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Research Article

Safe Combination of Cisplatin and Metformin Reverts the Malignant Ascites in a Mouse Model to a Solid Tumor by Downregulation of ΔNp63 and Induces Tumor Dormancy via m TOR/ p21 Mechanism

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Abstract

Currently, combination therapy has become the cornerstone of cancer treatment. The combination of different anti-cancer mechanisms can induce tumor cell quiescence. However, toxicity to normal tissue is the major limitation of existing combined drugs. In this study, Ehrlich ascites carcinoma (EAC) inoculated into mice was targeted with just one dose of cisplatin and later doses of metformin, a safe anti-diabetic drug with an anti-cancer effect, to maintain EAC cells in the quiescent state and secure a longer survival time without tumor recurrence. The group that underwent dual therapy had developed a delayed solid tumor instead of a malignant ascites. Induction of chemo-quiescence in the EAC cells was proven by downregulation of mechanistic target of rapamycin (*mTOR*) and upregulation of cyclin- dependent kinase inhibitor 1 (*p21*) expressions. Intriguingly, the conversion of free neoplastic cells into a solid tumor was associated with a significant decrease in ΔNp63 immunostaining in EAC cells. Taken together, a single dose of cisplatin followed by metformin doses could overcome the aggressiveness of malignant ascites by the conversion into a solid tumor, induction of chemo-quiescence and extension of survival time.

Keywords

Chemo-quiescence; Ehrlich ascites carcinoma; mTOR; Metformin; Cisplatin; ΔNp63.

Abbreviations

EAC: Ehrlich Ascites Carcinoma; *mTOR*: mechanistic Target Of Rapamycin; *p21*: Cyclin dependent kinase inhibitor.

Introduction

By 2040, 27.5 million new malignancy cases are expected every year worldwide if the recent rate of cancers incidence and population development continues in the future [1]. In many types of malignancy, ascites is a prognostic sign of advanced stage, with only 11% of patients survive to more than six months. [2]. Combination of therapeutic modalities has become the cornerstone of cancer therapy [3]. Basically, the combined agents work in a synergistic or additive effect, and thus, the required therapeutic dose of each agent is low [4]. The treatment with multiple drugs increases the opportunity of targeting all cancer cells including cancer stem cells that accused of drug resistance and cancer recurrence [5]. In contrast, the treatment with a constant single compound activates alternative salvage pathways in the cancer cell which confers a subsequent drug resistance [6]. Unfortunately; the majority of existent combined chemotherapeutic drugs for cancer treatment are still limited by their toxicity to healthy cells [7]. Another challenge is that many chemotherapeutic drugs induced tumor dormancy, where cancer cells are in the quiescent state, i.e. G0. They tend to resume proliferation when the general environment becomes available, resulting in tumor relapse. To overcome these problems, there is a strategy called "locked-in", which pharmacological agents can be used to maintain cancer cells in the quiescence (G0) state to prevent further tumor growth, recurrence, and/or metastasis throughout the lifetime of a patient [8]. The pursuit of safe and alternative chemo-quiescence adjuvants work in different anti-cancer mechanisms for combination therapy becomes necessary.

Currently, there is a tendency to using a category of pharmaceutical agents as anti-cancer drugs although they are primarily used for other therapeutic purposes [9]. Fortunately, a great benefit is associated with such an approach because existing drugs would have already undergone FDA procedures of drug safety and have identified pharmacokinetic properties [10]. Metformin, the first line drug for diabetes type 2, can acts as a safe anti-cancer agent through decreasing glucose utilization, the fuel for tumor initiation and growth [11]. Also, it suppresses *mTOR* activity, a major regulator of cell growth, proliferation, and survival, which is highly expressed in malignant tissue [12,13]. There are initial indications of activating cellular quiescence by metformin, the mechanism that reduces glucose uptake by malignant cells leading to cell cycle arrest [14].

Cisplatin is the alkylating agent that has been used for decades to treat many cancers such as ovary, neck, lung, testis, head, and breast cancer. However, it causes toxicity to bone marrow, hair, stomach [15]. The main dose-limiting toxicity of cisplatin is nephrotoxicity [16]. Furthermore, drug resistance has been observed in many cisplatin-treated patients who have relapsed in later years after remission [17]. Correspondingly, the combination therapy of cisplatin has become the mainstream of several cancer treatments to reach the desired therapeutic effect with low toxicity and resistance possibility [18].

