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The water soluble ruthenium(II) organometallic compound [Ru(*p*-cymene)(bis(3,5 dimethylpyrazol-1-yl)methane)Cl]Cl suppresses triple negative breast cancer growth by inhibiting tumor infiltration of regulatory T cells

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ABSTRACT

Ruthenium compounds have become promising alternatives to platinum drugs by displaying specific activities against different cancers and favorable toxicity and clearance properties. Here, we show that the ruthenium(II) complex [Ru(*p*-cymene)(bis(3,5-dimethylpyrazol-1-yl)methane)Cl]Cl (UNICAM-1) exhibits potent *in vivo* antitumor effects. When administered as four-dose course, by repeating a single dose (52.4 mg kg-1) every three days, UNICAM-1 significantly reduces the growth of A17 triple negative breast cancer cells transplanted into FVB syngeneic mice. Pharmacokinetic studies indicate that UNICAM-1 is rapidly eliminated from kidney, liver and bloodstream thanks to its high hydrosolubility, exerting excellent therapeutic activity with minimal side effects. Immunohistological analysis revealed that the efficacy of UNICAM-1, mainly relies on its capacity to reverse tumor-associated immune suppression by significantly reducing the number of tumor-infiltrating regulatory T cells. Therefore, UNICAM-1 appears very promising for the treatment of TNBC.

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1. Introduction

Breast cancer is a heterogeneous disease classified into molecular subtypes with distinctive gene expression signatures. Of all the molecular subtypes, triple negative breast cancer (TNBC) has the worst negative outcome and prognosis [1,2]. TNBCs occur most frequently in young women and tend to exhibit aggressive metastatic behavior [3]. TNBCs are estrogen receptor (ER) and progesterone receptor (PR)-negative and also lack high expression/amplification of HER2, limiting targeted therapeutic options [4]. New therapies against this breast cancer subtype are therefore an urgent unmet medical need. Cisplatin (*cis*-[PtIICl2(NH3)2]) is well established as an effective drug for the treatment of testicular cancer and, in combination with other chemotherapeutic agents, for ovarian, cervical, brain, bladder, lung, and breast cancers [5,6]. Recently, preclinical and clinical data have revealed encouraging anticancer activity of cisplatin as single-agent in patients with TNBC [7,8]. Despite the success of platinum-based drugs, their continued use is greatly limited by severe dose limiting side effects and intrinsic or acquired drug resistance [9–11]. In the search for anticancer agents containing nobel metals other than platinum, ruthenium compounds have turned out to be a cutting-edge class of anticancer compounds [12–18]. Accordingly, two ruthenium(III)-based compounds, namely

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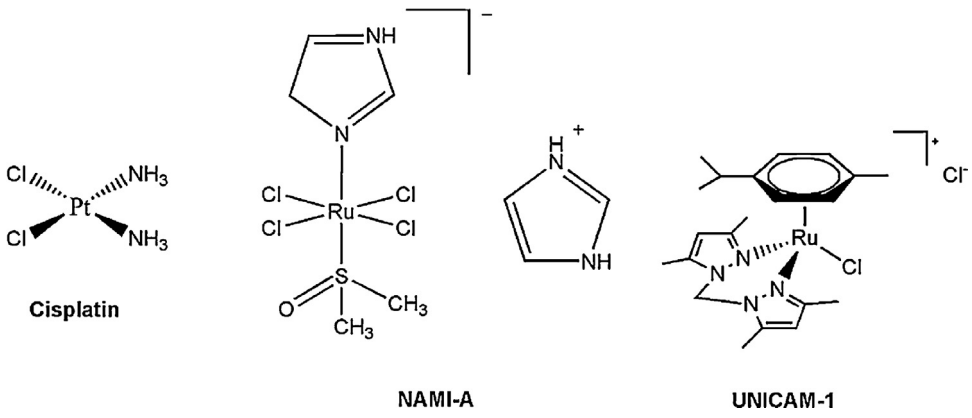

Fig. 1. Chemical structures of cisplatin, NAMI-A and UNICAM-1.

