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Forskolin improves sensitivity to doxorubicin of triple negative breast cancer cells via Protein Kinase A-mediated ERK1/2 inhibition

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ABSTRACT

Triple negative breast cancer (TNBC) is an invasive, metastatic, highly aggressive tumor. Cytotoxic chemotherapy represents the current treatment for TNBC. However, relapse and chemo-resistance are very frequent. Therefore, new therapeutic approaches that are able to increase the sensitivity to cytotoxic drugs are needed. Forskolin, a natural cAMP elevating agent, has been used for several centuries in medicine and its safeness has also been demonstrated in modern studies. Recently, forskolin is emerging as a possible novel molecule for cancer therapy. Here, we investigate the effects of forskolin on the sensitivity of MDA-MB-231 and MDA-MB-468 TNBC cells to doxorubicin through MTT assay, flow cytometry-based assays (cell-cycle progression and cell death), cell number counting and immunoblotting experiments. We demonstrate that forskolin strongly enhances doxorubicin-induced antiproliferative effects by cell death induction. Similar effects are observed with IBMX and isoproterenol cAMP elevating agents and 8-Br-cAMP analog, but not by using 8-pCPT-2′-O-Me-cAMP Epac activator. It is important to note that the forskolin-induced potentiation of sensitivity to doxorubicin is accompanied by a strong inhibition of ERK1/2 phosphorylation, is mimicked by ERK inhibitor PD98059 and is prevented by pre-treatment with Protein Kinase A (PKA) and adenylate cyclase inhibitors. Altogether, our data indicate that forskolin sensitizes TNBC cells to doxorubicin via a mechanism depending on the cAMP/PKA-mediated ERK inhibition. Our findings sustain the evidence of anticancer activity mediated by forskolin and encourage the design of future *in-vivo*/clinical studies in order to explore forskolin as a doxorubicin sensitizer for possible use in TNBC patients.

1. Introduction

Breast cancer is considered a very complex disease with heterogeneous morphological characteristics and unrelated clinical behavior, and is the leading cancer in women. Among breast cancers, the triple-negative breast cancer (TNBC) subtype constitutes approximately 20% of all diagnosed breast cancer cases, is associated with a highly aggressive nature and metastatic development with very frequent relapse and poor prognosis [1]. Loss of the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) represent genetic fingerprint of TNBC, its histological features are high proliferative index, absence of infiltrative margins, focal areas of necrosis, absence of gland formation, presence of central scar/fibrotic foci and lymphoplasmacytic infiltration [2–4]. TNBC is considered an orphan disease because no effective targeted-therapies are available. Currently, it is managed with a combination of surgery, radiation therapy and cytotoxic chemotherapy. However, relapse and chemo-resistance happen very frequently and distant metastases occur especially in the lungs, bone, liver and brain. For these reasons, in terms of disease-free and overall survival, TNBC is considerate a poor prognosis disease [5,6]. Thus, novel therapeutic options for the TNBC patients are needed.

In order to increase the therapeutic index of anticancer drugs, and in a contemporary way to reduce the side effects, recently, chemotherapy combination is receiving more attentions [7–9]. Doxorubicin is a longstanding potent chemotherapeutic agent and still represents a pillar of many cancer treatment protocols, including TNBC [10,11]. Although the antitumor activity of the doxorubicin is very robust, its effectiveness is generally limited by drug-resistance and dose-dependent side effects, especially cardiotoxicity [12]. Therefore, to identify an innovative combination chemotherapeutic strategy, in which one novel compound is able to reduce the dosage of doxorubicin, required to obtain

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antitumor effects, would definitely advantage cancer patients. In this contest, naturally occurring molecules constitute a possible attractive candidates [13,14].

Considering all possible natural compounds that are used in medicine, forskolin can be proposed as one of the most promising molecules for potential application in cancer therapy [15]. Forskolin is a diterpene that is produced by the roots of Coleus forskohlii, a characteristic Indian plant [16]. Notably, the natural compound forskolin has been used has been used for several centuries in medicine and its safeness has also been demonstrated in different studies [17–20]. Forskolin directly activates the adenylate cyclase enzyme, which generates cAMP from ATP, thus raising intracellular cAMP levels [21]. It is widely known that cAMP, either via protein kinase A (PKA)-dependent or PKA-independent mechanisms, affects many cellular functions and is considered very relevant to cancer [22–25]. Importantly, it has recently been shown that the cAMP elevating agent forskolin has numerous relevant anticancer effects, such as the induction of mesenchymal-to-epithelial transition, the inhibition of proliferation, motility and migration in many types of cancer cells, and also the enhancement of the sensitivity to conventional antineoplastic drugs [26–34].

The present study has been designed to investigate the possible effects of forskolin on chemosensitivity of TNBC cells to doxorubicin and the underlying mechanisms.