Combating cancer cells with serial non-toxic therapeutic agents that differ in the mechanisms of action enhances the treatment efficacy with a low chance of tumor recurrence in later years and extending the survival rate. Herein, we targeted EAC- bearing mice with one dose of cisplatin and subsequent doses of metformin, to induce EAC quiescence and improve the survival time.

Materials and Methods

Drugs and chemical reagents

Cisplatin was purchased from Mylan (10 mg/10 mL vial; Saint-Priest, France). Metformin from (Sigma-Aldrich, St. Louis, MO, USA)

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was dissolved in sterile water to make a solution of a concentration of 0.15 M to be used in the experiment. All other chemicals/reagents were of analytical grade.

Animal care and handling

A total of 75 female Swiss albino mice aged 6–8 weeks and weighed 18–25 g was purchased from National Research Center (Cairo, Egypt). In wire cages, mice were undergone to one-week acclimatization at identical conditions (27 ± 2 °C; 70–80 % humidity; 12-h light/darkness cycle) and supplied with standard pellet diet and water ad libitum. All performed experiments followed the guidelines for the care and use of laboratory animals approved by the University Animal Ethical Committee.

Tumor cell line

Ehrlich ascites carcinoma (EAC) is developed from a high grade of malignant mouse breast adenocarcinoma. As the mice were acclimatizing, EAC cells (1×10^6 cells) obtained from National Cancer Institute (Cairo, Egypt) were transplanted into the peritoneal cavity of a mouse to propagate. After 10 days, ascitic fluid containing EAC cells had developed and cells viability was testedfor in vivo experiments by trypan blue dye exclusion method and counted by a hemocytometer. The percentage of viable cells = [(total number of cells – number of trypan blue positive cells)/total number of cells] *100 [19].

Tumor transplantation and Experimental Design

On day zero, all mice were divided into five groups, 15 mice/group. Four groups were inoculated with 2.5×10^6 EAC cells (0.2 ml PBS/mouse) and one group was left as a normal healthy group and injected intraperitoneally by 200 μL saline for 14 days. 24 hour later, the inoculated groups with Ehrlich cells were classified according to the treatment mode as following: EAC control group was tumorized mice. EAC+Cis group was given one dose of cisplatin. EAC+Met group treated with metformin for three consecutive days. EAC+Cis+Met was represented the group received the dual-combination treatment which involved one dose of cisplatin then two days interval followed by metformin doses for the next three days. All tested therapeutic agents are injected intraperitoneally. Cisplatin was injected at dose of 3.5 mg/kg while the metformin dose was 200 mg/kg. All groups treated daily with 0.2 ml saline solution after 24 hourss of the last therapeutic dose until 14th day post tumor inoculation..

Sampling

On 15th day, six mice from each group were anesthetized, blood was collected via cardiac puncture, and then mice were killed by cervical dislocation. Peritoneal fluids were collected from the groups that had ascitic fluids for immediate measurement of tumor growth parameters (volumes of ascitic fluids and cells viability), and then EAC cells were isolated by centrifugation (2,000 rpm for 10 minutes at 4 °C) and divided for later experiments. Also, organs were preserved in 10% neutral buffer formalin for the histopathological investigation. The remaining mice in each group (n = 9 mice/ group) were left alive to estimate the mean survival time (MST) [20]. On 40th day, 3 mice from the group that had developed a solid tumor were dissected to collect solid tumors for cell cycle analysis, immunohistochemistry and histopathological examination. Total experimental period was 50 days.

QPCR analysis

On the 15th day, 4×10^6 cells were isolated from groups had ascitic fluid, washed and suspended in a cold 1ml PBS for immediate gene expression analysis. Total RNA was extracted from 4×10^6 cells using a TRIzol™ Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA, cat. no. 12183555) according to the manufacturer's instructions. The extracted total RNA was quantified by measuring the optical density at 260 nm using a spectrophotometer. The expression of p21, mTOR mRNA and GAPDH mRNA as a housekeeping gene was estimated according to the manufacturer's instructions of The One-Step RT-PCR Kit (Power SYBR® Green RNA-to-CTTM 1-Step Kit, Applied Biosystems, USA). The following primer pairs were designed using online Oligoperfect Designer Software (Thermo Fisher Scientific, USA) and their specificity was checked by BLAST analysis. (p21 forward, 5'-ACGGTGGAACTTTGACTTCG-3' and reverse, 5'-GAGTGCAAGACAGCGACAAG-3'; *mTOR* forward, 5'-CGTCACAATGCAGCCAACAA-3' and reverse, 5'-TGCCTTTCACGTTCCTCTCC-3'. GAPDH forward 5'-ATGGTGAAGGTCGGTGTGAAC-3' and reverse, 5'-TTGATGTTAGTGGGGTCTCGC-3'. The relative expression of the gene amplification product was calculated using the using $2^{-\Delta\Delta Ct}$ method [21].

