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ARTICLE TYPE

NAMI-A is Highly Cytotoxic Toward Leukaemia Cell Lines: Evidence of Inhibition of KCa3.1 Channels

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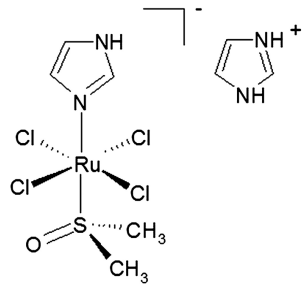
We report here that the established anticancer ruthenium(III) complex NAMI-A induces potent and selective cytotoxic effects in a few leukaemia cell lines. These results sound very surprising after 20 years of intense studies on NAMI-A, commonly considered as a "non-cytotoxic" antimetastatic agent. In addition, evidence is given for selective inhibition of KCa3.1 channels. The implications of these findings are discussed.

Since the discovery of cisplatin, platinum based drugs have played a central role in cancer treatment. Yet, intense efforts are currently devoted to the identification of novel anticancer metallodrugs that might overcome the limitations of platinum compounds, by exploiting the rich chemistry of several other metal centers (e.g. ruthenium, gold, tin, etc.).[1]

NAMI-A is a well-known mixed ligand ruthenium(III) anticancer complex discovered and developed in the 90's[2-3] and intensively investigated afterward. NAMI-A consists of a ruthenium(III) ion coordinated to four equatorial chloride ligands and to DMSO and imidazole as axial ligands within an overall distorted octahedral geometry (Scheme 1).



Scheme 1 [ImH]trans-[RuCl4(Im)(dmso-S)] (NAMI-A, Im= imidazole).

Many studies were dedicated to NAMI-A because of its favourable and peculiar biological properties[4-6]. Chemical studies characterised its solution behaviour and the progressive release of some of the original ligands, in particular at least two chlorides and dmso-S[7], under physiological conditions. From a biological standpoint, NAMI-A turned out to have little cytotoxicity towards many cancer cell lines, but pronounced antimetastatic effects in several animal cancer models[8-13]. This effect is attributed to the formation of adducts with membrane and cytosolic proteins[15-18], whereas DNA is not considered a major molecular target[14]. Owing to its overall pharmacological profile, low systemic toxicity and significant antimetastatic properties, NAMI-A was proposed for clinical trials in oncology. A phase I clinical trial was successfully completed a few years ago[19] while the results of a phase 1/2 combination study with gemcitabine are due to appear soon. Because of its peculiar pharmacological properties and eventual admission to clinical trials, NAMI-A is now intensely studied in the "Metals in Medicine" research community, as a prototype of a class of metal compounds showing poor cytotoxicity but remarkable anticancer properties in vivo. Notably, NAMI-A does not fully fit the established NCI criteria for new cancer drug discovery and would have been discarded in standard screening procedures due to its scarce cytotoxicity. Moreover, the molecular mechanisms through which NAMI-A induces its pharmacological effects remain largely unknown, although several hypotheses have been advanced [4, 19-23].

We studied the antiproliferative effect of NAMI-A on a panel of leukaemia cell lines, both myeloid (K562, FLG 29.1, HL60) and lymphoid (REH and 697). The effects of increasing NAMI-A concentrations (range 0-20 μM) were tested by the Trypan blue exclusion assay at different incubation times (24, 48, 72 hours). To our great surprise, and in sharp contrast to expectations, we found that NAMI-A strongly inhibited proliferation in all tested leukaemia cell lines, even at low micromolar concentrations. The growth inhibition profiles are shown in Figure 1 (panel A for myeloid, panel B for lymphoid cell lines) while the corresponding LD50 values (*i.e.,* the dose that caused the apoptosis of 50% of leukaemia cells), at different incubation times, are in the table of Figure 1C'. Following a single treatment at time zero, NAMI-A concentrations around 5 μM strongly inhibited both myeloid and lymphoid cell proliferation (Figure 1A and B). However, after about 48 hours, cells started to proliferate again. When NAMI-A was re-added after 24 and 48 hours of incubation, treatment nearly completely abolished cell proliferation. Data relative to K562 cells are shown in Figure 1B'. These results suggest that NAMI-A is degraded relatively rapidly in the medium[24-25]. For comparison, NAMI-A was also tested against a representative solid tumour cell line (IGROV-1), previously reported to be poorly sensitive to this ruthenium compound[26]. Consistent with literature, an extremely high LD50 value (429.87±59.76 μM) was determined (Figure 1C and 1C'). At difference from what reported for cisplatin[27], the cytotoxic effects of NAMI-A in

