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Anticancer Ruthenium(III) Complex KP1019 Interferes with ATP-Dependent Ca^{2+} Translocation by Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA)

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Sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), a P-type ATPase that sustains Ca^{2+} transport and plays a major role in intracellular Ca^{2+} homeostasis, represents a therapeutic target for cancer therapy. Here, we investigated whether ruthenium-based anticancer drugs, namely KP1019 (indazolium [*trans*-tetrachlorobis(1*H*-indazole)ruthenate(III)]), NAMI-A (imidazolium [*trans*-tetrachloro(1*H*-imidazole)(*S*-dimethylsulfoxide)ruthenate-(III)]) and RAPTA-C ([Ru(η^6-*p*-cymene)dichloro(1,3,5-triaza-7-phosphaadamantane)]), and cisplatin (*cis*-diammineplatinum(II) dichloride) might act as inhibitors of SERCA. Charge displacement by SERCA adsorbed on a solid-supported membrane was measured after ATP or Ca^{2+} concentration jumps. Our results show that KP1019, in contrast to the other metal compounds, is able to interfere with ATP-dependent translocation of Ca^{2+} ions. An IC_{50} value of 1 μM was determined for inhibition of calcium translocation by KP1019. Conversely, it appears that KP1019 does not significantly affect Ca^{2+} binding to the ATPase from the cytoplasmic side. Inhibition of SERCA at pharmacologically relevant concentrations may represent a crucial aspect in the overall pharmacological and toxicological profile of KP1019.

Sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is a member of the P-type ATPase family that includes enzymes differentiated for the transport of various cations, such as Na^+/K^+, H^+/K^+, and Ca^{2+}/H^+.[1,2] SERCA has a molecular weight of approximately 110 KDa. It is a membrane-bound protein sustaining Ca^{2+} transport and involved in cell Ca^{2+} signaling and homeostasis.[3] SERCA hydrolyzes one molecule of ATP to transport two Ca^{2+} ions against their electrochemical potential gradient from the cytoplasm to the lumen of sarcoplasmic reticulum (SR) in muscle cells. SERCA transport activity plays an essential role in lowering cytosolic calcium concentration as required for muscle relaxation. The catalytic function and molecular structure of SERCA has been overviewed extensively in several Reviews.[4–7] The SERCA transport cycle includes initial enzyme activation triggered by Ca^{2+} binding, followed by ATP utilization to form a phosphoenzyme intermediate. The free energy derived from ATP is then utilized by the phosphoenzyme for a conformational transition that favors displacement and release of bound Ca^{2+} against its concentration gradient. Ca^{2+} ions are delivered to the intravesicular lumen in exchange for lumenal protons, which are translocated across the membrane to the cytosolic side during subsequent enzyme dephosphorylation. Hydrolytic cleavage of the phosphoenzyme is the final step, which allows the enzyme to undergo a new transport cycle.

A number of studies revealed that various heavy metal ions, for example, Cd^{2+}, Hg^{2+}, Pb^{2+}, Cu^{2+} and Zn^{2+}, can affect SERCA activity in different types of membranes, thus acting as potent SERCA inhibitors.[8] Such inhibition typically results in a dramatic elevation of cytosolic calcium concentration, endoplasmic reticulum stress, and eventual cell death through apoptosis. In spite of the numerous studies carried out so far on SERCA, only a few reports are available in the literature concerning possible inhibition of SERCA by anticancer metal-based drugs. A study by Muscella et al. showed that the Pt^{II} complex [Pt(O,O′-acac)(γ-acac)(DMS)], where acac is acetylacetonate and DMS is dimethyl sulfide, decreases the activity of plasma membrane Ca^{2+} ATPase (but not SERCA activity) and Ca^{2+} membrane permeability in MCF-7 breast cancer cells, thereby increasing intracellular Ca^{2+} concentration and triggering rapid apoptosis.[9]

As it is known that anticancer metallodrugs typically behave as prodrugs and are able to release charged metallic fragments—or even free metal ions—we were prompted to establish whether these metal species might cause appreciable SERCA inhibition. Reasonably, SERCA inhibition may constitute one of the mechanisms through which anticancer metal-based drugs produce their antiproliferative effects. These arguments led us to investigate whether three established anticancer ruthenium compounds, namely NAMI-A, KP1019 and RAPTA-C, and the reference anticancer drug cisplatin, might cause appreciable SERCA inhibition in vitro.

