The Cytotoxic Effect of Metformin on RD Cell Line.

Ahmed Wahhab Mohammed*, Inam Samih Arif **, Ghaith Ali Jasim**
Babylon Health Office, Ministry of Health, Iraq
*** *Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Iraq***

DOI: https://doi.org/10.32947/ajps.19.01.0396

Article Info:

Received 5 DeC 2018
Accepted 24 Feb 2019
Published 1 Mar 2019

Corresponding Author email:
Pharm.ghaithali@uomustansiriyah.edu.iq
orcid: https://orcid.org/0000-0001-5153-4094

Abstract:

Background: Cancer is a pathogenesis that happens when modification in collections of normally occurring cells inside the human body occurs leading to non-controlled growth causing a lump called the tumor; this applies to all types of cancers except leukemia (cancer of the blood). Doxorubicin (DOX) is one of the highly effective anti-neoplastic drugs of the anthracycline's family used to treat many pediatric and adult cancers, e.g. solid tumors, lymphomas, leukemia and breast cancer. Doxorubicin is known to produce severe cytotoxicity. Metformin (Met) is a biguanide used for type 2 diabetes mellitus. Metformin have cytoprotective effect in addition to reducing basal and postprandial levels of glucose by decreasing the production of ROS, maintaining energy homeostasis and apoptosis regulation by its activation of adenosine monophosphate-activated protein kinase (AMPK). Met has also the ability to increase apoptotic factors and suppression of proliferation thus MET consider as cytotoxic and anti-proliferative drug.

Objectives: This study was designed to investigate the cytotoxic and antiproliferative effect of Met comparing to DOX as a control in RD cell, also combination of both.

Materials and Methods: Cell lines that used (Epithelial cells as a normal cell line and RD cell line was taken from human breast cancer) cultured in suitable media potentiated with different concentrations of heat-inactivated human serum. MTT assay (3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) using for detection of cytotoxicity in Epithelial and RD cell lines for duration 72hrs.

Results: The results showed that treatment with DOX, MET and combination DOX with MET there is significant ($p \leq 0.5$) cytotoxic, apoptotic and antiproliferative effect and there is potentiation effect between MET and DOX on tumor cells when treated by combination DOX with MET also Met showed a valuable cytotoxic effect through the detection of IC50.

Conclusion: From the results, it can be concluded that Metformin have a good cytotoxic and antiproliferative effect on tumor cell lines.

Key words: Doxorubicin, Metformin, IC50, RD cell line.

التأثير السمي للمتفورمين على خط الخلية السرطانية Rd

احمد وهاب محمد*، إنعام سامح عارف، غيث علي جاسم****
دائرة صحة بابل ، وزارة الصحة ، العراق
*** فرع الادوية والسموم ، كلية الصيدلة ، الجامعة المستنصرية ، العراق*