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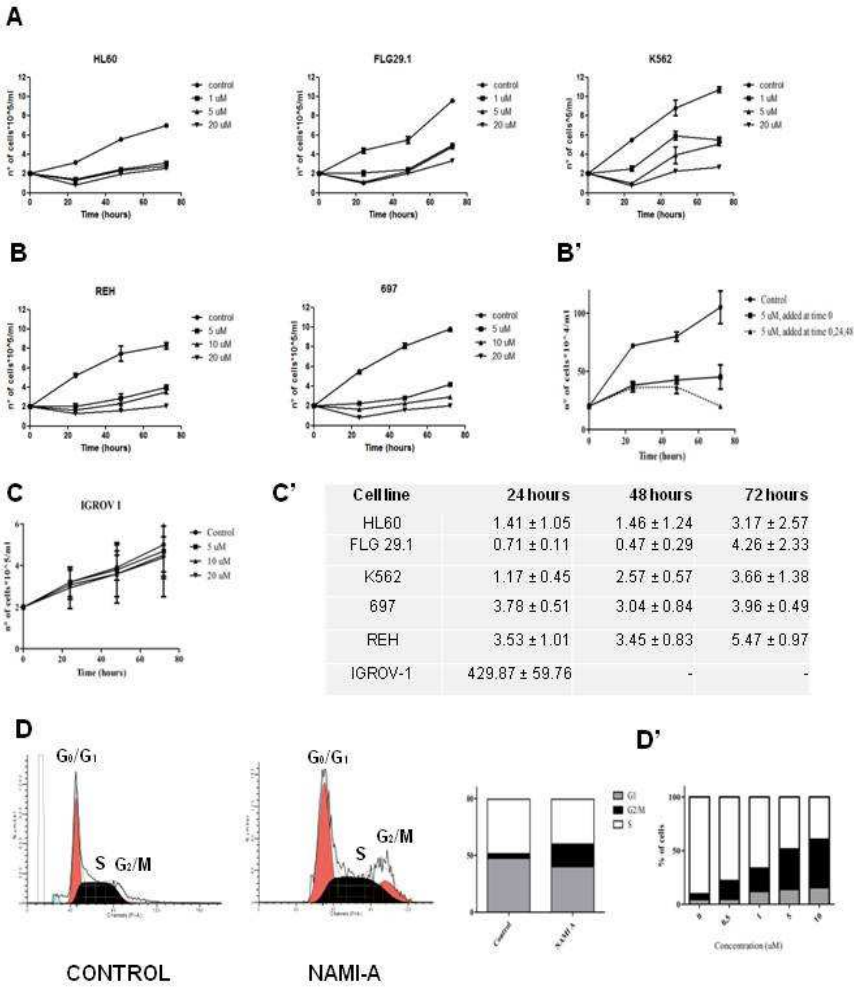
leukaemia cells occurred quite early after administration. This fact indicates that NAMI-A is a stronger cytotoxic drug in leukaemia cells compared to cisplatin, and suggests a different mechanism of action for the two drugs.

We also confirmed in K562 cells (Figure 1D) previous studies, which documented the induction of a G2/M arrest by ruthenium compounds[28-29]. A significant increase of the percentage of cells in the G2/M phase was observed after 24 hours of treatment, indicating that NAMI-A blocks cell cycle in leukaemia cells. Since the activation of specific apoptosis pathways as a consequence of a G2/M block[30-31] has been reported, we analysed the apoptotic profile of K562 cells treated for 48 hours with NAMI-A (0.5 to 10 μM). The percentages of early apoptotic (A+/PI-), late apoptotic (PI+/A+) and viable cells (PI-/A-) are reported in Figure 1D' and Supplementary Figure 1. On the whole, NAMI-A induced a significant cellular apoptosis at concentrations above 5 μM.