2. Materials and methods

2.1. Antibodies and chemical reagents

Chemical reagents: Propidium iodide (PI) (Sigma Life Science, Milan, Italy), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Life Science, Milan, Italy), bovine serum albumin (BSA) (Microtech, Naples, Italy), 8-Br-cAMP, cAMP analogue 8-pCPT-2′-O-Me-cAMP, forskolin, 3-isobutyl-1-methylxanthine (IBMX), isoproterenol, Protein Kinase A inhibitor KT5720, ERK inhibitor PD98059, adenylate cyclase inhibitor SQ22,536, doxorubicin and DMSO were purchase from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Taxol and 5-fluorouracil were obtained as a generous gift from prof. Michele Caraglia. Primary antibodies used for immunoblotting: Anti-tubulin antibody (CP06, Oncogene-Calbiochem, La Jolla, CA), anti-p-ERK (#5726), anti-ERK (#9102), anti-p-CREB (Ser133, #9198) and anti-CREB (#9197) (Cell Signaling Technology, Danvers, MA, USA), anti-p-p38 (ab4822) and anti-p38 (ab31828) (Abcam, Cambridge, UK). All other antibodies were obtained from Santa Cruz Biotechnology (San Diego, CA, USA). Secondary horseradish peroxidase (HRP) conjugated antibodies used for immunoblotting: goat anti-rabbit (GtxRb-003-DHRPX) and goat anti-mouse (GtxMu-003-EHRPX.0.05) (Immunoreagents Inc., Raleigh, NC, USA). Solutions and buffers were prepared with ultra-high quality water.

2.2. Cell culture and treatments

MDA-MB-231 and MDA-MB-486 human triple negative breast cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). MDA-MB-231 and MDA-MB-486 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma Life Science, Milan, Italy) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 2 mM glutamine (Gibco, Grand Island, NY) and 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco, Grand Island, NY). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Doxorubicin was prepared as a solution in ddH_2O, stored at 4 °C, while forskolin was dissolved in DMSO. Both compounds were added to culture medium in order to obtain a final concentration as required by single experiments. Negative Control cells were treated with an equal volume of DMSO (< 0.1% v/v). Typically, cells were split (3×10^5/6 cm plates) and grown in 10% FBS for 24 h, then media was removed and cells were incubated with fresh medium supplemented or not with doxorubicin and forskolin, alone and/or in combination. Times and concentrations are indicated in "Results" section. After treatment, floating and adherent cells were collected (respectively by centrifugation and trypsinization) and counted. Floating and adherent cells were also used for flow cytometry-based assays (cell-cycle progression and cell death) experiments.

2.3. Cell viability assay

The procedure has been described elsewhere [34]. Briefly, cells were seeded in a 96-well plates (3×10^3 cells/well) and grown in 10% FBS for 24 h, then they were treated as described in Section 2.2. After treatments, MTT solution was added in each well (final concentration of 0.5 mg/mL) and incubated at 37 °C for 3 h. MTT-formazan crystals were solubilized in isopropanol/hydrochloric acid 4% 1 N at room temperature on a horizontal table shaking for 20 min. The absorbance was measured at 570 nm using a Bio-Rad 550 microplate reader (Bio-Rad Laboratories, Milan, Italy). Cell viability experiment was expressed in "% of control", that is the percentage of absorbance respect to the control (100%). MTT experiments were repeated at least 4 times.

2.4. Evaluation of sub-G1 and cell cycle phases by flow cytometry

Cells were fixed in 70% ice-cold methanol/PBS and incubated overnight at 4 °C. The day after cells were spun-down at 400 × *g* for 5 min, washed two times with ice-cold PBS, centrifuged again and re-suspended in 1 mL PI staining solution (50 μg/mL PI and 100 μg RNase A in PBS). At this time, samples were moved to 5-mL BD-Falcon tubes and stored at 4 °C until assayed. Flow cytometry analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson), at least 20K events for each samples were acquired. ModiFIT Cell Cycle Analysis software was used to define the percentage of G1, S, G2/M regions and also for the sub-G1 events.

2.5. Cell death assay by propidium iodide uptake and flow cytometry analysis

Cell death was measured as previously described [34]. Changing of plasma membrane permeability represents the essential condition to allow to Propidium Iodide (PI) to bind DNA. Due to mechanisms of death (apoptosis, necrosis, autophagy etc.) plasma membranes generally become permeable. To compare PI uptake, in different cells populations or in the same one, is used as a method to discriminate dead cells from live cells. Briefly, cells were seeded in 6-well plates (10×10^4 cells/well) and grown for 24 h. After that, medium was changed and cells were treated for times, concentrations and modalities indicated in "Results" section. As previously described in Section 2.2, cells were recovered and incubated with PI-FACS buffer containing 0.5 mg/ml of PI in PBS and analyzed by flow cytometry.

2.6. Preparation of cell lysates

RIPA buffer: 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS , 10 μg/ml aprotinin, 1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were re-suspended in 3–5 vol of RIPA buffer and incubated on ice for 1 h. Later, samples were spun-down at 18,000 × *g* in a table top centrifuge for 15 min at 4 °C. Supernatant (SDS total extract) was recovered in order to determine proteins concentration (using Bradford Method) and to prepare samples for immunoblotting (adding Laemmli buffer 4× and boiling).

2.7. Immunodetection of proteins

20–40 μg of proteins from whole extracts were loaded in a polyacrylamide gel (Bio Rad Laboratories, Hercules, CA, USA), separated by SDS-PAGE and transferred on nitrocellulose membrane (Sigma-Aldrich,