Cell cycle analysis by flow cytometry

2.5×10^6 EAC cells were collected from groups that had ascitic fluids on the 15th day, besides solid tumors that excised from (EAC+Cis+Met) group on 40th day were fixed in 1ml ice cold absolute alcohol and preserved at +4°C for cell cycle analysis according to the Vindeløv's method [22]. The samples were run in a FACS Calibur system (BD, Sunnyvale, CA, USA). Data analysis was conducted using DNA analysis program MODFIT (verity software and CELLQuest software (version 3.3; Becton Dickinson).

Immunohistochemical analysis

EAC cells collected on the 15th day and Ehrlich solid tumors excised on the 40th day were fixed in formalin and paraffin embedded for Immunohistochemistry [23]. Three-micron of tissue sections were tested for the immune-histochemical detection of antigens with anti- ΔNp63 antigen (Clone 4A4, BioGenex,

USA, cat.no. AM418-5M). Tissue sections visualized with Ultra vision LP detection system: HRP polymer/DAB plus Chromogen (TL-015-HD; Thermo scientific). Images were obtained with a light microscope (Binocular, Olympus Microscope; Shinjuku, Tokyo, Japan) for IHC quantification. The percentage of yellow ΔNp63 staining was evaluated with image J software (version 1.48, 32 bit).

Biochemical assays in serum and tumor homogenate

Serum samples were obtained by centrifugation at 3000 rpm/min for 20 min and stored at −20 C° for biochemical analysis. Creatinine, urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total proteins were estimated in serum using commercial kits (Biodiagnostic Company for Laboratory Services, Giza, Egypt). Also, the levels of glutathione (GSH) and superoxide dismutase (SOD) were measured in the collected EAC cells using assay kits (Biodiagnostic Company for Laboratory Services, Giza, Egypt).

Histopathology analysis

Cytological changes in lungs, kidneys and liver were studied by hematoxylin and eosin staining according to the method of Bancroft [24]. Organs were collected from all mice groups wither those were killed on the 15th or 40th day washed in PBS and to remove the blood. Tissues were then fixed in 10% neutral buffered formalin for 24 hours blocks of tissues were prepared, sectioned, and stained

with hematoxylin–eosin then examined with a light microscope (Binocular, Olympus Microscope; Shinjuku, Tokyo, Japan).

Statistical analysis

All data are expressed as mean ± SD. One-way analysis of variance (ANOVA) followed by a Tukey's test was used to assess significant differences among all groups using GraphPad Prism Software 5 (LaJolla, CA, USA). *P values were considered statistically significant at* $P < 0.05$.

Result

Metformin enhances the anti-tumor potential of one dose of Cisplatin on EAC cells

