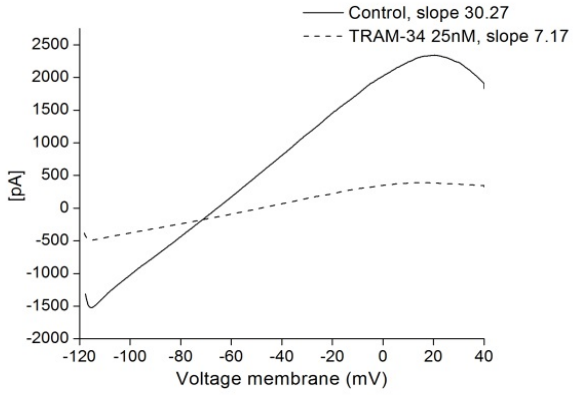

Figure S4. KCa 3.1 currents elicited in HEK 293 KCa 3.1 transfected cells and the effect of TRAM 34 25 nM (dashed line).

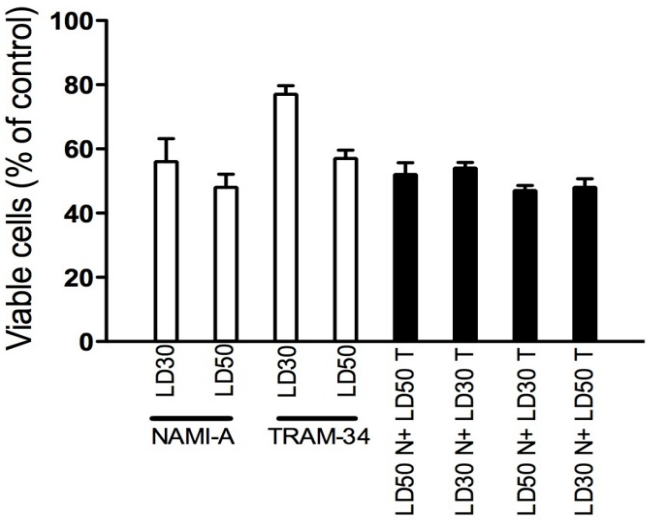

Figure S5. Effect of NAMI-A and TRAM-34 alone and in combination on leukemic cells. FLG 29.1 cell were incubated for 24 hours with NAMI-A (LD50 and LD30) and TRAM-34 (LD50 and LD30) or combinations of the two drugs and proliferation was evaluated with Trypan Blue assay. Values are reported as percentage of control untreated cells (mean±SEM). LD50 and LD30 value of TRAM-34 in FLG 29.1 cells: 6.02 μM and 1.1 μM. LD50 and LD30 value of NAMI-A in FLG 29.1 cells: 0.71 μM and 0.20 μM.

Materials and methods

Cell cultures.
We maintained the ALL cell lines REH, 697 and the AML cell lines FLG 29.1, HL60, and K562 in RPMI 1640 medium supplemented with 2mM L-glutamine, 10% bovine calf serum (HyClone) and maintained at 37°C in a humidified atmosphere in 5% CO2 in air. HEK 293 cells were cultured in DMEM high glucose (Euroclone, Milan, Italy), plus 10% FBS and 4% L-glutamine and were transfected with pcDNA3.1 KCa 3.1 with Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Transfected cells were selected by the addition of 800mg/ml G418 (Life Technologies, Carlsbad, USA)

Pharmacology experiments.
To test drug cytotoxicity against ALL and AML cells, cells were serum starved for 16 h in RPMI medium, then seeded in a 96-well flat-bottomed plate (Corning-Costar, Corning, NY, USA) at a cell density of 2 x105 cells per well (100 ml), in RPMI containing 1% FCS. NAMI-A was dissolved in DDW at the concentrations 5mM. NAMI-A was added at time zero and used at the final concentrations indicated in Figures and in the legends to Figures. After 24, 48 and 72 hours, viable cells (determined by Trypan blue exclusion see below) were counted in triplicate using a hemocytometer. Each experimental point represents the mean of four samples carried out in three separate experiments. The LD50 value (ie, the dose that caused the apoptosis of 50% of leukemic cells) was calculated by fitting the data points with a sigmoidal curve using Origin 6 software. CI values were calculated using CalcuSyn software Version 2 (Biosoft). CI > 1, antagonisms; CI = 1, additivity; CI < 1, synergy.

Trypan Blue Assay.
Cell viability was assessed by Trypan blue exclusion assay. In brief, 20 µl of 0.4% trypan blue solution was added to 20 µl cell suspension in culture medium. The suspension was gently mixed and applied to haemacytometer. Viable and dead cells were identified and counted under light microscope. Blue cells failing to exclude dyes were considered nonviable, and transparent cells were considered viable. The percentage of viable cells was calculated on the basis of total number of cells (viable plus nonviable).

Apoptosis analysis.
Apoptosis analysis.
We undertook studies with annexin V/propidium iodide (Annexin-V-FLUOS staining kit; Roche) to measure apoptosis. Leukemic cells (seeded and treated as described in “Pharmacology experiments”) were washed twice with PBS and then resuspended in 100 μL of binding buffer and incubated with FITC-conjugated annexin V and propidium iodide. The mixture was incubated at room temperature for 15 minutes before flow cytometric analysis with a FACSscan (Becton Dickinson).