الخلاصة:
الخلفية: السرطان هو مرض يحدث عندما يتم التعديل في مجموعات من الخلايا التي توجدعادة داخل جسم الإنسان مما يؤدي إلى نمو غير خاضع للرقابة يسبب تكتل يسمى الورم ؛ هذا ينطبق على جميع أنواع السرطان باستثناء سرطان الدم (سرطان الدم).
دوكسوروبيسين (دوكس) هو واحد من الأدوية الفعالة للغاية المضادة للأورام من عائلة أنثراسيكلين المستخدمة لعلاج العديد من سرطانات الأطفال والبالغين ، على سبيل المثال الأورام الصلبة والأورام اللمفاوية وسرطان الدم وسرطان الثدي. .من المعروف أن الدوكسوروبيسين ينتج سمية خلوية شديدة.
يستخدم لمرض السكري من النوع 2. الميتفورمين له تأثير واقي للخلايا بالإضافة إلى biguanide المتفورمين (ميت)هو ، والحفاظ على توازن الطاقة وتنظيم ROS خفض مستويات الجلوكوز القاعدية وما بعد الأكل عن طريق خفض إنتاج يمتلك ميت أيضًا القدرة على .(AMPK) موت الخلايا المبرمج من خلال تنشيطه كيناز البروتين المنشط أحادي الفوسفات .زيادة عوامل موت الخلايا المبرمج وقمع الانتشار وبالتالي يعتبر دواءًا سامًا للخلايا ومضاد للتكاثر
الأهداف: تم تصميم هذه الدراسة للتحقيق في التأثير السام للخلايا ومضاد للتكاثر لدواء ميت مقارنة دوكس كعنصر تحكم في خلية RD وكذلك عندما يتم مزج الاثنين معا.
المواد والأساليب: خطوط الخلايا التي استخدمت (الخلايا السطحيه كخط خلايا طبيعي وخط خلية RD مأخوذة من سرطان الثدي البشري) تم تربيتها في وسائط مناسبة معززة بتركيزات مختلفة من مصل الإنسان المعطل بالحرارة .
-4،5 -3)ديميثيلثيازول -2-بيل) بروميد رباعي الإيثيلوليثيل 2،5-ديفينيل يستخدم للكشف عن السمية الخلوية في خطوط الخلايا السطحيه و RD لمدة 72 ساعة.
النتائج: أظهرت النتائج أن العلاج باستخدام دوكس و ميت والجمع بينهما له تأثير كبير ($p\leq 0.5$) سام للخلايا ، وموت الخلايا المبرمج ومضاد للتكاثر ، وهناك تأثير قوي بين ميت و دوكس على الخلايا السرطانية عند معالجتها عن طريق الجمع بينهما أيضًا وكذلك أظهر ميت تأثيرًا سامًا للخلايا من خلال الكشف عن.IC50
الخلاصة: من النتائج ، يمكن استنتاج أن الميتفورمين له تأثير سام سام للخلايا ومضاد للتكاثر على خطوط الخلايا السرطانية.
الكلمات المفتاحية: خط خليةRD، IC50 ، دوكسوروبيسين، ميتفورمين.

Introduction:

Cancer is a pathogenesis that happens when modification in collections of normally occurring cells inside the human body occurs leading to non-controlled growth causing a lump called the tumor; this applies to all types of cancers except leukemia (cancer of the blood). [1]. A tumor in a simple form a space-occupying lesion (something that should not be there, that is; a "lump") occurs by abnormal cell replication (medically, the word "tumor" literally means "swelling). While cancer is a disease in which cells division is *totally out of control* and there is destruction to the genes (DNA) which normally *stop* cell division when it needs to be stopped [2].

Cancer considers the second causable factor of death in the world and was leaded to for 8.8 million death case in 2015. Around 70% of death cases occurs due to cancer in middle- and low- income countries. Cancer treatment may involve surgery, radiation and/or chemotherapy. Most people may receive combination of chemotherapeutic agents [3].

Chemotherapy is a type of cancer therapy that uses drugs to kill cancer cells these cancer therapy that uses drugs to damage cancer cells. It is also named "chemo." there are many different types of chemotherapy such as antibiotics group (doxorubicin). Doxorubicin is effective anticancer drugs that used to treat many pediatric and adult cancers, e.g. solid tumors, lymphomas, leukemia. [4].

Doxorubicin reduced to doxorubicinol which also have biological activity

Doxorubicin also undergo reduction to a semiquinone radical by several intracellular oxido reductases. Reoxidation of semiquinone radical yield reactive oxygen species (ROS). The ROS production considered as one mechanism of its anticancer and antibiotic capabilities. Doxorubicin excretion is done by the liver and kidney through a biphasic half-life of 5 minand30–40 hrs [5].

The drug of first-line regime in treating of type 2 diabetes is currently MET. Besides its glucose-declining effect, there is important effect of the drug of potential relevance to cardiovascular and cancer diseases. Metformin's mechanism of action in diabetes and may also be of importance in cardio vascular and cancer diseases [6].

MET by mechanisms thought to involve the activation of AMPK (AMP-activated protein kinase) [7] and/or suppressing of adenylate cyclase [8] in response to energetic stress, and/or the direct inhibition of mitochondrial glycerol phosphate dehydrogenase [9] lead to decreases blood glucose.