Cell line	24 hours	48 hours	72 hours
HL60	1.41 ± 1.05	1.46 ± 1.24	3.17 ± 2.57
FLG 29.1	0.71 ± 0.11	0.47 ± 0.29	4.26 ± 2.33
K562	1.17 ± 0.45	2.57 ± 0.57	3.66 ± 1.38
697	3.78 ± 0.51	3.04 ± 0.84	3.96 ± 0.49
REH	3.53 ± 1.01	3.45 ± 0.83	5.47 ± 0.97
IGROV-1	429.87 ± 59.76	-	-

Fig. 1 Effects of NAMI-A on cell proliferation, cell cycle distribution and apoptosis. (A) Effects of NAMI-A (range 0-20 μM) on myeloid leukaemia cells (HL60, FLG 29.1, K562 respectively) proliferation, after a single treatment at time 0, given as number of Trypan Blue negative cells. (B) Effects of NAMI-A on lymphoid leukaemia cells (REH, 697) proliferation, after a single treatment at time 0 given as number of Trypan Blue negative cells. (B') Threefold treatment with NAMI-A on K562 cells at time 0, 24 and after 48 hours given as number of Trypan Blue negative cells. (C) Effects of NAMI-A on ovarian cancer cells proliferation (IGROV-1 cell line), after a single treatment at time 0 given as number of Trypan Blue negative cells. (C') LD50 Values (μM) for NAMI-A in a panel of leukaemia cell lines and a solid tumour cell line, IGROV-1. The cell lines were exposed to increasing concentrations of NAMI-A in the range 0-20 μM. After 24, 48 and 72 hours, viable cells (determined by Trypan blue exclusion) were counted in triplicate using a haemocytometer. Each experimental point represents the mean of four samples carried out in three separate experiments. The LD50 value (i.e., the dose that caused the apoptosis of 50% of leukaemia cells) was calculated by fitting the data points with a sigmoidal curve using Origin 6 software (Microcal Software). (D) Panels on the left: Leukaemia cells (K562 cell line) were exposed to NAMI-A (10 μM) for 24 hours, a representative example is reported. Panel on the right: Histogram relative to the mean percentage of K562 cells in the cell cycle phases (G1=grey, G2/M= black, S=white). (D') Leukaemia cells (K562 cell line) were exposed to increasing concentrations of NAMI-A (range 0-10 μM) for 48 hours. The percentage of Annexin V+/Propidium Iodide- cells was measured. Values are means± SEM of two independent experiments. The histogram relative to the mean percentage of living cells with low Annexin and low Propidium Iodide staining (white bar), of early apoptotic cells with high Annexin V and low Propidium Iodide staining (black bar) and of late apoptotic cells with high Annexin V and high Propidium Iodide staining (grey bar).