NAMI-A and KP1019 are two innovative ruthenium(III) compounds that are currently undergoing clinical evaluation; in preclinical experiments, NAMI-A showed a high selectivity against the formation of metastases,[10,11] and KP1019 was found to be active against colorectal tumors[12] and has successfully completed phase I clinical trials.[13,14] RAPTA-C is a ruthenium arene complex currently under advanced preclinical investigation.[15] Several studies have been performed to investigate the mode of action of these ruthenium-containing agents and their structure–activity relationships. However,

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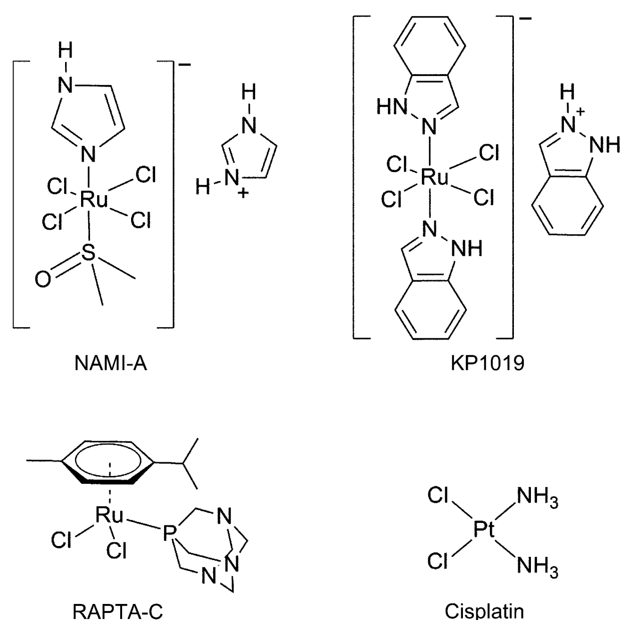

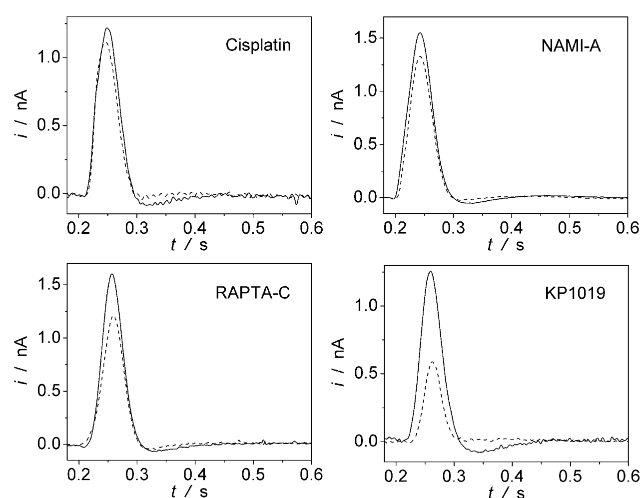

Figure 1. Current transients induced by 100 μM ATP concentration jumps in the presence of Ca^{2+} ions were recorded in the absence (——) and in the presence (-----) of 10 μM metallodrug.

many aspects of the tumor-inhibiting action displayed by these complexes are still largely unknown.

