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Efficacy of metformin in mediating cellular uptake and inducing apoptosis activity of doxorubicin

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ABSTRACT

The clinical use of doxorubicin (DOX) is limited due to its systemic side effects and drug resistance. Recent evidence suggests that metformin prevents and controls certain but not all types of cancer. The beneficial use of metformin in combination with some chemotherapeutic agents has been reported. The aim of this study is to investigate the influence of metformin on DOX-induced effects in human prostate DU145 cancer cells and clarify its molecular mechanisms. For this purpose, DU145 cells were treated with DOX or metformin, either alone or in combination with each other. The proliferation of DU145 cells was inhibited by DOX-alone and metformin-alone treatment in a time and dose-dependent manner. Metformin could enhance the cytotoxicity of DOX by increasing DOX cellular uptake and cell cycle arrest at G1/S checkpoint which is associated with the enhancement of p21 protein expression. Moreover, metformin could elevate DOX-induced apoptosis in DU145 cells in a concentration-dependent manner and DOX-induced caspase-3 activity. These findings suggest that the combined treatment of metformin with DOX potentiates the anticancer efficacy of DOX in DU145 cells via inhibiting ABCB1 function, cell cycle arrest at G1/S transition and apoptosis induction.

1. Introduction

Doxorubicin (DOX) is one of the well-known chemotherapeutic drugs with therapeutic activity against hematopoietic cancers and solid tumors such as prostate cancer (Minotti et al., 2004; Pollakis et al., 1983; Tyagi et al., 2002; Tacar et al., 2013). The mechanisms of action of DOX are DNA intercalation, topoisomerase II inhibition, reactive oxygen species (ROS) generation and ultimately cell cycle arrest (Lupertz et al., 2010). Like other chemotherapeutic agents, its serious side effects have limited its clinical applications (Tacar et al., 2013; Carvalho et al., 2009). Based on the low therapeutic index of DOX and resistance to this agent, there has been significant research interest in developing new effective strategies that decrease DOX effective dose and improve its therapeutic index.

Metformin belongs to the biguanide class of oral hypoglycemic agents and is widely used as an antidiabetic drug in the clinic to treat type 2 diabetes. The anti-proliferative effects of metformin have been reported in several cancers including breast, colon, glioma, ovarian, pancreatic and prostate cancers (Rattan et al., 2011). An inverse relationship has been found between the progress of prostate cancer and type 2 diabetes mellitus in patients who use metformin (Hankinson et al., 2017; Wright and Stanford, 2009). Several suggested mechanisms for the antineoplastic action of metformin include (i) activation of p53, (ii) promotion of apoptosis, (iii) activation of adenosine monophosphate activated protein kinase (AMPK) pathway, (iv) inhibition of tumor migration, (v) downregulation of cyclin D1, (vi) induction of cell cycle arrest, and (vii) suppression of HER2 expression. (Vazquez-Martin et al., 2009; Zhuang and Miskimins, 2008; Buzzai et al., 2007; Sahra et al., 2008). Metformin has revealed its antiproliferative activity through arresting G0/G1 or G2/M cell cycle and inducing caspase-dependent apoptosis (Rattan et al., 2011; Sahra et al., 2008; Cantrell et al., 2010; Isakovic et al., 2007; Janjetovic et al., 2011a). Metformin-induced cell cycle arrest was found to be mediated through both p21-dependent and p21-independent mechanisms (Gartel and Radhakrishnan, 2005; Templeton and Ramesh, 2014; Miyamoto-Yamasaki et al., 2007). Recently, some epidemiologic studies have revealed that metformin use does not reduce the risk of several types of cancers and it could not be recommended as a monotherapy drug for treatment of cancer (Kowall et al., 2015). The use of metformin as a neoadjuvant anticancer agent with traditional chemotherapeutic drugs has been suggested to reduce their dose and decrease the side effects related to high dose administration (Zhang and Guo, 2016). Depending

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Abbreviations

ABCB1	ATP-binding cassette B1
AMPK	Adenosine monophosphate activated protein kinase
BSA	Bovine serum albumin
CSC	Cancer stem cells
DAPI	4′, 6-diamidino-2phenylidole
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
ECL	Enhanced chemiluminescence
ESCC	Esophageal squamous cell carcinomas
EB/AO	Ethidium bromide and Acridine orange
FBS	Fetal bovine serum
FT-IR	Fourier Transform infrared spectroscopy
GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
MTT	3-(4,5-dimrthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OCT1	Organic cation transporter 1
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PI	Propidium iodide
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RPMI	1640 W/L Glutamate Roswell Park Memorial Institute 1640 with L Glutamate
SDS	Sodium dodecyl sulfate
TBS (T)	Tris-buffered saline (and Tween 20)

on the chemotherapeutic drug, metformin uses different molecular mechanisms to exert its combination effect and combats tumors (Peng et al., 2017).