NAMI-A (imidazolium *trans*-[tetrachloro(dimethylsulfoxide)(1H-imidazole)ruthenate(III)]) [19] and NKP1339 sodium *trans*-[tetrachloridobis(1Hindazole)ruthenate(III)] [20], are currently ongoing in phase II clinical trials. The different toxicity profiles between platinum- and ruthenium-based compounds could probably due to different targets. It is widely accepted that the antineoplastic properties of platinum compounds rely on their interaction with DNA, which, in turn, activates cell death [21]. Instead, the mechanisms by which ruthenium-based drugs exert their anticancer effects remain to be fully elucidated but recent evidence suggests that ruthenium compounds are most likely to be multitargeted [22,23]. Thus, they could represent a valid therapeutic alternative to platinum-based drugs which are often associated with an unfavorable toxicity profile. Among the ruthenium(II) organometallic complexes, the half sandwich arene–ruthenium subgroup, in particular, offers a great promise in the field of cancer therapy. Two prototypical compounds reported by Aird et al. [24] and RAPTA-C developed by Scolaro et al. [25] have shown relevant therapeutic potential. We have recently reported an extensive study on the coordination chemistry of ruthenium arene fragments with bis(pyrazol-1-yl)methan ligands [26]. In this work we extend our investigation to the *in vivo* antitumor activity of the prototype compound, [Ru(*p*-cymene)(bis(3,5-dimethylpyrazol-1-yl)methane)Cl]Cl, termed UNICAM-1 (Fig. 1). The results presented here reveal that UNICAM-1 has significant anticancer activity in a murine model of TNBC and results to be well tolerated, showing considerably reduced side-effects when compared to cisplatin and NAMI-A. The analysis of tumor immune infiltrate suggests that the response to this new chemotherapeutic agent relies mainly on its capacity to elicit an anticancer immunosurveillance.

2. Material and methods

2.1. Compounds

NAMI-A was prepared according to a patented procedure [27]. Cisplatin was obtained by Sigma Chemical Co. (St. Louis, MO). UNICAM-1 was prepared as previously described [26] and its analytical and spectroscopic data have been reported in the Supplementary materials.

2.2. Cell cultures, cell proliferation assay and lysates preparation

A17 cells were established from spontaneous lobular carcinomas that arose in a FVB/neuT mice transgenic for the activated isoform of rat HER-2/neu oncogene (FVB/neuT233), as previously described [28,29]. A17 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA). Human breast cancer MDA-MB 231 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C. UNICAM-1 effect on cell viability, respect to cisplatin and NAMI-A, was evaluated by seeding 2.5×10^3 cells/well (A17 cells) or 7×10^3 cells/well (MDA-MB-231 cells) in octuplicate in 96 well plates in complete medium. The day after, fresh medium containing appropriate concentrations of UNICAM-1, NAMI-A and cisplatin (all dissolved in isotonic solution, 0.9% $NaCl_{(aq)}$) were added. After 72 h cell viability was determined using an MTT (Sigma Aldrich, St. Louis, MO) assay, as previously described [30]. The cytotoxicity of the compounds was reported as percentage of viable cells relative to control cells. All the experiments were repeated three times. For cell lysates preparation, 4×10^5 cells/well (A17 cells) or 8×10^5 cells/well (MDA-MB-231 cells) were seeded in 6-well plates, treated with UNICAM-1, NAMI-A and cisplatin drugs for 48 h, harvested, and lysed in RIPA buffer (1% NP40, 0.5% Na-deoxycolic acid and 0.1% SDS in PBS) with fresh protease inhibitors (Protease Inhibitor Cocktail, Sigma Aldrich, St. Louis, MO).

2.3. Animals

Female FVB/NCrl mice were obtained from Charles River S.r.l. (Lecco, Italy), and housed under controlled conditions. Mice were treated according to the European Community guidelines. The Animal Research Committee of the University of Camerino authorized the experimental protocol.

2.4. Treatments and tumor growth

10^5 A17 cells were inoculated in 200 μl of PBS into mammary fat pad of 8-week-old FVB females. 10 days after tumor challenge, mice were randomly divided in 4 groups (10 mice per group) and treated with UNICAM-1, NAMI-A, cisplatin or isotonic solution (vehicle) by intraperitoneal injection (*via* i.p) in accordance with two different dosage and treatment schedules (protocol q3 × 4 or q1 × 6) as previously reported [31]. According to protocol q1 × 6, 35 mg/kg/day of ruthenium complexes (210 mg/kg/day final amount) and 2 mg/kg/day of cisplatin (12 mg/kg/day final amount) were administered for six consecutive days. According to protocol q3 × 4, 52.5 mg/kg/day of ruthenium complexes (210 mg/kg/day final amount), and 3 mg/kg/day of cisplatin (12 mg/kg/day final amount) were administered four times, once every three days. Body weight of mice receiving UNICAM-1, NAMI-A and cisplatin was checked once a week and compared with untreated controls. Tumor

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