We evaluated the effects of these metal-based drugs on the transport cycle of SERCA through a novel electrical method, which makes use of a solid-supported membrane (SSM). Charge transfer in P-type ATPases has previously been investigated by using the SSM technique to obtain insight into the ion transport mechanism.[16–19] The technique allows direct measurement of charge displacements yielding information about movements of charged substrates within the transport protein.[20,21] The SSM technique is also well-suited for the analysis of drug interactions with membrane transporters. In this respect, the effects of various inhibitors showing a variable affinity for SERCA were investigated using the SSM technique.[22–24] It is worth mentioning that the SSM sensor combined with robotized instrumentation is expected to become an attractive platform technology for drug screening and development.[25,26]

To gain information on the interaction of these antitumor compounds with SERCA and its possible inhibition, we performed current measurements on native SR vesicles containing SERCA adsorbed on an SSM. After vesicle adsorption on the SSM, SERCA was incubated in a buffer solution containing the metallodrug (1 or 10 μM for 5 min). A 100 μM ATP concentration jump was then carried out in the presence of each metallodrug, and the corresponding ATP-induced current transient was compared to that measured in the absence of the antitumor compound, taken as a control measurement (Figure 1). It is worth mentioning that the charge obtained by numerical integration of the ATP-induced current transient is attributed to an electrogenic event corresponding to translocation of bound Ca^{2+} through the ATPase after utilization of ATP.[22] The resulting current transients are comparatively shown in Figure 1, and the corresponding charges, obtained by numerical integration, are reported in Table 1.

Table 1. Normalized charges (Q_n) following 100 μM ATP concentration jumps in the presence of the metallodrug at 1 or 10 μM.

Drug	Q_n [%][a] 1 μM	10 μM
Cisplatin	98 ± 2	93 ± 2
NAMI-A	100 ± 5	94 ± 6
RAPTA-C	97 ± 2	80 ± 2
KP1019	74 ± 3	50 ± 5

[a] Charges are normalized with respect to the maximum charge measured in the absence of the metallodrug; data represent the mean ± SE of three independent measurements.

Remarkably, we noticed that KP1019 strongly decreases the ATP-induced peak current (Figure 1) and the related displaced charge (Table 1) both at 1 and 10 μM concentration. We also verified whether the organic cation indazolium (the counter ion in KP1019) has an effect on the ATP-induced current transient. We noticed no significant effect on the current transient and related charge following an ATP concentration jump in the presence of 10 μM indazolium chloride (data not shown), thereby confirming that the observed decrease in peak current can be specifically ascribed to the interaction of KP1019 with SERCA. Therefore, we can conclude that KP1019 exerts an inhibitory effect on SERCA by interfering with translocation of bound Ca^{2+} upon ATP utilization. A far weaker decrease in the peak current and related charge was found with 10 μM NAMI-A, RAPTA-C, or cisplatin (Figure 1 and Table 1), and virtually no effect was observed at 1 μM concentration (Table 1).

The interaction of KP1019 with SERCA was therefore characterized in greater detail. To evaluate the concentration dependence of the KP1019 effect, we performed ATP concentration jumps in the presence of KP1019 at increasing concentration. In these experiments, the incubation time was extended to 15 min. Indeed, we noticed a more potent effect of KP1019 on

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SERCA when the enzyme was incubated with the ruthenium compound for 15 min. In particular, the charge measured in the presence of 10 μM KP1019 was decreased to approximately 38% with respect to the value recorded in the absence of the drug (as compared with 50% after 5 min incubation). This behavior may be ascribed to the "prodrug" nature of KP1019 and to the slow kinetics of its ligand exchange reactions.[27]

Figure 2 shows the curve obtained by plotting the measured charge as a function of KP1019 concentration. The charge was normalized with reference to the maximum charge attained in the absence of the metallodrug. From this curve, an IC_{50} value

