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Resiniferatoxin induces death of bladder cancer cells associated with mitochondrial dysfunction and reduces tumor growth in a xenograft mouse model

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ABSTRACT

Bladder cancer (BC) is the fifth most common non-cutaneous malignancy and the most common form of BC in Western countries is transitional cell carcinoma. Resiniferatoxin (RTX) has found therapeutic usefulness for the treatment of bladder dysfunction but no data are available on its use as chemotherapeutic agent. The aim of this work is to evaluate the use of RTX as new anti-cancer drug in BC therapy. The effects of RTX on cell viability and cell death were evaluated on T24 and 5637 BC cell lines by MTT assay, cell cycle analysis, Annexin-V/PI staining and agarose gel electrophoresis of DNA. Mitochondrial depolarization and ROS production were assessed by flow cytometry. ADP/ATP ratio was measured by bioluminescence and caspase 3 cleavage by Western blot. For *in vivo* experiments, athymic nude mice, xenografted with T24 cells, received subcutaneous administrations of RTX. Tumor volumes were measured and immunohistochemistry was performed on tumor sections. Our data demonstrated that RTX influences cell cycle and induces necrotic cell death of BC cells by altering mitochondrial function, leading to depolarization, increase in ADP/ATP ratio and ROS production. Moreover, RTX is able to reduce tumor growth in a xenograft mouse model. Overall, we demonstrated that RTX induces necrotic cell death of BC cells and reduces tumor growth in a xenograft mouse model of BC, suggesting RTX as a new potential anti-cancer drug in BC chemotherapy.

1. Introduction

Bladder cancer (BC) is among the five most common malignancies worldwide. There are over 70,000 new cases of BC each year in the United States alone [1] and the most common form of BC in Western countries is transitional cell carcinoma (TCC) [2]. Approximately 30–40% of patients with high-risk non-muscle-invasive TCC of the bladder will progress to a more advanced disease within 5 years and up to 34% of them will ultimately die of bladder cancer [3]. Overall, only 20–40% of patients with advanced TCC have a 5-year survival rate, despite aggressive multimodal therapy and radical cystectomy remain the mainstay of treatment of muscle-invasive disease [4,5]. To ameliorate patient life expectancy, improvement of current chemotherapeutic regimens and development of novel chemotherapeutic strategies are necessary. Recently, different therapeutic approaches based on targeting tumor mitochondria have been proposed [6], with the expectation that this novel class of agents could reduce tumor cells viability with an acceptable therapeutic index [7].

Resiniferatoxin (RTX) is a diterpene found in the latex of the cactus *Euphorbia resinifera* containing a homovanillic acid ester, a

Abbreviations: Ab, antibody; BC, bladder cancer; CCCP, carbonyl cyanide chlorophenylhydrazone protonophore; DMSO, dimethyl sulfoxide; ΔΨm, mitochondrial transmembrane potential; H&E, hematoxylin/eosin; I-RTX, 5′-iodoresiniferatoxin; PI, propidium iodide; ROS, reactive oxygen species; RTX, resiniferatoxin; TCC, transitional cell carcinoma; TRP, transient receptor potential channels; TRPV, TRP vanilloid; TUR, transurethral resection.

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key structural motif of capsaicin, displaying analgesic activity and functioning as an ultrapotent capsaicin analog. RTX has found therapeutic usefulness in the urologic field in the treatment of bladder dysfunctions and painful bladder [8,9]. Very few data are available on its use as chemotherapeutic agent. The anticancer activity of vanilloids such as capsaicin and dihydrocapsaicin can be mediated through both a direct pathway, independent of transient receptor potential vanilloid receptor 1 (TRPV1), the receptor for vanilloids, and an indirect pathway, through the interaction with TRPV1 and the subsequent intracellular calcium overload [10–16]. In regard to RTX, there are indications that mitochondria could be involved in the TRPV1-independent vanilloids-induced cell death. In pancreatic cancer tissue [13], squamous cell carcinoma and non-small lung cancer cell lines [14,17] RTX causes non-vanilloid receptor mediated cell death.

This work is aimed to evaluate the potential use of RTX as new therapeutic strategy against BC through *in vitro* and *in vivo* preclinical experiments.

2. Materials and methods

2.1. Cell lines

The p53 mutant T24 TCC and 5637 grade II BC cell lines, purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), were maintained in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum, 2.5 mM HEPES, 2 mM L-glutamine, 100 IU/ml of penicillin, 100 g/ml of streptomycin (Lonza) at 37 °C, 5% CO_2 and 95% humidity. Normal human urothelial cells (NHUC) were purchased from ScienceCell Research laboratories (Carlsbad, CA, USA) and cultured in Urothelial Cell Medium supplemented with 5 ml (100x) of urothelial cell growth supplement (UCGS, ScienceCell Research laboratories) 100 IU/ml of penicillin, 100 μg/ml of streptomycin (Science Cell Research laboratories) at 37 °C, 5% CO_2 and 95% humidity.