To assess the anti-tumor effects of metformin and cisplatin against EAC cells, changes in tumor volume, tumor cells viability and mean survival time of treated groups were observed **Figure 1**. EAC-bearing mice treated with the combination therapy showed no ascitic fluid volume on the day 15 post tumor inoculation compared to the other groups **Figure 1A**. There was a delayed tumor recurrence on day 25 as a solid tumor instead of developing peritoneal fluid containing tumor cells. Regarding cells viability, cisplatin and metformin displayed a significant decrease in EAC cells ($P < 0.05$) reached to 83.39 ± 1.64% and 97.16 ± 1.15% respectively compared to the EAC control group **Figure1B**. Subsequently, the treatment with the combination treatment showed a significant increase in MST to 50 days compared to the control and other treated groups.
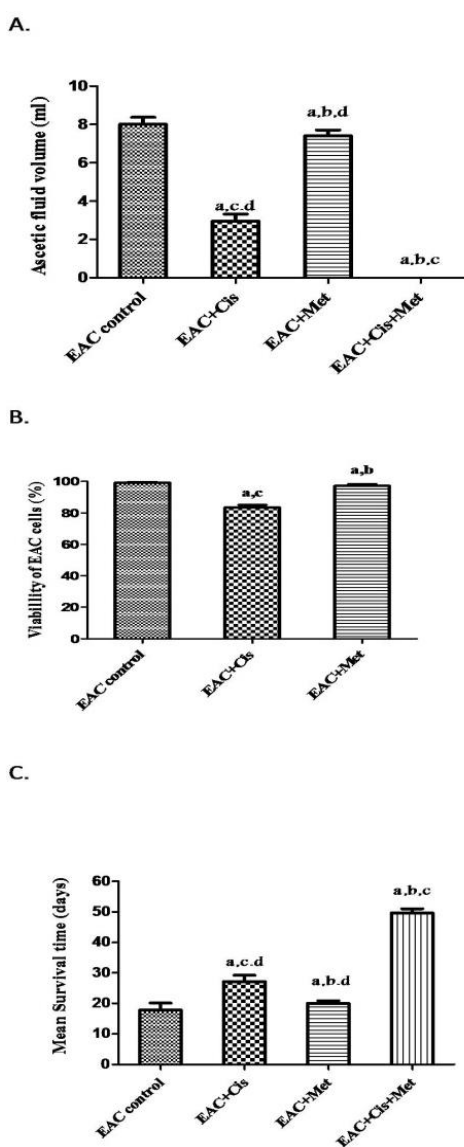

Figure 1: Effect of metformin and cisplatin as single drugs or in combination on the tumor progression parameters of EAC-bearing mice. A. Ascetic fluid volumes (ml) collected from the groups that had ascetic fluids on the 15th day post tumor inoculation. B. EAC cells viability% in peritoneal fluids from the control and treated groups. C. Mean survival time (MST) of the treated groups within the experimental period (50 days). Data presented as a mean±SD of six animals .P ≤ 0.05 compared to all groups. Cis, cisplatin. Met, metformin
a : significant versus EAC control group.b: significant versus EAC+Cis group.
c: significant versus EAC+Met group.
d. significant versus EAC+Cis+Met group

Effect of metformin and cisplatin on relative expression of mTOR and P21 genes in EAC cells

There was a reversal relationship between *p21* and *mTOR* expressions after treatment with cisplatin or metformin. Cisplatin that was injected as one dose and metformin caused a long-term significant increase of *p21* expression and a significant decrease in *mTOR* expression compared to EAC control group **Figure 2**.

Metformin and Cisplatin induce cell cycle arrest in G0-G1 phase

The used treatments significantly increased arrest in the G0/G1 phase compared to EAC control group. Cisplatin arrested 75.4% of EAC cells in G0/G1, while metformin arrested 57.7% of EAC cells and 19.2% of cells were apoptotic cells **Figure 3A, B, C**. After 40 days of tumor inoculation, the analysis of cell cycle of the solid tumor showed just 32.2% of tumor cells remained in the quiescent state, with 37.2% proliferating cells and highest percentage of apoptotic cells among the other treated groups reached to 28.3% **Figure 3D**.

Inhibitory effect of metformin and/or Cisplatin on ΔNp63 level

In **Figure 4**, immunohistostaining analysis of ΔNp63 in EAC cells showed a strong positive yellow immunolabelling for ΔNp63 in multiple EAC control cells **Figure 4A**. Mild was seen in cisplatin-treated cells and moderate in few EAC cells treated with metformin **Figure 4B and C**. However, after 40 days of tumor inoculation, Cis+Met group showed a strong positive immunolabelling for ΔNp63 in the capsule of the solid tumor **Figure 4D**, surrounding muscle

Figure 4E and adipose tissue **Figure 4F**.

Metformin and cisplatin effect on antioxidant enzymes In EAC cells

Cisplatin and metformin as single drugs inhibited SOD and GSH levels significantly ($P<0.05$) in the EAC cells compared to that of EAC control group **Figure 5**. The effect of cisplatin and metformin as a combination therapy would be higher.

Assessment of kidney and liver functions after treatment with metformin and cisplatin.

Data presented in **Table 1** demonstrated that serum of Cis+Met group showed most significant changes in the tested serum parameters compared to other groups. There was an elevation in ALT, AST and creatinine concentrations and a decrease in total proteins and urea ($P<0.05$) compared to other groups. In general, serum of EAC-bearing mice showed a significant change compared to the normal mice ($p<0.05$).

