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TREATMENT OF MCF-7 AND MDA-MB-231 HUMAN BREAST CANCER CELL LINES WITH ERYTHROPOIETIN, DOXORUBICIN AND THEIR COMBINATION

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By

ESAM M. RADWAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for Master of Science

January-2015

DEDICATION

I wish to dedicate this thesis to my mother (Marhumah. Mabroka) and father (Marhum. Hajj. Muhammad) for their love and giving me the genes for research. They have always believed in me and have always encouraged me not only during this master period but throughout life.

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

TREATMENT OF MCF-7 AND MDA-MB-231 HUMAN BREAST CANCER CELL LINES WITH ERYTHROPOIETIN, DOXORUBICIN AND THEIR COMBINATION

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The cancer chemotherapies are formulated to target the rapidly proliferating cancer cells more efficiently than those normal cells of low proliferating rate. In cancer therapies erythropoietin (EPO) is often used in combination with chemotherapeutic drugs to treat the cancer-associated anemia. In this study, it is hypothesized that EPO does not modify the cytotoxic effect of doxorubicin (DOX). Thus the objective of the study was to determine the effect of DOX-EPO combination treatment on human breast cancer cell lines. The cytotoxicity of DOX (1 µg/mL) alone or in combination with EPO (1 µg/mL DOX – 1 IU/mL EPO) against two human breast cancer cell lines, the MCF-7 and MDA-MB-231 cell, was determined by 3-4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide cell viability assay, neutral red uptake and lactate dehydrogenase assays. The activity caspases and morphology of the treated cells were also determined. Doxorubicin inhibited MCF-7 cells with IC50 of 0.217±0.05 and 0.127±0.01 µg/mL when determined by the MTT and NR assay respectively, at 72 hours.

On the MDA-MB-231 cells the IC50 of DOX was 0.12±0.09 µg/mL and 0.118±0.04 µg/mL by the MTT and NR assay, respectively, at 72 h of treatment. Doxorubicin in combination with EPO did not exhibit any notable difference in cytotoxicity to the cell lines when a compared to DOX alone. The anti-proliferative effect of DOX alone was obvious on the MCF-7 and MDA-MB-231 cell, showing decline in cell counts from 27.4 and 27.3 × 105 to 2.1 and 7.0 × 105 cells respectively, after 72 h of treatment. Light microscopic examination of DOX-treated MCF-7 and MDA-MB-231 cells at 72 h demonstrated apoptotic changes in cellular morphology characterized by cell rounding followed by a loss of adherence with subsequent cell shrinkage and blebbing.

The mechanism of cell death was determined through the caspases-3 and -9 activities of the treated cells. The results demonstrated that caspases-3 and-9 activities were significantly ($p<0.05$) elevated early in DOX-treated MCF-7 MDA-MB-231 cells after 24 h, suggesting the apoptotic effect of DOX is via the mitochondrial pathway. Erythropoietin did not cause any change in caspase level in the treated cells. This study shows that EPO did not modify the cytotoxic effect of DOX on MCF-7 and MDA-MB-231 cells and thus is safe to be used with DOX in the treatment of breast cancers in patients with concurrent anemia.

Keywords: doxorubicin, erythropoietin, MCF-7, MDA-MB-231, doxorubicin-erythropoietin combination, apoptosis

Abstrak tesis yang dikemukakan Kepada Senat Universiti Putra Malaysia sebagaimemenuhi keperluan untuk ijazah Master Sains

PERLAKUAN TITISAN SEL MCF-7 DAN MDA-MB-231 PAYUDARA MANUSIA DENGAN ERITROPOIETIN, DOKSORUBISIN DAN GABUNGANYA

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Kemoterapi kanser dirumus untuk mensasar sel kanser memproliferat cepat secara lebih berkesan daripada sel normal yang lambat memproliferat. Dalam terapi kanser, eritropoieitin (EPO) kerap diguna secara gabungan dengan drug kemoterapi lain untuk merawat anemia terkait kanser. Dalam kajian ini adalah dihipotesiskan yang EPO tidak mengubah suai kesan sitotoksik doksorubisin (DOX). Justeru, objektif kajian ini ialah untuk menentukan kesan perlakuan gabungan DOX-EPO terhadap titisan sel kanser payudara manusia. Kesitotoksikan DOX (1 µg/mL) secara bersendirian atau secara gabungan dengan EPO (1 µg/mL DOX – 1 IU/mL EPO) terhadap dua titisan sel kanser payudaya manusia, sel MCF-7 dan MDA-MB-231, telah ditentukan melalui asai kebolehhidupan sel (3-4,5-dimetiltiazol-2-il-2,5-difenil tetrazolium bromide) (MTT), pengambilan merah neutral (NR) dan laktat dehidrogenase.Aktiviti kaspase dan morfologi sel terperlaku juga ditentukan. Doksorubisin merencat MCF-7 masing-masing pada IC50 0.217±0.05 dan 0.127±0.01 µg/mL apabila ditentukan melalui assai MTT dan NR assay pada jam 72 perlakuan.

Terhadap sel MDA-MB-231, IC50 DOX masing-masing adalah 0.12±0.09 μg/mL dan 0.118±0.04 μg/mL mengikut assai MTT dan NR, pada jam 72. Doksorubisin secara gabungan dengan EPO tidak menunjukkan perbezaan jelas dalam kesitoktoksikan terhadap titisan sel tersebut apabila dibanding dengan DOX secara bersendirian. Kesan antipemproliferatan DOX secara bersendirian terhadap sel MCF-7 dan MDA-MB-231 adalah jelas dengan menunjukkan penurunan bilangan sel masingmasing daripada 27.4 and 27.3 × 105 kepada 2.1 and 7.0 × 105 sel selepas 72 jam perlakuan. Pemeriksaan mikroskopi cahaya terhadap sel MCF-7 dan MDA-MB-231 terperlaku DOX selama 72 jam menunjukkan perubahan apoptosis dalam morfologi sel yang dicirikan oleh pembulatan diikuti hilang daya pelekatan dan seterusnya pengecutan dan pembleban sel. Mekanisme kematian sel ditentukan melalui aktiviti kaspase-3 dan -9 sel terperlaku.