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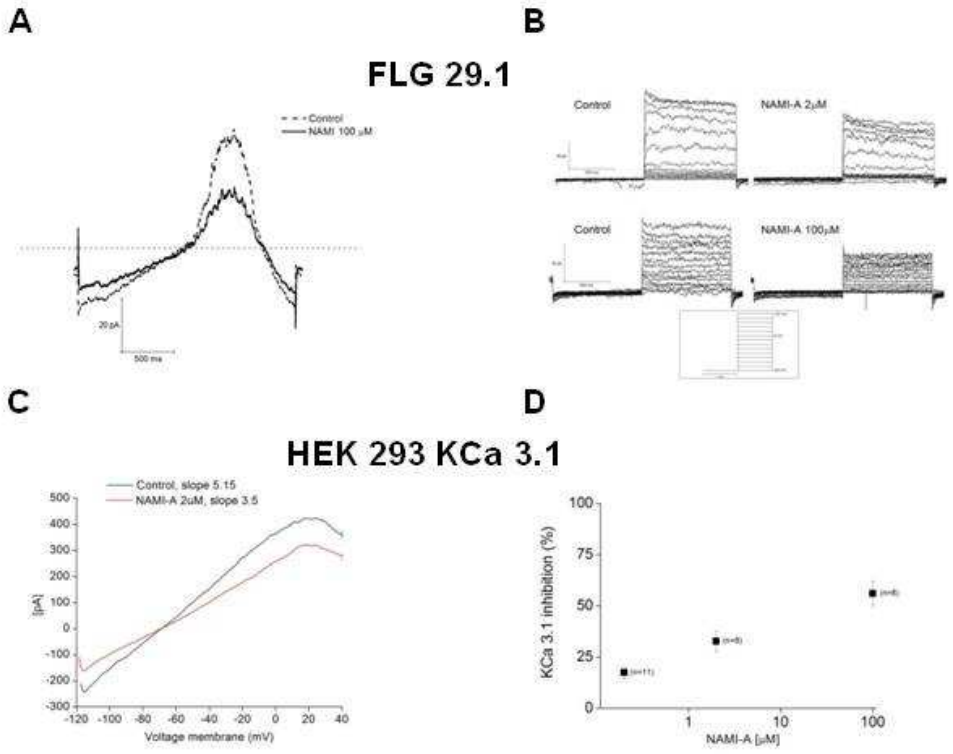
We then analysed the possible mechanism underlying the cytotoxic effect of NAMI-A in leukaemia cells. To assess the role of a differential metal uptake in leukaemia cells compared to solid tumours, we determined the intracellular ruthenium content. The amount of ruthenium taken up by K562 cells was higher than in IGROV-1 cells by only about 30% (Supplementary Table 1). This relatively small difference in ruthenium uptake cannot explain the differences observed in the induced antiproliferative effects. We also noticed that, consistent with previous reports[32], only a small percentage of ruthenium is able to enter cancer cells even after a long incubation time (the intracellular Ru concentration being less than 10% of ruthenium concentration in the medium)[29,33]. Hence, the greater cytotoxic effect of NAMI-A in K562 cells cannot be traced back to a greater ruthenium uptake nor to a direct concentration-dependent effect on DNA.



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	% of cell vitality	Combination Index (CI)
NAMI-A (LD_{50}=0.71 µM)+TRAM-34 (LD_{50}=6.02 µM)	52.0±3.7	>1
NAMI-A (LD_{50}=0.71 µM)+TRAM-34 (LD_{30}=1.10 µM)	54.3±1.8	>1
NAMI-A (LD_{30}=0.20 µM)+TRAM-34 (LD_{50}=6.02 µM)	47.5±1.6	>1
NAMI-A (LD_{30}=0.20 µM)+TRAM-34 (LD_{30}=1.10 µM)	48.2±2.7	>1

Figure 2

Fig. 2. NAMI-A affects plasma membrane ion channels. (A) Representative current obtained in FLG 29.1 cells with a voltage ramp protocol in the absence (dashed line) and in presence of 100 µM NAMI-A (continuous line). The holding potential of -70 mV rules out the contribution of hERG channels, which are fully deactivated at this Vm. (B) Outward currents elicited in FLG 29.1 cell line and effects of NAMI-A 2 µM (top) or 100 µM (bottom). The stimulation protocol is shown in the inset. (C) Currents elicited in HEK 293 cells transfected with KCa 3.1 in the presence or absence of NAMI-A 2 µM. The stimulation protocol is detailed in Materials and Methods section. (D) Inhibitory effect of NAMI-A (0.2, 2 and 100 µM) on KCa 3.1 currents. The fold decrease of slope conductance was taken as a measure of channel block. (E) Effect of NAMI-A and TRAM-34 alone and in combination on FLG 29.1 cells. Cells 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. CI values for NAMI-A (at the LD50 dose and at the LD30 dose) in combination with the specific inhibitor of KCa3.1 channels TRAM-34 (at both LD50 and LD30 dose) in FLG 29.1 cells. Original data are from Supplementary Figure 6. CI values were calculated using CalcuSyn software Version 2 (Biosoft). CI > 1, antagonisms; CI = 1, additivity; CI < 1, synergy.