Histopathology findings

In our histopathological results, as the cytotoxicity of drugs increases, the number of infiltrative tumor cells increase. Cis+Met treated group showed a high EAC metastasis compared to a single drug-treated groups in liver and lungs and kidney of the treated group **Figure 6, 7, 8**. The other treated groups showed no tumor infiltration. In general, the number of infiltrative tumor cells and damaged tissue structure had not been increased on the 40th day after tumor inoculation.
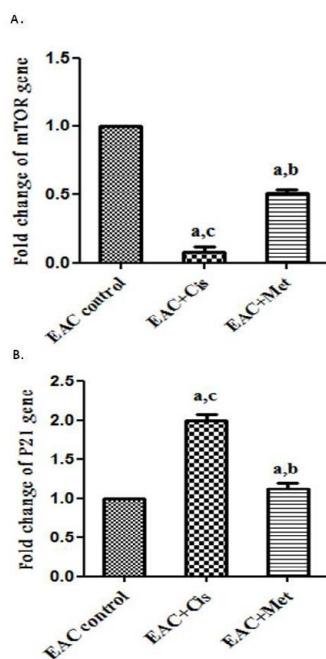

Figure.2: Synergistic Effect of metformin and cisplatin on the relative expression of mTOR and p21 genes EA*C cells*. A. fold change of *mTOR* and B. *p21* in EAC cells collected on the 15th day post tumor inoculation the data are normalized to GAPDH (internal control). the results are expressed as a mean± SD of six animals. P ≤ 0.05 compared with all groups. Cis, cisplatin. Met, metformin.

a : significant versus EAC control group. b: significant versus EAC+Cis
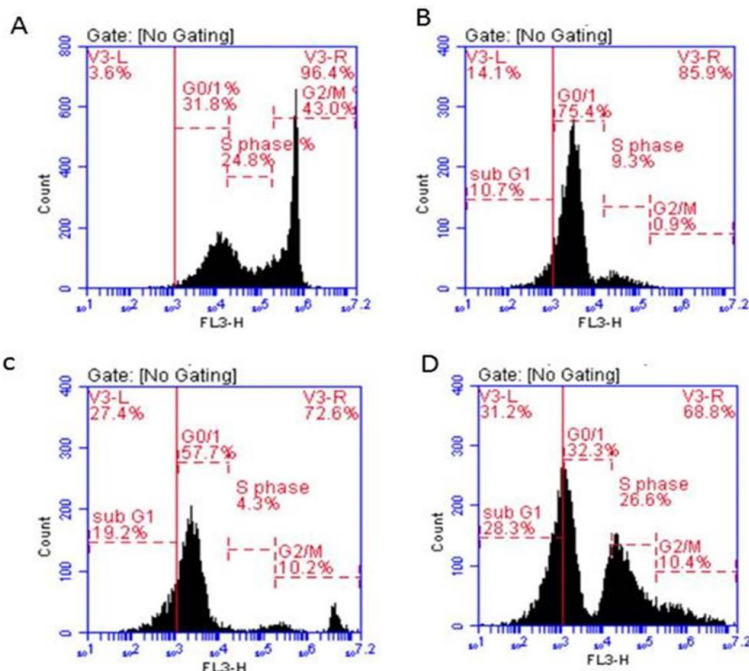
group.c: significant versus EAC+Met group.