It is impossible to uniformly kill a group of cells with high heterogeneity and thus difficult to obtain a good outcome through administration of a single anticancer agent because the group of cells is likely to comprise cells that are responsive to the treatment as well as those that are resistant. As a result, therapy combining multiple agents that have different mechanisms of action has evolved, i.e., combination chemotherapy. The goal of combination chemotherapy is to eradicate tumor cells through potent therapy before the appearance of resistant cells or an elevation in the number of the resistant cells [10].

Identification and development of safe and potent combination therapy composed of multiple drug resistance reversing agents such as biguinide (MET) with a potent chemotherapeutic agent such as anthracyclin (DOX) is still a big deal among researchers, most of studies showed there is a good targeting correlation can be represented through potentiation or synergism effect to one of them of combining therapy lead to obtaining the desired effect [11].

Apoptosis is over twenty times faster than mitosis. Apoptotic cells are undergoing engulfment and destruction by neighboring cells without a trace [12].

Apoptosis is represented by specific morphologic features, including lossing of plasma membrane structure and binding, plasma membrane blebbing, the cytoplasm and nucleus condensation, and inter nucleosomal cleavage of DNA [13].

Lossing of plasma membrane structure consider one of the earliest features of apoptosis. In apoptosis, the cellular membrane phospholipid phosphatidy-lserine (PS) translocation from the inner to the outer leaflet of the cellular plasma membrane, there by exposing PS to the outer cellular environment [14].

Apoptosis and mitosis shared in common of morphological features such as chromatin condensation, cell shrinkage and membrane blabbing. Thus, the equilibrium between apoptosis and cell proliferation must be strictly maintained to support tissue homeostasis [15].

Cell proliferation, cell differentiation and cell death are important processes in multicellular organisms, and many lines of event link apoptosis to proliferation, uncontrolled proliferation can be associated with a high level of apoptosis [16]

Apoptosis can happen at any stage of the cell cycle, as the metabolic machine responsible for induction of caspase-3. When caspase-3 activated by Tumor Growth Factor B1 (TGFB1) lead to cleaves p21, p27andPARP(poly ADP-ribose polymerase) that lead to induction of cell cycle arrest that may be initiate the conversion of cell-cycle arrest to apoptosis .The cyclin-dependent kinase1 also known as P21, participate in an important role in a many of cellular processes including cell

cycle regulation, apoptosis, and autophagy [16] .

Materials and Methods

Epithelial cell line and RD cancer cell line in falcon was obtained from the Biotechnology center/ Al-Nahrain University. The cell culture medium was DMEM (Dulbecco ‘s modified Eagle ‘s medium). The cells were incubatedat 37°C at a humidified atmosphere 5% CO2 a Cells were passaged in to new T25 or T50 cell culture flasks every 4 days by washing with Phosphate Buffer Saline (PBS) followed by harvesting via Trypsin EDTA. All cell cultures were incubated in a humidified incubator supplemented with 5% CO2 and 95% air at 37ºC.

Study Design

Two cell lines have been used Epithelial cell as normal cell and RD cancer cell treated by MET (Samaraa company), DOX (Saba company) and combination DOX with MET for duration 72hrs and MTT (3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) assay in Epithelial and RD cell lines 72hrs. The % of growth inhibition calculated by the equation [14]:
% growth inhibition =1-% cell viability ×100.

Cytotoxic Activity Assay (MTT):

The two cell lines (Epithelial and RD) accounted by cell counter technique where (1 x 10^2/µl) in 96 well microplate with 100µl in each well and incubated for 24 hours in starved media at 37°C.

Cytotoxicity of Doxorubicin.

Six serial dilutions of doxorubicin (Saba comany Turky) was prepared by dissolving crystalline powder of DOX in DMSO and (40µM, 20µM, 10µM, 5µM, 2.5µM, 1.25µM) was added to normal epithelial cell line and cancer RD cell lines to determine the cytotoxic effect of DOX after 72hrs incubation periods.

Cytotoxicity of Metformin.

Six serial dilutions of Metformin (Samara company Iraq) was prepared by dissolving crystalline powder in distilled water and (40mM, 20mM, 10mM, 5mM, 2.5mM, 1.25mM) and added to normal epithelial cell line and RD cancer cell lines to determine the cytotoxic effect of MET after 72hrs incubation periods.

