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Forskolin attenuates doxorubicin-induced accumulation of asymmetric dimethylarginine and s-adenosylhomocysteine *via* methyltransferase activity in leukemic monocytes

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ABSTRACT

Doxorubicin (DOX) is an antitumor drug, associated with cardiomyopathy. Strategies to address DOX-cardiomyopathy are scarce. Here, we identify the effect of forskolin (FSK) on DOX-induced-asymmetric-dimethylarginine (ADMA) accumulation in monocytoid cells. DOX-challenge led to i) augmented cytotoxicity, reactive-oxygen-species (ROS) production and methyltransferase-enzyme-activity identified as ADMA and s-adenosylhomocysteine (SAH) accumulation (SAH-A). However, except cytotoxicity, other DOX effects were decreased by metformin and FSK. FSK, did not alter the DOX-induced cytotoxic effect, but, decreased SAH-A by > 50% and a combination of three drugs restored physiological methyltransferase-enzyme-activity. Together, protective effect of FSK against DOX-induced SAH-A is associated with mitigated methyltransferase-activity, a one-of-a-kind report.

1. Introduction

Chemotherapy (CT) is a prominent anticancer strategy practiced in all stages of cancer treatment. However, CT has significant limitations such as development of drug resistance and lack of sensitivity to anticancer drugs besides the other side effects. Reason being that CT damages normal cells in different organ systems, bone marrow and hair follicles that divide rapidly, thus these cells become sensitive to antimitotic drugs [1]. This leads to the prevalent dose-dependent side effects of CT including cardiomyopathy, arrhythmias, nephrotoxicity, dementia and pulmonary toxicity, among the others.

DOX (PubChem CID-31703), a cytotoxic anthracycline antibiotic is a frequent CT medication used to treat both solid and hematologic malignancies. It prevents DNA replication by intercalation as well as elevates free radical production, thus it confers cytotoxicity. However, its therapeutic limitation includes cumulative cardiotoxicity, manifested as acute and chronic events [2]. While molecular mechanism(s) of DOX-induced antineoplastic activity is well established, influence of DOX in chronic cardiotoxicity remains unclear. Previous studies have demonstrated that multiple mechanisms are involved in DOX-induced heart failure, although increased oxidative stress (OS) in cardiomyocytes is a well-known fact. Likewise, DOX-induced cardiotoxicity is associated with different cellular events including increase in iron accumulation, DNA disruption and reduction in eNOS (endothelial nitric oxide synthase) activity *via* asymmetric dimethylarginine (PubChem CID-123831) build-up [3,4]. ADMA is a metabolic by-product formed during protein methylation, while s-adenosylmethionine (SAM, PubChem CID-34756)-dependent enzyme, protein arginine methyltransferase-1 (PRMT1) catalyses ADMA biosynthesis in the cytoplasm of all cells [5]. Synthesized free ADMA then proceeds into the blood plasma *via* the extracellular space. Increased ADMA level reduces nitric oxide production through competitive inhibition of eNOS [6], hence ADMA is associated with different pathologies including cardiovascular and metabolic diseases. In this regard, ADMA lowering strategies have

Abbreviations: ADMA, asymmetric dimethylarginine; cAMP, cyclic AMP; CVD, cardiovascular disease; CT, chemotherapy; DOX, doxorubicin; DDAH, dimethylarginine diaminohydrolase; eNOS, endothelial nitric oxide synthase; FSK, forskolin; HCY, homocysteine; HTRF, homogenous time-resolved fluorescence; L-arg, L-arginine; L-cit, L-citrulline; MET, metformin; NAD+, nicotinamide adenine dinucleotide; OS, oxidative stress; PRMT1, protein arginine methyltransferase1; ROS, reactive oxygen species; SAH, s-adenosylhomocysteine;; SAH-A, SAH accumulation; SAHH, s-adenosylhomocysteine hydrolase; SAM, s-adenosylmethionine; SIRT1, sirtuin1

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clinical applications. While DOX-induced cardiotoxicity is associated with ADMA accumulation [4] and increased ROS production, strategies to mitigate DOX-induced detrimental effects are less explored. Therefore, a competent approach that will tactfully antagonize DOX-induced cardiotoxicity as well as preserve the anti-cancer effect of DOX needs to be identified. Such an enticing strategy can be exploited to mitigate cardiovascular disease (CVD)-induced mortality in cancer patients.

Forskolin (PubChem CID- 47936) is classified as a potent non-adrenergic activator of adenylyl cyclase and cAMP as well as an anti-oxidant, besides the fact that FSK activates SIRT1 *via* cAMP-PKA pathway independent of NAD^+ [7]. Impressive characteristics of FSK include being an antihypertensive, a platelet aggregation inhibitor, a smooth muscle relaxant, a vasodilator and a cardiotonic agent [8]. Yet, the effect of FSK in regulating ADMA metabolism remains unexplored. Should FSK modulate intracellular ADMA levels, a novel trait of FSK, over-and-above the existing vasodilatory nature might be revealed. Similar to FSK, experimental and clinical studies have demonstrated that metformin (PubChem CID 4091) mitigates ADMA accumulation by activating the deacetylating enzyme, SIRT1, as well as increasing DDAH (dimethylarginine diaminohydrolase) activity; while DDAH is known to metabolize ADMA to L-citrulline (L-cit) [9]. In support, we have previously reported that SIRT1 potentially deacetylates PRMT1 wherein deacetylation alleviates the enzyme activity of PRMT1 [10].