Figure 3: Cisplatin and metformin arrest the majority of EAC cells in the quiescent phase G0/G1.DNA histograms show EAC cells distribution in cell cycle phases in the control group (A), groups treated with cisplatin (B), metformin (C) and the combined drug (D) . Numeric data shows the proportions of cells in different cell-cycle phases.
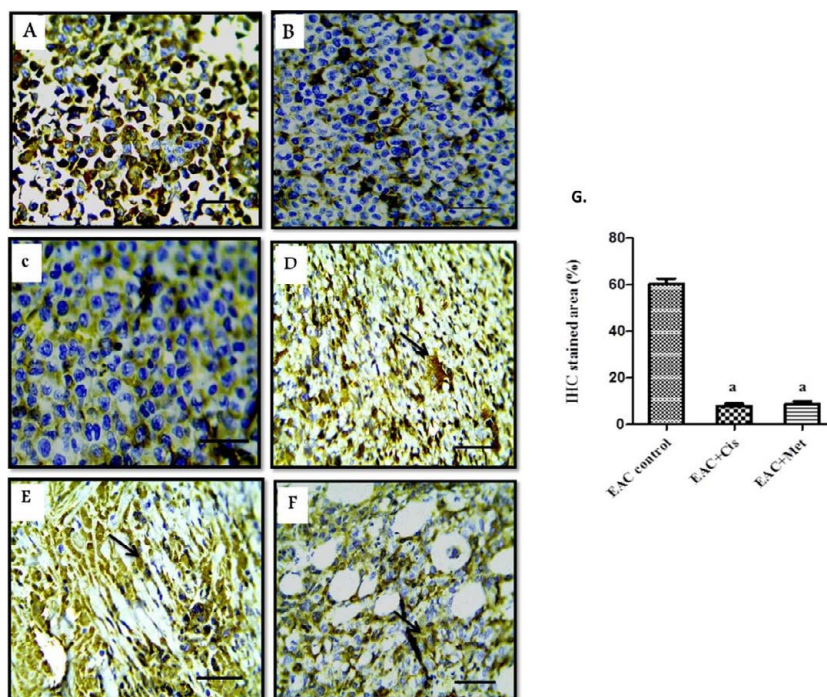

Figure 4: Low ΔNp63 immunostaining induced by metformin and cisplatin in Ehrlich ascites on the day 15 or solid tumor on day 40. A.an EAC control group with a strong positive yellow immunolabelling in multiple EAC cells. B. group treated with cisplatin shows a mild positive yellow immunolabelling for ΔNp63 in aggregation of EAC cells.C. EAC cells treated with metformin has a moderate positive immunolabelling for ΔNp63 in few EAC cells. D. solid tumor from a group received the combined treatment had a strong positive immunolabelling for ΔNp63 is seen in capsule of EAC cells (arrows). E. in surrounding muscle (arrows). F. and in adipose tissue (arrows). IHC counterstained with Mayer's hematoxylin X 200 bar 100. G. Comparison between the stained areas of ΔNp63 in EAC control group and the groups that has ascitic fluids on day 15 using Image J. Stars means significant when p<0.05.
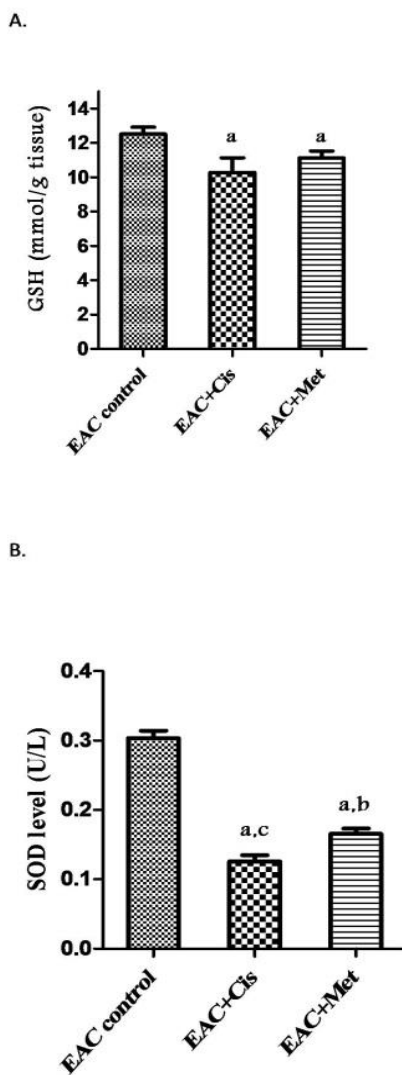
Figure 5: Cisplatin and metformin inhibit SOD and GSH enzymes in EAC cells.

GSH and B. SOD levels in EAC cells treated by metformin or cisplatin that collected on the 15th day after tumor inoculation. Data presented as mean ± SD of six animals. $P \leq 0.05$ compared to all groups. Cis, cisplatin. Met, metformin. GSH, reduced glutathione. SOD, superoxide dismutase.

a significant versus EAC control group.

b: significant versus EAC+Cis group.

d: d: significant versus EAC+Met group.