The present study, for the first time, investigates the influence of metformin on the cellular uptake of DOX in human prostate cancer DU145 cells as an in vitro model for the advanced hormone-refractory prostate cancer (Stone et al., 1978). Hormone-refractory prostate cancer has a low therapeutic response to conventional chemotherapy (Kageyama, 2008). DOX has been used as a treatment option for hormone-refractory prostate cancer (Minotti et al., 2004; Pollakis et al., 1983; Tyagi et al., 2002; Tacar et al., 2013). Moreover, its effect on DOX-induced cytotoxicity and apoptosis is evaluated.

2. Materials and methods

2.1. Cell culture

Human prostate DU145 cancer cell line was maintained at 37 °C in a humidified incubator containing 5% CO_2 in RPMI-1640 (Roswell Park Memorial Institute 1640 with L Glutamate) complete medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Biosera, UK) and penicillin-streptomycin (Biosera, UK). Then, 1.7 mM and 121 mM stock solutions of DOX (Sigma) and metformin (Sigma), respectively, were made in deionized water and were maintained at 4 °C.

2.2. Assay of anticancer activity

Cytotoxic effects of metformin and DOX were assessed using MTT (3-(4,5-dimrthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. DU145 cells at a density of 1×10^4 cells cm^{-2} that were seeded in 96-well plates in complete medium. After 48 h, the growth medium was replaced with 200 µl medium containing different concentrations of metformin (0.5–40 mM) or DOX (0.0005–5 µM) for 24, 48, and 72 h. Based on the results, the influence of metformin (5 mM and 10 mM) on DOX cytotoxicity (0.0005 µM and 0.05 µM) in DU145 cells was investigated after 48 h of treatment. MTT assay was used to assess the cytotoxicity of DOX alone and in combination with metformin. Next, 20 µl of MTT solution (5 mg ml^{-1}) was added to each well and it was incubated for 4 h. After removing the culture medium and adding 150 µl of dimethyl sulfoxide (DMSO), the absorbance was measured at 570 nm using an ELx800 Absorbance Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA).

2.3. Cell cycle analysis

DU145 cells with a density of 1×10^4 cm^{-2} were cultured in 6-well plates in complete medium for 48 h and were then treated with metformin (10 mM) and DOX (0.05 µM and 0.0005 µM) either alone or in combination for 48 h. At the end of the treatment, the cells were washed in phosphate-buffered saline (PBS) and were treated with propidium iodide (PI) solution (10 µg ml^{-1} sodium citrate, 1000 µg ml^{-1} propidium iodide, 10 µg ml^{-1} Triton X-100, and 3.3 µg ml^{-1} RNase in PBS) for 1 h in the dark. Then, the cellular DNA content was assessed by flow cytometry (Becton Dickinson FACSCaliburTM; BD Biosciences, San Jose, CA) and the flow cytometry data were analyzed using the CellQuest software.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells treated for 48 h with metformin (10 mM) and DOX (0.0005 and 0.05 µM) alone or in combination using the TRIzol reagent (Roche, Germany). RNA (1 µg) was pretreated with RNase-free DNase I and the reverse transcription reaction was carried out using the M-MLV reverse transcriptase (Genet Bio, Korea) according to the manufacturer's instruction. Glyceraldehyde 3- phosphate dehydrogenase (GAPDH) was used as the control. Primers spanning each exon-exon junction were employed to detect the mRNA level of p21 and GAPDH (Table 1). The p21 and GAPDH primers were designed using the Beacon Designer software 7.5. The 25 µl PCR reaction mixture contained 1 µl of cDNA, 50 µM of dNTP, 0.15 U Taq DNA polymerase, 2.5 mM $MgCl_2$, and 10 pmol of each primer. The first denaturation and the final extension steps were performed for 5 min at 95 °C and 10 min at 72 °C, respectively. PCR amplification was done using a thermal cycler (Biometra, Germany). Amplicons were electrophoresed on 1% agarose gel and were visualized by ethidium bromide staining.

2.5. Fluorescence microscopy method

DU145 cells (1×10^4 cm^{-2}) were cultured in 6-well plates. After 24 h of incubation, the cells were exposed to DOX alone (10 µM) and

Table 1
Primers sequences and characteristics.

Gene names	ID NCBI	Sequencing Primers (5′→3′)	Amplicon length (bp)	T_a (°C)
p21	NM000389	F: AGACCAGCATGACAGATTTC R: GAGACTAAGGCAGAAGATGTAG	141	64
GAPDH	NM00204604	F:GTCGGAGTCAACGGATTTGG R:GGCAACAATATCCACTTTACCAGAG	81	57