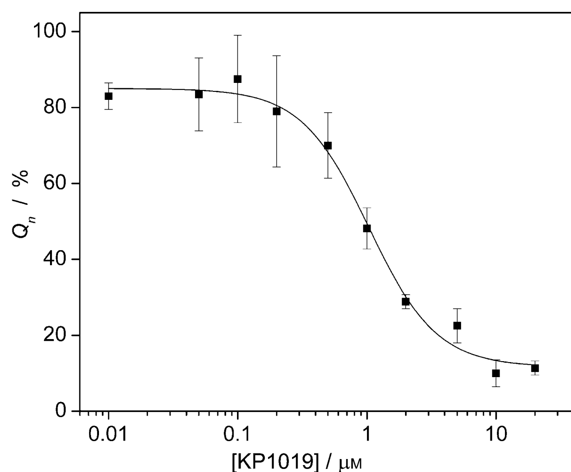

Figure 2. Normalized charges (Q_n) related to ATP concentration jumps in the presence of Ca^{2+} ions as a function of KP1019 concentration. The charges are normalized with reference to the maximum charge attained in the absence of the metallodrug. The solid line represents the fitting curve to the ATP-induced charges ($IC_{50}=1.0\pm0.1$ μM). The error bars represent the standard error (SE) of three independent measurements.

of 1.0 ± 0.1 μM could be determined. It is interesting to compare the IC_{50} value determined for KP1019 with the IC_{50} values of various compounds demonstrating different degrees of potency and specificity towards SERCA.[28,29] These compounds include the highly specific and potent inhibitor thapsigargin (TG; IC_{50} ~ 0.1 nM[30,31]), as well as cyclopiazonic acid (IC_{50} ~ 10–20 nM)[32] and 2,5-di-(*tert*-butyl)-dihydroxybenzene (IC_{50} ~ 1 μM),[33] which have been used for protein crystallization and structural studies.[34] Clotrimazole (IC_{50} ~ 35 μM),[23] curcumin (IC_{50} ~ 15 μM),[35] and 1,3-dibromo-2,4,6-tri(methylisothiouronium)benzene (IC_{50} ~ 15 μM)[36] are less specific inhibitors with medium affinity. As a point of pharmacological interest, TG derivatives have been considered for treatment of prostate cancer[37,38] and cyclopiazonic acid for myocardial ischemia.[39]

We also examined whether KP1019 can interfere with cytoplasmic Ca^{2+} binding to SERCA. To this aim, Ca^{2+} concentration jumps in the absence of ATP and in the presence of KP1019 were carried out. The charge obtained by numerical integration of the Ca^{2+}-induced current transient is attributed to an electrogenic event related to binding of Ca^{2+} ions to SERCA from the cytoplasmic side in the absence of ATP.[22] Figure 3 shows the normalized charges following 10 μM free Ca^{2+} con-

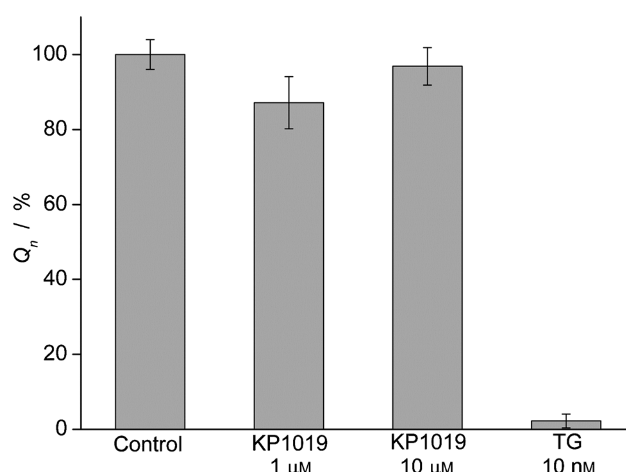

Figure 3. Normalized charges (Q_n) following 10 μM free Ca^{2+} concentration jumps in the absence of ATP and in the absence or presence of KP1019 (1 or 10 μM), or in the presence of 10 nM thapsigargin (TG). The charges are normalized with respect to the maximum charge measured in the absence of the metallodrug (control). The error bars represent the standard error (SE) of three independent measurements.