Table 1: the effect of cisplatin and metformin as single drugs or in combination on the level creatinine, urea, ALT, AST, and total proteins in serum of the different groups.

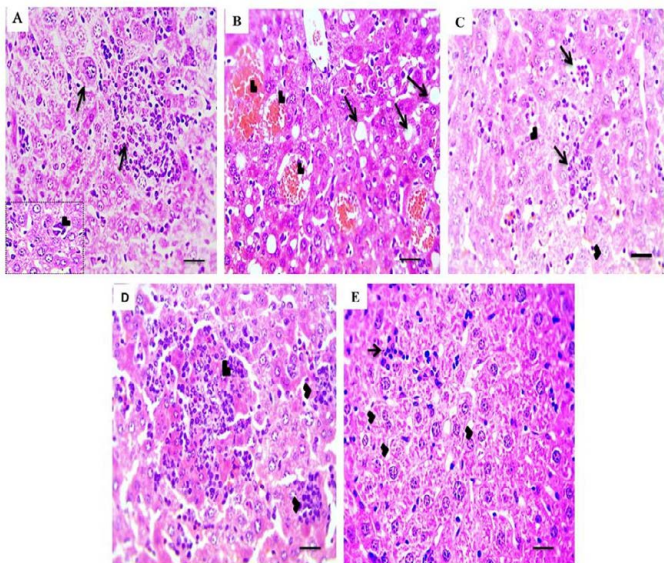| EAC+Cis+Met	EAC+Met	EAC+Cis	EAC control	Normal	
2.79±0.15[a,b,c]	2.1±0.07[a,b,d]	2.48±0.26[a,c,d]	1.53±0.09[#]	0.54 ± 0.06	**Creatinine (g/dl)**
56.53±0.85 [a,b,c]	63.06±0.14[b,a,d]	66.65±0.80[a,c,d]	62.43±0.56[#]	43.76±2.21	**Urea(mg/dl)**
3.75±0.12 [a,b,c]	4.66±0.07[a,b,d]	5.00±0.54[a,c,d]	4.18±0.39[#]	6.08±0.16	**Total protein (g/dl)**
81.58±0.38 [a,b,c]	71.00±0.62 [a,b,d]	68.27±0.46[a,c,d]	76.66±0.46[#]	27.44±0.9	**ALT(U/L)**
141.30±0.96[a,b,c]	130.00±1.73[a,b,d]	125.22±1.05[a,c,d]	138.7±0.57[#]	93.85±0.52	**AST(U/L)**

Figure 6: Assessment the effect of metformin and/or cisplatin on liver histopathology after 15 days and 40 days of tumor inoculation.

A. Liver of untreated EAC group shows activation of kupffer cells with eosinophilic intranuclear inclusions (arrowhead), sinusoidal infiltration of single and clumps of tumor cells mixed with lymphocytes (arrows).

B. Liver of cisplatin treated mice shows cytoplasmic vacuolation (arrows) sinusoidal congestion (arrowheads).

C. liver of metformin treated group shows dilation of sinusoids with clumps of tumor cells (arrows) with hydropic degeneration of hepatocytes (arrowheads).

D. On the 15th day, liver of Cis+Met group shows focal areas of necrosis infiltrated with mononuclear cells, neutrophils and clumps of EAC cells (arrowheads) H&E X: 200 bar 50.

E. On the 40th day, liver treated with the dual therapy shows aggregation of tumor cells and leukocytes in sinusoids (black arrow) and mild hepatocytes swelling due to degeneration (arrowheads). H&E X:400 bar 50
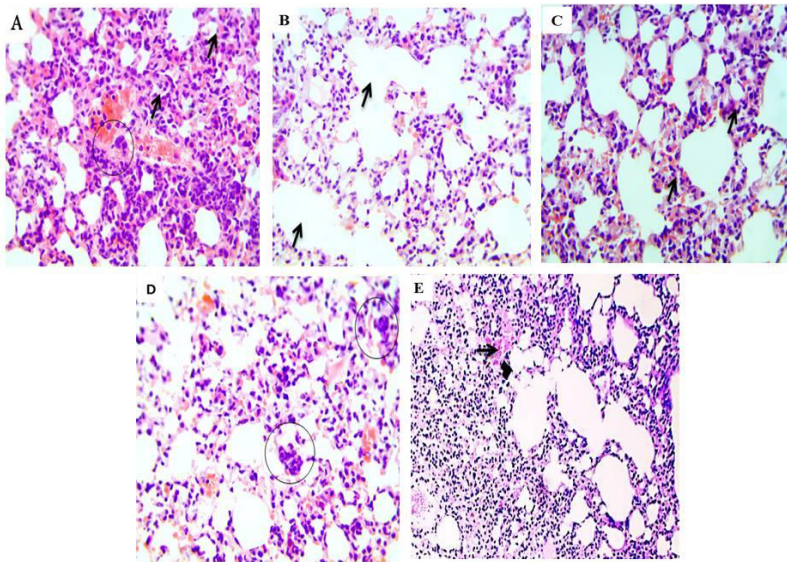