Cytotoxicity of Combination (Doxorubicin with Metformin)

Six serial dilutions (1.25µM, 2.5µM, 5µM, 10µM, 20µM, 40µM) of DOX were prepared in combination with (10 mM) of MET then added to the two cell lines to determine the cytotoxic effect [17].

Statistical Analysis

Throughout this research, the experiments were performed three times independently and results were expressed as the mean ± the standard deviation and compared using analysis of variance of one way (ANOVA) followed by post hoc LSD software. p value < 0.05 was considered as statistically significant, IC_{50} was calculated according to the regression equation (y = mx+b) where y: % of inhibition, m: mean of regression, x: dose, b: cell viability [18].

Results

Cytotoxicity of Doxorubicin, Metformin and combination of Doxorubicin with Metformin on epithelial cell line.

The results showed significant dose dependent inhibition of growth in normal epithelial cell line induced by different concentration of DOX, MET and the combination of DOX with MET (10mM), as shown in Table (1) and Fig (1) during 72hrs.

Table (1) Effect of DOX, MET and DOX with MET on Epithelial Cell line after 72hr.

conc. µM,mM	% Growth Inhibition of DOX ± SD	% Growth Inhibition of MET ± SD	% Growth inhibition of Combination ± SD
1.25	38±0.07	26±0.01#	43±0.03*
2.5	45±0.03	38±0.05#	52±0.04*
5	53±0.08	49±0.02#	62±0.1*
10	65±0.04	57±0.08#	72±0.07*
20	74±0.01	68±0.07#	79±0.1*
40	80±0.1	72±0.2#	86±0.04*

Each value expressed as mean ±SD. The statistical analysis done by using one-way ANOVA followed by Post Hoc, LSD.
Significant difference ($p < 0.05$) when compared between DOX with MET. #
* Significant difference ($p < 0.05$) when compare combination with either DOX or MET alone.

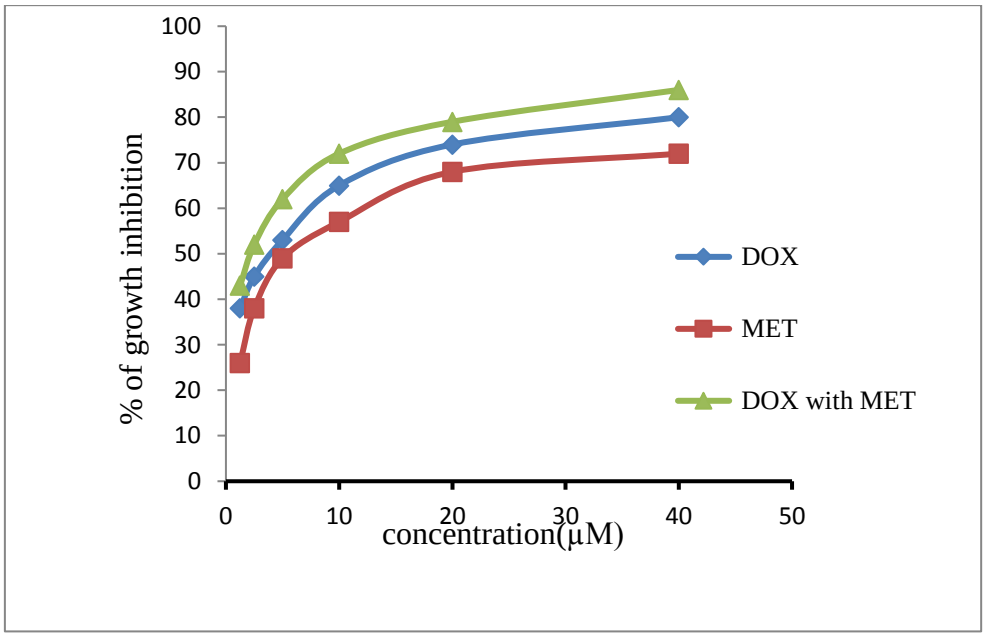

Figure (1) Effect of DOX, MET and DOX with MET 10 mM on Growth Inhibition of Epithelial Cell line after 72hr.