centration jumps in the absence or presence of KP1019 (1 or 10 μM), or in the presence of 10 nM TG for comparison. Here again, the charges were normalized with respect to the charge measured in the absence of the metallodrug (control). As shown in Figure 3, the displaced charge is not significantly affected by KP1019, thereby indicating that KP1019 does not interfere with Ca^{2+} binding to the ATPase transport sites. It is worth noting that KP1019 behaves differently from TG, which prevents cytoplasmic Ca^{2+} binding to SERCA, as previously reported.[22]

Finally, we investigated the effect of KP1019 on the ATPase hydrolytic activity by measuring steady state inorganic phosphate (P_i) production by SERCA, with ATP as a substrate at 37 °C. As in the current measurements described above, the protein was incubated with KP1019 for 15 min prior to the addition of ATP. The ATPase activity was found to be 2.9 ± 0.1 μmol P_i/(h mg protein) in the absence of KP1019 and 2.3 ± 0.1 μmol P_i/(h mg protein) in the presence of 10 μM KP1019, corresponding to a decrease in SERCA activity of approximately 20% in the presence of KP1019. Therefore, we can conclude that KP1019 has a moderate inhibitory effect on the ATPase hydrolytic activity.

In conclusion, we have explored here whether some anticancer metallodrugs might cause significant inhibition of SERCA and thus alter intracellular calcium homeostasis. While NAMI-A, RAPTA-C and cisplatin were found to be scarcely effective in influencing SERCA response, a remarkable inhibitory effect was highlighted in the case of KP1019. In fact, we observed that KP1019 is able to interfere with ATP-dependent translocation of Ca^{2+} ions by the ATPase, with an IC_{50} value of 1 μM. On the other hand, we discovered that KP1019 does not interfere with Ca^{2+} binding to the enzyme from the cytoplasmic side and has a moderate inhibitory effect on ATPase hydrolytic activity. These observations suggest that an active ruthenium species may bind SERCA in a site that is distinct

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from the two high-affinity calcium binding sites, which are located in the transmembrane region.[6,7] We propose that a ruthenium-containing species coordinates to exposed amino acid residues (for example, histidine) of SERCA. We may speculate that such an interaction produces uncoupling of ATP hydrolysis with transport of Ca^{2+} ions, thus resulting in decreased calcium translocation across the SR membrane.

KP1019 was formerly reported to be cytotoxic in various cancer cell lines, with IC_{50} values in the order of 10–30 μM.[40] On the ground of the present findings, it emerges that KP1019 at pharmacologically relevant concentrations is capable of interfering strongly with SERCA function. Alterations of intracellular calcium metabolism consequent to SERCA inhibition might represent one of the mechanisms through which KP1019 produces its biological effects and triggers apoptotic cancer cell death.

Experimental Section

Compound synthesis and preparation: Imidazolium [*trans*-tetrachloro(1*H*-imidazole)(*S*-dimethylsulfoxide)ruthenate(III)] (NAMI-A),[41] [Ru(η^6-*p*-cymene)dichloro(1,3,5-triaza-7-phosphaadamantane)] (RAPTA-C)[42] and indazolium [*trans*-tetrachlorobis(1*H*-indazole)-ruthenate(III)] (KP1019)[13] were synthesized as described in the literature. Characterization data were in agreement with previously reported values and the %purity of all compounds, used in our biological assays, was significantly greater than 95% as determined by CHN analysis. Cisplatin (*cis*-diammineplatinum(II) dichloride) was purchased from Sigma–Aldrich. Stock solutions of test compounds in the millimolar concentration range were prepared in ultrapure water (obtained by purifying deionized water, 18.2 MΩcm at 25 °C).