2.2. MTT assay

T24 and 5637 BC cells (6×10^3/well) were seeded into 96-well plates and cultured with different doses of RTX (Tocris Bioscience, Bristol, UK, 0.1–50 μM) alone or in combination with the TRPV1 antagonist 5′-iodoresiniferatoxin (I-RTX) (50 nM, Tocris Bioscience), dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, USA) or respective vehicles for 24 h. At the end of treatment, samples were processed as described previously [12]. Four replicates were used for each treatment and data were represented as the average of at least three separate experiments. In some experiments MTT assay was performed using NHUCs treated with RTX (50 μM) for 24 h. IC50 was mathematically determined using Graph Pad Prism 5 software.

2.3. Cell-cycle analysis and Annexin-V staining

Cells (3×10^5/well) were plated in six-well culture dishes and treated for 12–24 h with 20 μM RTX or vehicle. Cells were fixed by adding ice-cold 70% ethanol and then washed with staining buffer (PBS, 2% FBS and 0.1% NaN_3). Next, the cells were treated with 100 μg/ml ribonuclease A solution (Sigma Aldrich), incubated for 30 min at 37 °C, stained for 30 min at room temperature with Propidium Iodide (PI) 20 μg/ml (Sigma Aldrich) and finally analyzed by flow cytometry and the Cyflogic software (CyFlo Ltd, Finland). Phosphatidylserine exposure on BC cells, treated with RTX (20 μM) for 12 h was detected by Annexin-V-FITC (Enzo Life Sciences, Farmingdale, USA) and analyzed by the FACScan Flow Cytometer with the CellQuest software (Becton Dickinson, San Jose, USA).

2.4. Western blot

T24 and 5637 cells, untreated or treated with 20 μM RTX or vehicle for 12 h, were lysed and protein samples were subjected to Sodium Dodecyl Sulfate–PolyAcrylamide Gel Electrophoresis (14%) and transferred onto Hybond-C extra membranes (GE Healthcare, Uppsala, Sweden) as described [12]. Membranes were blotted with rabbit polyclonal anti-caspase 3 Ab (1:1000, Cell Signaling Technology, Denver, USA) according to the manufacturer's instructions followed by HRP-conjugated donkey anti-rabbit Ab (1:2000, GE Healthcare) and with mouse monoclonal anti-GAPDH Ab (1:5000, Sigma Aldrich). GAPDH protein levels were used as loading control. Immunostaining was revealed by enhanced ECL Western Blotting analysis system (GE Healthcare). Densitometric analysis was performed by ChemiDoc using the Quantity One software (Bio-Rad).

2.5. DNA fragmentation assay

Cells (1.5×10^6) were treated with RTX (20 μM) or vehicle for 24 h and genomic DNA was extracted using DNA extraction kit (Qiagen, Milan, Italy). DNA fragmentation, used as a criterion to distinguish necrosis from apoptosis, was assessed by electrophoresis on 1.7% agarose gel and ethidium bromide staining. Ultraviolet spectroscopy at 302 nm was used to report results.

2.6. Mitochondrial transmembrane potential ($\Delta\Psi_m$)

Mitochondrial transmembrane potential ($\Delta\Psi_m$) was evaluated by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraehylbenzimidazolylcarbocyanineiodide (JC-1) staining. Cells (4×10^4/well) were seeded into 24-well plates and treated with 20 μM RTX or vehicle for different times and then incubated with 10 μg/ml of JC-1 [12]. Carbonyl cyanide chlorophenylhydrazone protonophore (CCCP, 50 μM, Sigma Aldrich), a mitochondrial uncoupler that collapses $\Delta\Psi_m$, was used as positive control. Samples were analyzed using the FACScan cytofluorimeter with the CellQuest software.

2.7. Measurement of ADP/ATP ratio

ADP/ATP ratio was measured in BC cells treated with vehicle or RTX (20 μM) by the EnzyLight ADP/ATP Ratio Assay Kit (BioAssay Systems, Hayward, CA, USA) following the manufacturer's instructions. Bioluminescence was acquired by FluoStar OMEGA luminometer (BMG LABTECH GmbH, Ortenberg, Germany).

2.8. Reactive oxygen species (ROS) production

The flow cytometric detection of ROS production was assessed by using the CellROX® Flow Cytometry Assay Kits (Life Technologies, CA, USA) Briefly, BC cells (4×10^4/well) were seeded into 24-well plates and cultured for different times with RTX (20 μM) or vehicle. Then, cells were stained for 30 min at 37 °C, 5% CO_2, protected from light with the CellROX® Detection Reagent and analyzed by flow cytometry; fluorescence intensity was expressed in arbitrary units on a logarithmic scale.

2.9. Grafting of T24 cells into immunodeficient mice and RTX treatment protocol

Athymic nude (nu/nu) 6-week-old male mice (Harlan Laboratories, San Pietro al Natisone, Italy) were housed in pathogen-free conditions on a 12 h light/dark schedule. Twenty mice