Cytotoxicity of Doxorubicine, Metformin and Combination of Doxorubicin with Metformin on RD cell line.

The results showed significant dose dependent inhibition of growth in RD cell line induced by DOX, MET and the combination of DOX with MET (10Mm), as shown in Table (2) and Fig (2) during 72hrs.

Table (2) Effect of DOX, MET and DOX with MET 10 µM on RD Cell Line after 72hr.

conc. µM,mM	% Growth Inhibition of DOX± SD	% Growth Inhibition of MET ± SD	% Growth Inhibition ± SD
1.25	21±0.07	18±0.002#	30±0.07*
2.5	37±0.01	31±0.02#	39±0.03*
5	49±0.04	40±0.4#	51±0.2*
10	60±0.001	52±0.06#	67±0.07*
20	71±0.05	61±0.001#	75±0.02*
40	83±0.2	76±0.1#	89±0.4

Each value expressed as mean ±SD. The statistical analysis done by using one-way ANOVA followed by Post Hoc, LSD.

Significant difference ($p < 0.05$) when compared between DOX with MET#
*Significant difference ($p < 0.05$) when compare combination with either DOX or MET alone

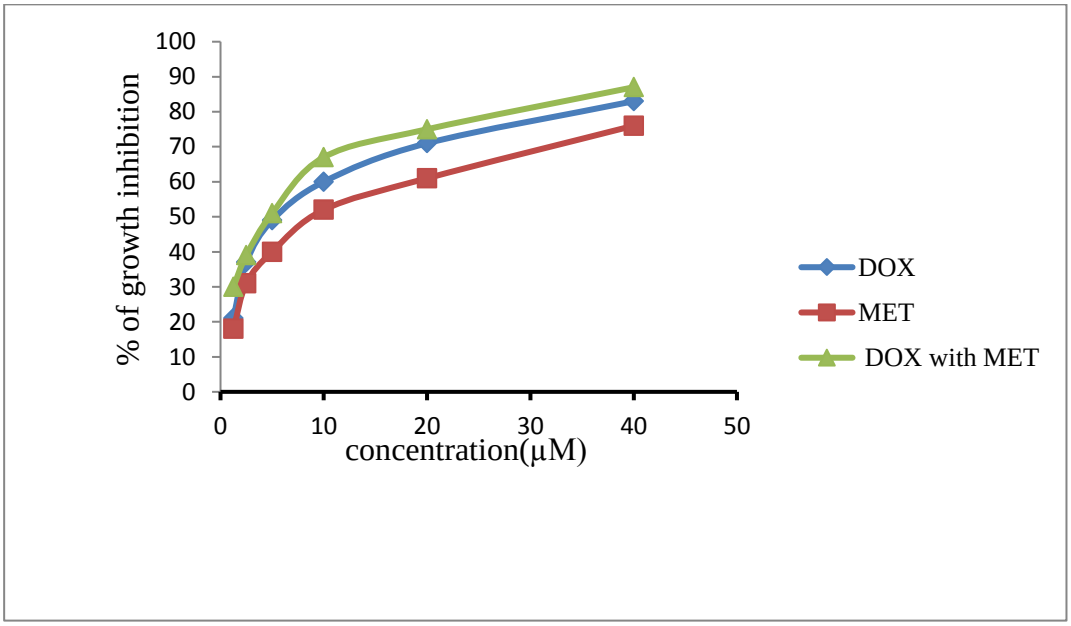

Figure (2) Effect of DOX, MET and DOX with MET 10mM on Growth Inhibition of RD Cell Line after 72hrs.

Based on the IC_{50} values, the cells showed a wide variation in sensitivity of cancer cell line (RD) to the treatment (DOX, MET and DOX with MET) more than sensitivity of normal cell (epithelial) cell line.
MTT assay revealed significant dose dependent effects after 72hrs of incubation periods for DOX, MET and combination (DOX with MET).
However, at lower than 1.25 µM no more effect produced even after incubation for a longer time.

The IC_{50} values after application of DOX on epithelial and RD cell lines after 72hr time intervals can be illustrated in (Table 3):

Table (3) IC_{50} of DOX for Epithelial and RD cell line during 72hrs.