ATPase preparation and biochemical measurements: Sarcoplasmic reticulum (SR) vesicles were obtained by isolation from the fast twitch hind leg muscle of New Zealand white rabbit, as previously described.[43] New Zealand white rabbits were purchased from Harlan (Italy). Animal manipulations were carried out according to the Italian Guidelines for Animal Care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and approved by the local Advisory Committee for Ethical and Juridical Control of the Center for Housing of Laboratory Animals of the University of Florence (Italy). All efforts were made to minimize animal sufferings. Protein concentration was determined by the Lowry method using bovine serum albumin as a standard.[44] The total protein content of SR vesicles was 8.4 mg mL^{-1}. SERCA (isoform 1a) accounts for approximately 50% of the microsomal protein.[45]

SERCA hydrolytic activity was determined following inorganic phosphate (P_i) production by a colorimetric method.[46] The reaction mixture contained 20 mM 4-morpholinepropanesulfonic acid (MOPS) (pH 7.0), 80 mM KCl, 3 mM $MgCl_2$, 0.2 mM ethylene glycol tetraacetic acid (EGTA), 0.2 mM $CaCl_2$, 7.5 μg of SERCA/mL, 2 μM A23187 ionophore, and 5 mM NaN_3. The reaction was started by addition of 1 mM ATP. The incubation temperature was 37 °C. Measurements of ATPase activity were carried out in the absence and presence of 10 μM KP1019.

Electrical measurements: Current transients were measured following adsorption of native SR vesicles incorporating SERCA on a solid-supported membrane (SSM). The SSM consists of an alkanethiol monolayer covalently bound to a gold electrode via the sulfur atom, and a phospholipid monolayer on top of it.[16,17] After adsorption, SERCA was activated by a concentration jump of a suitable substrate, that is, ATP or Ca^{2+}. If the substrate concentration jump induces charge displacement across the vesicular membrane, a current transient can be recorded due to the capacitive coupling between vesicle membrane and SSM.[17]

In ATP concentration jump experiments, the non-activating solution contained 150 mM choline chloride, 25 mM MOPS (pH 7.0), 0.25 mM EGTA, 1 mM $MgCl_2$, 0.25 mM $CaCl_2$ (10 μM free Ca^{2+}) and 1 mM dithiothreitol (DTT); the activating solution contained, in addition, 100 μM ATP. In Ca^{2+} concentration jump experiments, the non-activating solution contained 150 mM choline chloride, 25 mM MOPS (pH 7.0), 0.25 mM EGTA, 1 mM $MgCl_2$ and 1 mM DTT; the activating solution contained, in addition, 0.25 mM $CaCl_2$ (10 μM free Ca^{2+}). Free Ca^{2+} concentration was calculated with the computer program WinMAXC Ca-Mg-ATP-EGTA Calculator version 1.0 (http://maxchelator.stanford.edu/).[47] Unless otherwise stated, 1 μM of the calcium ionophore A23187 was used to prevent formation of a Ca^{2+} concentration gradient across the SR vesicles.

The concentration jump experiments were performed by employing the SURFE^{2}R^{One} device (Nanion Technologies, Munich, Germany). The temperature was maintained at 22–23 °C for all the experiments. To verify the reproducibility of the current transients on the same SSM, each measurement was repeated six times and then averaged to improve the signal-to-noise ratio. Standard deviations did not exceed 5%. Each set of measurements was usually reproduced using 2–3 different SSM electrodes.

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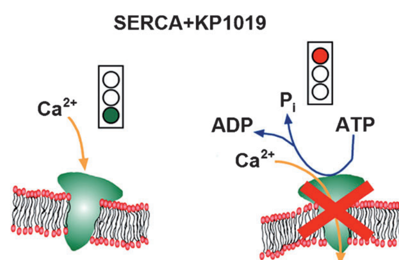
Anticancer Ruthenium(III) Complex KP1019 Interferes with ATP-Dependent Ca^{2+} Translocation by Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA)



Heavy metal! KP1019 (*trans*-[$RuCl_4$(1*H*-indazole)$_2$]) along with other ruthenium(III)-based anticancer agents was evaluated for its ability to inhibit sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), a P-type ATPase that sustains Ca^{2+} transport and plays a major role in intracellular Ca^{2+} homeostasis. KP1019 was found to interfere with ATP-dependent Ca^{2+} translocation by SERCA ($IC_{50}=1$ μм), but was shown not to affect cytoplasmic Ca^{2+} binding to the ATPase.

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