In the present study, we tested whether FSK, through decreasing methyltransferase activity, could reduce ADMA accumulation without modulating the anti-cancer effect of DOX, *in vitro*. Reasons for using human monocytoid cells (THP1) for the experiments are that i) these cells are involved in two diseases – cardiovascular disease (ability to produce ADMA, SAH (PubChem CID-439155) and express eNOS) as well as cancer (leukemia), ii) the specific study targets such as methyltransferase and ADMA are also involved in these two pathologies. We found that FSK treatment on monocytoid cells attenuated DOX-induced i) intracellular accumulation of ADMA and SAH and ii) ROS generation while preserving the DOX-induced cytotoxicity.

2. Materials and methods

2.1. Culture and treatment of cells

Human monocytoid cells (THP1) (purchased from National Center for Cell Science, India) were cultured in RPMI-1640 medium containing 10 mM/L HEPES, 0.1 mM/L sodium pyruvate, 2 mM/L glutamine and 50 mM/L 2-mercaptoethanol, supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a 95% air and 5% CO_2 incubator. Cells (1×10^6/ml) in logarithmic growth phase were treated with different concentrations of the drugs (DOX-2 µM, FSK-10 µM, MET-1 mM) or RPMI-1640.

2.2. Cytotoxicity assay

Cell viability was assessed using MTT assay [11], which is based on the reduction of MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to a colored formazon. Levels of reduced MTT were determined by measuring the difference in absorbance at 595 and 650 nm. About 1×10^6 THP1 cells/ml were exposed to increasing concentrations of i) DOX (0.125, 0.25, 0.5, 1, 2µM) ii) MET (0.01, 1, 10 mM) and iii) FSK (5, 10 20 µM) for 24 h in CO_2 incubator at 37 °C. After incubation, cell viability was determined. For MTT assay, cells were exposed to 0.25 mg/ml MTT in PBS for 30 min at 37 °C then solubilized in 200 µl of 20% (w/v) SDS in 50% (v/v) N, N′-dimethylformamide, pH 4.5. Samples were assayed in triplicate, and the mean value for each experiment was calculated. The obtained results are expressed as a percentage of control, which is considered to be 100%.

2.3. DPPH radical scavenging assay

Free radical scavenging activity of test compounds was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay. Fresh 1 mM DPPH solution was prepared in methanol and 3 ml of this solution was mixed with 100 µl of different concentrations (3.125, 6.25, 12.5, 50, 100, 200 µg/ml) of the test compounds. Ascorbic acid was used as reference. Samples were incubated for 30 min at 37 °C and absorbance was measured by spectrophotometer at 517 nm.

2.4. SAH conversion assay

Methyltransferase activity was measured from the conversion of SAM to SAH, as reported previously [12,13]. Cells were treated with DOX, MET or FSK, independently or in combinations, for 24 h at 37 °C. Cells were homogenized in 50 mM Tris-HCl (pH 7.4) and centrifuged at $500 \times g$ for 10 min at 4 °C. Supernatant was utilized for the homogenous time-resolved fluorescence (HTRF) assay (Cisbio kit) [14,15]. Cell lysate, as an enzyme source, was reacted with the methyl donor SAM and incubated for 45 min at room temperature (RT). This facilitates SAH formation, a by-product of transmethylation reaction, through binding of SAM with methyltransferase enzyme in lysate. Then SAH-d_2 and SAH-antibody labeled with Lumi4-Tb (anti-SAH-Lumi4-Tb cryptate) were added to the lysate mix and incubated for 1 h at RT. Due to the competition between native SAH and SAH-d_2 to bind with SAH antibody, and when SAH-d_2 binds with SAH antibody, light emission occurs at 620 nm. However, when SAH produced from the enzyme reaction binds with SAH antibody, due to loss of fluorescence resonance energy transfer (FRET), light emission occurs at 665 nm. Hence, the concentration of SAH formed is inversely proportional to the intensity of fluorescence signals. Three controls were used in the assay as such i) buffer control comprising detection and enzymatic buffers; ii) negative control (no enzyme, no-activity control) had reaction mix without cell lysate; and iii) maximum HTRF ratio control comprising SAH antibody and SAH-d_2 with other reagents. Difference in the intensities of HTRF signals in different experimental conditions were measured using a Spectramax i3x (Molecular Devices) multimode plate reader at 665 or 620 nm.

2.5. Measurement of ADMA by HPLC

Monocytoid cells were exposed to DOX, MET or FSK, as previously described. Treated cells were washed thrice, centrifuged at $200 \times g$, 5 min at 4 °C and sonicated in ice-cold 50 Mm Tris-HCl (pH 7.4) followed by ultra-filtration through 3-kDa MWCO filters to remove proteins, as described [16]. The LC ESI-MS analyses were performed with a LC ESI-MS 2020 system equipped with LC10ADVP binary pump (Shimadzu, Japan). Shimadzu HPLC system was coupled on-line with an MS single quadruple ion trap. Identification of ADMA in the lysate was performed using reference ADMA and mass spectrometry, while quantification of cellular ADMA was carried out by HPLC. Samples were separated on a Inertsil NH_2 column (250 mm × 4.6 mm, 5 µm Inertsil NH_2 column) and detected using a photodiode array detector set at a wavelength of 205 nm. Mobile phase consisted of methanol:water (45:55) run at ambient temperature with a flow rate of 0.5 ml/min. Mass (MS) compartment consisted of single quadruple mass spectrometer with electrospray ionization (ESI) source and nitrogen gas was used to assist nebulization with a flow rate of 1.5 ml/min. Temperature was set for curved desolvation line (CDL) and heat block at 280 °C and 320 °C. Measurements were performed from peak area of the LC chromatogram. Quantification was achieved with the calibration curves obtained from standard solutions prepared at a concentration of (0, 6.25, 12.5, 25, 50, 100, 200 µg/ml). Retention time of ADMA peaks were found to be at 3.7 min [17]. Data were collected and processed using Lab Solution Software (Version 7.1, Shimadzu).