IC_{50} of DOX (µM)	**72hrs**
Epithelial	3
RD	1

Table (3) showed the low values of IC_{50} at 72hrs, this is due to DOX is trafficked

straight to the nuclear area without any accumulation in the cytoplasm up to 24hrs. Some trace amounts do appear in the cytoplasm at 48hrs and increase accumulation in 72hrs, cytoplasmic DOX mechanism of action associated with oxidative stress as a result of reactive oxygen species production. To determine the cytotoxicity of MET, MTT assay has been used and the effects after 72hrs of incubation in vitro was studied.

The IC_{50} values after application of MET on epithelial and RD cell lines after 72hr time intervals can be illustrated in (Table 4).

Table (4) IC_{50} of MET for Epithelial and RD cell lines during 72hrs.

IC_{50} of MET (µM)	**72hrs**
Epithelial	6
RD	2

Table (4) showed the low values of IC_{50} during 72hrs refers to sensitivity of cells to MET that means the MET induced DNA synthesis alteration, the highly cytotoxic effect of MET and strong reduction in DNA synthesis in all experimental cultures after 72 hours of incubation.

This study was showed the combination of (DOX and MET) has cytotoxic effect on epithelial and RD cell lines *in vitro* and MET has synergism effect to DOX on all cell lines, all that can be shown through percent of inhibition values that is can be illustrated by table (5) below:

Table (5) IC_{50} of DOX with MET for Epithelial and RD cell line during 72hrs.

IC_{50} of DOX with MET (µM)	**72hrs**
Epithelial	2
RD	2

Table (5) showed the low values of IC_{50} refers to high sensitivity during 72hrs that is refer to potentiation effect of DOX by MET.

Discussion

In the present study, we observed that combining MET, with DOX leads to potentiation effect to DOX and decreasing the IC_{50} of DOX in all lines of cells *in vitro* that is occurs through Multi drug resistance shows to be one of the most challenging problems in chemotherapy agents [19]. Doxorubicin induces drug resistance by over expressing numerous drug transporters (e.g., *ABCB1*) to reduce intracellular drug accumulation. *ABCB1* encoded P gp is up regulated in various drug resistant tumors and accounts for thee efflux of different drugs. The reduction of Pgp expression or function is feasible to reverse multi drug resistance. The combination of DOX with MET shows synergistic effects in all cell lines. Since P gp consumes two ATPs to export one substrate, MET could weaken Pgp function by reducing ATP production. This leads to elevation intracellular and nuclear DOX accumulation, which facilitates DOX cytotoxicity. Furthermore, MET suppress *ABCB1* (Pgp) expression on the mRNA and protein levels, whereas DOX lead to enhancement the drug-resistant phenotype. This result suggested that MET may be reverse acquired multi drug resistance during prolonged DOX therapy. Additionally, it was found that MET could selectively eliminate cancer stem cells, which are responsible for intrinsic drug resistance and prolong remission in multiple cancer cell types [20].

The cell cycle represents a successive step which lead to proliferation of the cell under a strict control of many factors e.g. CDKs, p21, p27 and p53 which is the tumor suppressor factors [21].

Many of anticancer drugs display antiproliferative activity by targeted apoptosis in the cancerous cells, which might be through interference with DNA replication and the integrity of DNA [20].

After apoptosis induction, fast alterations in the organization of phospholipids in most cell types happens leadingto exposure of PS on the cell surface. Recognition of

PS by phagocytes *in vivo* results in the eliminates of cells programmed to die thus apoptosis not always associated with the local inflammatory response which associated with necrosis [22].

Externalized PS *In vitro* detection can be achieved through interaction with the annexin V. In case of calcium presence, fast high affinity binding of annexin V to PS happens. PS translocation to the cell surface preceding nuclear degradation. DNA fragmentation, and the expression of most apoptosis-associated molecules making annexin V binding a marker of early-stage apoptosis [23].

Previous researches showed that DOX may enhances apoptosis by enhancing production of H2O2 which will induces nitric oxide synthase (eNOS), Antisense eNOS depressed DOX-induced oxidative stress and apoptosis, in addition to p53 dependent pathways [24].