Cell Cycle Analysis.
After 24h of incubation, cells (seeded and treated as described in “Pharmacology experiments”) were collected and washed twice in PBS solution (1X) than incubated with propidium iodide solution (PI: 50 g/ml, RNase: 1 mg/ml) for 30 minutes at room temperature protected from light. The analysis was performed with cytofluorimetry using FACSscan (Becton Dickinson). At least 10,000 cells were collected. The cell cycle profiles were calculated by using the Modfit Cell Cycle software.

Samples preparation for uptake studies.
K562 and IGROV-1 cells were incubated with 10 μM of NAMI-A for 72h. After treatment, the supernatant was removed and cells were washed three times. Before the quantification of ruthenium content, cells were digested in PE vials by heating at 90°C for 24 hours with 1 ml aqua regia (HCl suprapure grade and HNO_3 sub-boiled in 3:1 ratio). After digestion samples were diluted to 5 ml with ultrapure water (≥18 MΩ), spiked with 4 ppm Ge used as internal standard and analyzed.
Ruthenium content quantification by ICP-AES analysis. The determination of ruthenium concentration in the samples was performed in triplicate by a Varian 720-ES Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES). Calibration standards were prepared by gravimetric serial dilution from mono standards at 1000 mg L-1. Wavelengths used for ICP-AES analysis of Ru and Ge were 267.876 and 209.426 nm, respectively. The operating conditions, optimized to obtain a maximum signal intensity, instrumental conditions and measurement are reported in Table S2. Within each sample, a rinse solution constituted by 2% v/v HNO_3 was used. The ICP torch, spray chamber, nebuliser, and sample introduction tubes were accurately cleaned and, prior to sample analysis, the instrument was purged for at least 1 hour with 2% v/v HNO_3 rinse solution.

Patch-clamp recordings.
Membrane currents were recorded in the whole-cell configuration of the patch-clamp technique, at room temperature (about 25°C). Electrodes were pulled from borosilicate glass capillaries (inside diameter 0.86 mm, outside diameter 1.5 mm; Harvard Apparatus, Holliston, MA, USA), using a PC-10 pipette puller (Narishige, Tokio, Japan). Electrodes typically had a resistance of 3.5-5 MΩ. Series resistance was always compensated up to approximately 80%. Currents were amplified and filtered using a Axopatch 1D (Molecular Devices, Sunnyvale, CA) interfacing with a Digidata 1440A (Molecular Devices) and a computer with pClamp 10.3 software (Molecular Devices). Currents were low-pass filtered at 2 kHz and digitized online at 10 kHz. Data were subsequently analyzed with pClamp and Origin 8.0 (Microcal Inc., Northampton, MA, USA) software.

Solutions.
For measurement of the outward currents the pipette solution contained (in mM): K+ aspartate 130, NaCl 10, MgCl2 2, CaCl2 2, HEPES 10, EGTA 10, titrated to pH 7.3 with KOH. During the experiments, cells were usually perfused with an extracellular solution containing (in mM): NaCl 130, KCl 5, CaCl2 2, MgCl2 2, HEPES 10, Glucose 5, adjusted to pH 7.4 with NaOH. For measurement of the KCa 3.1 currents in HEK 293 transfected cells internal pipette solution and external solution were prepared as in ref. 27.

Protocols.
For the ramp protocol, currents were elicited, from -70 mV holding, by 1 sec voltage ramps from -120mV to +50mV and then back to -120mV. Outward currents were elicited from a -80 mV holding by a 1 sec step at -80 mV, followed by a 1 sec pulses ranging from -80mV to +60 mV in 10 mV steps. KCa 3.1 currents were elicited by voltage ramps protocol as in [1]. The fold decrease of slope conductance was taken as a measure of channel block. NAMI-A was applied for at least 5 minutes (or until steady state inhibition was attained), with each concentration being tested on at least 5 different cells.

Total RNA extraction and Reverse Transcription (RT-PCR).
Total RNA was extracted from FLG 29.1 lines following the TRIzol® Reagent protocol (Life Technologies, Carlsbad, USA). 1μg of total RNA was reverse transcribed to cDNA, in a 20 μl reaction mixture, using Random Primers and SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, USA), following manufacturer's procedure.

Polymerase Chain Reaction.
cDNA was amplified by polymerase chain reaction using Platinum® PCR SuperMix (Invitrogen, Carlsbad, USA), following manufacturer's protocol. Primers final concentration was 200 nM (from a stock solution of 10 μM) in a 25 μl reaction mixture. For KCa 3.1, KV 1.3 and KV 1.5 the primers sequence are the following: KCa 3.1 forward CGGGAACAAGTGAACTCCAT - KCa 3.1 reverse ACTGGGGAAAGTAGCCTGGT; KV 1.3 forward CCCAGACCCCTTTCAAAAC - KV 1.3 reverse TGGGATTATTGTTCGTGGTG; KV 1.5 forward ACTTGCGGAGGTCCCTTTAT - KV 1.5 reverse GGGAGGAAAGGAGTGAAAGG. All the primers used in this experimental work were designed using the software Primer3.