Hasilnya menunujukkan aktiviti kaspase-3 dan -9 meningkat awal secara ketara ($P<0.05$) dalam sel MCF-7 dan MDA-MB-231 terperlaku DOX selepas 24 jam, menyarankan bahawa kesan apoptosis DOX adalah menerusi arah laluan mitokondrion. Eritropoietin tidak menunjukkan sebarang berubahan dalam aras kaspase sel terperlaku. Kajian ini menunjukkan EPO tidak mengubah suai kesan sitotoksik DOX terhadap sel MCF-7 dan MDA-MB-231 dan dengan ini adalah selamat untuk diguna bersama DOX dalam rrawatan kanser payudaya pesakit mengidap anemia iringan.

Kata kunci: doksorubisin, eritropoietin, MCF-7, MDA-MB-231, gabungan doksorubisin-eritropoietin, apoptosis

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Thank you.

I certify that a Thesis Examination Committee has met on 8 January 2014 to conduct the final examination of Esam M. Radwan on his thesis entitled "Treatment of MCF-7 and MDA-MB-231 Human Breast Cancer Cell Lines with Erythropoietin, Doxorubicin and their Combination" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

ATCC	The American Type Culture Collection
CO_2	Carbon Dioxide
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dUTP	2'-deoxyuridine 5'-triphosphate
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
EtOH	Ethanol
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
G_0	Resting phase
G_1	Gap between mitosis and DNA synthesis
G_2	Gap between DNA synthesis and mitosis
HCl	Hydrochloric acid
HepG2	Human hepatocellular liver carcinoma cell line
HRP	Horseradish Peroxidase
IC_{50}	Inhibition concentration at 50 percent
ICAM-1	Inter-Cellular Adhesion Molecule 1
KCl	Potassium Chloride
KH_2PO_4	Potassium dihydrogen phosphate
LDH	Lactate Dehydrogenase
M	Mitosis
mL	Milliliter

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide
NaHPO4	Disodium hydrogen phosphate anhydrous
NaOH	Sodium Hydroxide
PBS	Phosphate buffer saline
pH	Minus the decimal logarithm of the hydrogen ion activity in an aqueous solution
PI	Propidium iodide
S	DNA synthesis
STAT	Signal Transducers and Activators of Transcription
TDT	Deoxynucleotidyl Transferase
TP53	Tumor protein p53
TUNEL	TdT-mediated dUTP Nick End Labeling
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low-density lipoproteins
WNT	Proteins have roles in embryogenesis, cancer and in normal physiological processes
µg	Microgram

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Erythropoietin (EPO) is a heavily glycosylated glycoprotein, produced in theper tubular cells of the kidneys in response to hypoxia (Hale et al., 2006), regulates the production of EPO by the bone marrow. Each EPO molecule binds to two EPO receptors on the surface erythrocyte precursors to cause proliferation, differentiation and survival of erythroids (Fisher, 2003; Fu and Arcasoy, 2007; Lappin et al., 2002). Anemia is one of manifestations of cancers, which results from a complex interaction of various factors; thus making the treatment of renal failure alone to alleviate the condition a futile effort (Barrett-Lee et al., 2005; Cella, 1997).

Cancers are often associated with anaemia. The anemia in cancers may not be due to renal disease and in fact could be due to other causes to included blood loss, hemolysis, nutritional deficiencies, bone marrow disorders, chemotherapy and radiotherapy, and the anemia of cancer itself (Beguin, 1995; Beguin, 1998; Beguin and Vanstraelen, 2008; Mughal, 2004). It seems that in breast cancers, the circulating EPO concentrations are often very low, which could account for the anemia in these patients (Aapro et al., 2008; Leonard et al., 2005; Seal et al., 2006).

There are several anti-breast cancer drugs used today. However, doxorubicin (DOX) seems to the best among these drugs and it can be used on its own or in combination with other drugs like epirubicin, mitoxantrone, cisplatin, and etoposide (Ayers et al., 2004; Henderson et al., 2003). Erythropoietin is used as adjuvant therapy to alleviate the symptoms of anemia. However, previous studies showed that EPO has synergistic activity with tamoxifen and traxol against the MCF-7 or MDA-MB231 cell line (Gewirtz et al., 2006; Kim et al., 2008; Kokhaei et al., 2007; Szenajch et al., 2010). It is not known whether or not EPO has similar synergistic effect with DOX. Thus it is hypothesised in this study that EPO potentiates the cytotoxic effect of DOX on breast cancer cell lines, the MCF-7 and MDA-MB231 cells.

The general objective of this study is to determine the effect of combination treatment of EPO and DOX on breast cancer cell lines, the MCF-7 and MDA-DB231 cells.

Specific Objectives:

1. To determine the effect of DOX alone and in a combination with EPO on the viability and proliferation of MCF-7 and MDA-MB-231 cells.
2. To determine the effect of EPO-DOX combination treatment on MCF-7 and MDA-MB-231 cells.
3. To determine the possible mechanism of cytotoxicity induced by the EPO-DOX combination on MCF-7 and MDA-MB-231 cells.

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