The same protocol showed that MET, also , have the ability to produces dose dependent increase in the translocation ofPS to the extracellular membrane due to decline the activation of IRb, Akt and ERK1/2, increased pAMPK, FOXO3a, p27, Bax and cleavedcaspase-3, and decreased phosphorylation of p70S6K and Bcl-2 protein appearance also there's elevation in H2O2 that lead to elevation in oxidative stress which was associated with declining in cell numbers [25] , and combination of DOX with MET showed enhanced effect in a dose dependent that is due to MET and DOX co-treatment markedly decreased tumor volume, increased survival rate, and improved other parameters compared to single therapy effect was mediated by down regulation of cyclin D1 while elevating the level of the tumor suppressorgeneP53. The activity of AMPK and NF-κB was modulated so that they triggered apoptotic pathway rather than proliferative one [26].

The cell division relies on the activation of cyclins, which bind to CDKs to induce cell-cycle progression towards S phase and later to begin mitosis. Their function is tightly regulated by cell-cycle inhibitors such as p21 and p27 proteins. P21 is involved in growth arrest induced by cell-cycle checkpoints, senescence, or terminal differentiation. Following anti-mitogenic signals or DNA damage, p21and p27 bind to cyclin-CDK complexes to inhibit their catalytic activity and induce cell cycle arrest [27].

P21 consider as indicator of cell death by inhibition of proliferation and cell cycle arrest after application of DOX through highly expression of p21 from the inner to the outer membrane of both cancerous cells. This refers to the ability of DOX to inhibit proliferation of cells due to again strictly dependent on FHL2 expression, the protein responsible for an up- regulation of p21in cancer cells in response to DOX treatment [28].

Due to the expression of p21 impedes DOX-induced cell death by inactivation of cyclin-dependent kinase (CDK) activity, which in turn blocks the cell cycle at the G1 and G2 phases. The present findings here reinforced this idea by showing p21's ability of abrogating DOX-induced cell death correlated with its inhibition of cell cycle progression after reducing p65 in p53 cells [29].

A previous study showed that MET, also , have the ability to produces dose dependent increase in the expression of P21 to the extracellular membrane in both cancerous cells that is refer to the MET caused cell cycle arrest accompanied by decreased cyclin D1 and increased p21 protein expression also MET can indirectly shows antiproliferative effects as well as directly suppress cell proliferation through cell cycle modulation, up regulation of tumor suppressor genes and by inducing cell death mediated by increased oxidative stress[30] .

The combination of DOX with MET treatment shows enhanced effect in a dose dependent that refers to ability of combination to cause dysfunction of cell cycle checkpoint proteins can alter the progression of the cell cycle, also

dysfunction the G1/S phase proteins cyclin D1, CDK4 and p21. Cyclin D1 and CDK4, which are responsible for the transition from G0/G1 phase to S phase, were down regulated following combined treatment [31]. These results demonstrated that the combined treatment group arrested cancer cells in the G0/G1 phase [32].

Conclusion

From the results, it can be concluded that Metformin have a good cytotoxic and antiproliferative effect on RD cell lines.

Acknowledgment

The authors would like to thank all participants for providing the practice platform of this study.

Conflict of Interest

The authors report no conflicts of interest in this work.