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Seeking an alternative mechanism to explain the antiproliferative activity of NAMI-A in leukaemia cells, we tested whether this compound affects plasma membrane ion channels, as reported for other ruthenium-containing compounds[34-35]. To this end we carried out patch-clamp experiments in whole-cell mode on FLG 29.1 cells, perfused with an extracellular solution containing physiological ion concentrations (EK was -80 mV; ECl was -50 mV). We applied a voltage ramp protocol, in the absence or in the presence of saturating concentrations of NAMI-A (100 μM). NAMI-A blocked a whole-cell current with a reversal potential around -70mV (Figure 2A). The effect of the drug was very similar to that produced by 5mM TEA (Supplementary Figure 2), suggesting that NAMI-A mostly affects a K^+ current. The concentration-dependent effect of the drug was tested on outward currents, by applying voltage steps between -80 mV and +60 mV (the conditioning potential being -80 mV). Figure 2B shows that both 2 and 100 μM NAMI-A reduced the outward currents. In particular, 2 μM NAMI-A produced an approximately 36.5 ± 4.4% (n=9) block of the current inhibited by 100 μM NAMI-A, in broad agreement with results obtained on leukaemia cell proliferation.

Next, we sought to identify the K^+ channel type affected by NAMI-A. As detailed in the figure legend, no effect was observed on the hERG1 currents expressed by FLG 29.1 cells.[36] These cells also express mRNA encoding the Ca^{2+} dependent K^+ channel KCa 3.1 (Supplementary Figure 3). Hence, we tested whether NAMI-A might inhibit KCa 3.1 channels heterologously expressed in HEK 293 cells. In these cells, as expected, the specific KCa 3.1 inhibitor TRAM[34] produced a strong inhibition of the whole-cell currents, indicating that these are largely constituted by KCa 3.1 (Supplementary Figure 4). Moreover, 2 μM NAMI-A significantly reduced the KCa 3.1 currents (Fig. 2C). At the concentrations (1-2 μM) that reduce leukaemia cell proliferation, NAMI-A inhibited about 30% of the KCa 3.1 current (Figure 2D). Finally, there was no cooperativity on leukaemia cell vitality between NAMI-A and TRAM-34 (at the LD50 and LD30 values; for NAMI-A: 0.71 μM and 0.20 μM, for TRAM-34: 6.02 μM and 1.10 μM). The combination indexes (CI) regarding the different combinations of the drugs are reported in Figure 2E and Supplementary Figure 5. Altogether, we attribute most of the antiproliferative effect of NAMI-A in leukaemia cells to the inhibition of KCa 3.1 channels, encoded by the KCNN4 gene.

KCa 3.1 channels are overexpressed in a wide variety of cancer cells including leukaemias[37]. Recently, it has been reported that the block of KCa 3.1 in chronic lymphocytic leukaemia (CLL) reduced cell viability[38]. In line with our results, in leukaemia and lymphoma cells, KCa 3.1 currents mainly regulate cell proliferation, whereas they are pivotal regulators of cell migration in epithelial and glial cancer cells[37]. Such different functional roles of KCa 3.1 channels in different cell types appears to explain why NAMI A is strongly cytotoxic in leukaemia cells, whereas it produces relevant antimetastatic effects, with low cytotoxicity, in solid tumours.

Conclusions

In conclusion, we have demonstrated here that: (i) NAMI-A produces strong and unexpected cytotoxic effects in a panel of leukaemia cell lines; (ii) cell death occurs predominately through apoptosis; (iii) the time course of the observed cytotoxic effects is markedly different from the one observed with cisplatin[27], NAMI-A being much faster; iv) the relevant antiproliferative effects caused by NAMI–A in leukaemia cells most likely arise from the selective blockade of KCa 3.1 channels. .

Based on the above findings and on the reported high sensitivity of various leukaemia cell lines to this metallodrug, we suggest that NAMI-A is a promising candidate for further clinical studies on leukaemia treatment. Indeed, previous clinical trials showed that NAMI-A is safe even at relatively high doses[18] so that blood concentrations greater than 10 μM may be easily reached, which reinforces the above suggestion.

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Notes and references

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TOC

NAMI-A is Highly Cytotoxic Toward Leukaemia Cell Lines: Evidence of Inhibition of KCa3.1 Channels

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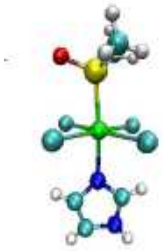
Table of Contents

The anticancer ruthenium(III) complex NAMI-A induces potent and unexpected cytotoxic effects in leukaemia cells causing selective inhibition of KCa3.1 channels.



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