References

1- Cancer Causes, Types, Treatment, Symptoms & Signs2015:28:216.
2- Cell Biology - What are the differences between cancer and tumour? Biology Stack Exchange 2012:8:82.
3- Types of Cancer Treatment [Internet]. National Cancer Institute. 2017.
4- Octavia Y, Tocchetti CG, Gabrielson KL, Janssens S, Crijns HJ, Moens AL. Doxorubicin-induced cardiomyopathy: from molecular mechanisms to therapeutic strategies. J Mol Cell Cardiol. 2012 Jun;52(6):1213–25.
5- Rachel J. Pharmacokinetics of Doxorubicin in Pregnant Women. Cancer Chemother Pharmacol. 2014 April; 73(4): 789–797.
6- Foretz M, Guigas B, Bertrand L, Pollak M, Viollet B. Metformin: from mechanisms of action to therapies. Cell Metab. 2014 Dec 2;20(6):953–66.
7- Shaw RJ . The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science. 2005 Dec 9;310(5754):16426.
8- Miller RA . Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. Nature. 2013 Feb 14;494(7436):25660.
9- Madiraju AK . Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. Nature. 2014 Jun 26;510(7506):542-
10- **Yasunao Kogashiwa.** Combination Chemotherapy for Solid Tumors in Head and Neck February 14, 2013. Journal of Drug Metabolism & Toxicology.
11- Ana Catarina Pinto etal. Combination Chemotherapy in Cancer:Principles, Evaluation and Drug Delivery Strategies 2016. Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra Portugal.
12- Apoptosis: A Review of Programmed Cell Death. [cited 2018 Oct 25].
13- Koopman G. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood. 1994; 84(5):1415-1420.
14- Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer. 2003 Oct;3(10):721–32.
15- Pucci B, Kasten M, Giordano A. Cell Cycle and Apoptosis. Neoplasia N Y N. 2000 Jul;2(4):291–9.
16- PDF) Links between apoptosis, proliferation and the cell cycle. ResearchGate.
17- Jimmy R.Thériault . Inhibition of the Unfolded Protein Response by metformin in renal proximal tubular epithelial cells. Biochemical and Biophysical Research Communications Volume 409, Issue 3, 10 June 2011, Pages 500-505.
18- Wilkes GM. Targeted Therapy: Attacking Cancer with Molecular and Immunological Targeted Agents.

Asia-Pac J Oncol Nurs. 2018;5(2):137–55.
19- Ying Li . Metformin synergistically suppress tumor growth with doxorubicin and reverse drug resistance by inhibiting the expression and function of P-glycoprotein in MCF7/ADR cells and xenograft models. Oncotarget. 2018 Jan 5; 9(2): 2158–2174.
20- Bruna Pucci . Cell Cycle and Apoptosis. Neoplasia. 2000 Jul; 2(4): 291–299.
21- Mohamed Hassan . Apoptosis and Molecular Targeting Therapy in Cancer. Biomed Res Int. 2014; 2014: 150845.
22- Susan Elmore . Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol. 2007; 35(4): 495–516.
23- Coxon KM . Purification of annexin V and its use in the detection of apoptotic cells. Methods Mol Biol. 2011; 731:293-308.
24- Patel N Silymarin modulates doxorubicin-induced oxidative stress, Bcl-xL and p53 expression while preventing apoptotic and necrotic cell death in the liver. oxicol Appl Pharmacol. 2010 Jun 1;245(2):143-52.
25- Kehn K, Deng L, de la Fuente C, Strouss K, Wu K, Maddukuri A, et al. The role of cyclin D2 and p21/waf1 in human T-cell leukemia virus type 1 infected cells. Retrovirology. 2004 Apr 13; 1:6.
26- Zhi-Peng Ji . Transcription activated p73-modulated cyclin D1 expression leads to doxorubicin resistance in gastric cancer. Exp Ther Med. 2018 Feb; 15(2): 1831–1838.
27- Nahla E El-Ashmawy . Metformin augments doxorubicin cytotoxicity in mammary carcinoma through activation of adenosine monophosphate protein kinase pathway. May 1, 2017.
28- Coqueret O . New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol. 2003 Feb;13(2):6570.
29- Li X . Targeting of cell cycle and let-7a/STAT3 pathway by niclosamide inhibits proliferation, migration and invasion in oral squamous cell carcinoma cells. Biomed Pharmacother. 2017 Dec;96:434-442.
30- ShenglinMa . Induction of p21 by p65 in p53 null cells treated with Doxorubicin. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. May 2008, Pages 935-940.
31- Bukholm IK . Protein expression of p53, p21 (WAF1/CIP1), bcl-2, Bax, cyclin D1 and pRb in human colon carcinomas. Virchows Arch. 2000 Mar;436(3):224-8.
32- Xianbin Cai . Metformin Induced AMPK Activation, G0/G1 Phase Cell Cycle Arrest and the Inhibition of Growth of Esophageal Squamous Cell Carcinomas In Vitro and In Vivo. PLoS One. 2015; 10(7).