Figure 7: Assessment of metformin and/or cisplatin effects on lungs histopathology after 15 days and 40 days of tumor inoculation.

A. On the 15th day, lung of untreated EAC-bearing mice displays a marked thickening of interstitial tissues with numerous areas of cellular infiltration, fibroblasts proliferation and congested blood vessels (arrows) and perivascular small clusters of tumor cells (circle).

B. Lung of cisplatin -treated mice shows focal alveolar emphysema (arrows).

C. Lung of metformin-treated mice showed mild interstitial thickening (arrows).

D. group treated with the dual treatment shows a mild thickening of interstitial tissues with presence of single tumor cells (circle) H&E X: 200 bar 50. E. On the day 40, a group received the dual treatment shows congestion (black arrows), alveolar emphysema (arrow head), H&E X:100 bar 100.
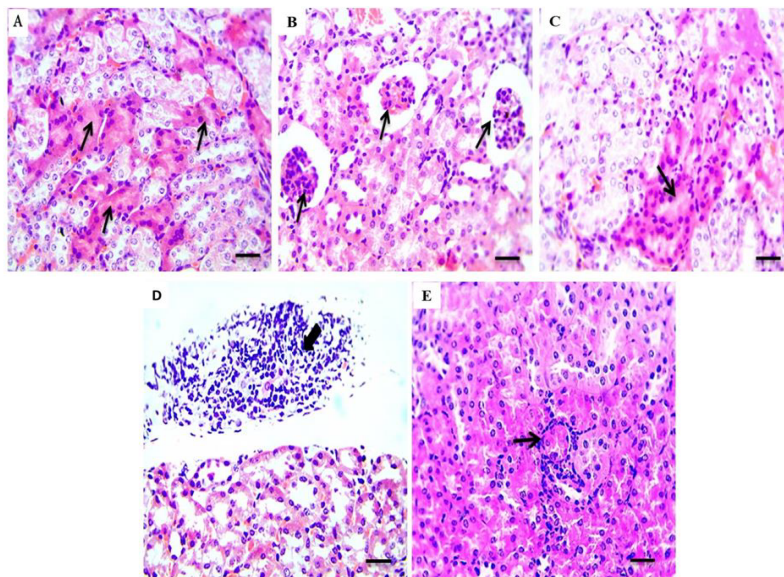
Figure 8: Assessment the effect of metformin and/or cisplatin on kidneys histopathology after 15 days and 40 days of tumor inoculation. On the 15th day,

A. kidney of EAC control group shows tubular necrosis (arrows), swollen and congested glomeruli (arrowheads). **B.** Kidney of cisplatin- treated mice shows shrunken collapsed glomeruli (arrows).

C. Kidney of metformin treated group show tubular necrosis.

D. Kidney of Cis+met- treated mice shows few tumor cells admixed with neutrophils and lymphocytes adherent to the capsule of kidney without invasion into parenchyma (arrow) H&E X: 200 bar 50.

E. On the 40th day, kidney treated with the dual therapy shows perivascular aggregation of tumor cells and leukocytes around renal tubule (black arrow) X: 400 bar 50

Discussion

Malignant ascites develops in last stage of several cancers as a sign of the treatment failure to control cancer progression [25]. Currently, combination therapy is a solution to overcome drug resistance and enhance treatment efficacy [26, 27]. However, the damage to healthy tissue and drug resistance associated by multiple doses of the existing anti-cancer drugs averts the combination therapy benefit [28]. Combining an effective one dose of the anti-neoplastic drug with safe agents that act in different anti-cancer mechanisms may provide the optimum treatment. In our study, just one dose of cisplatin followed by metformin doses could reverse the malignancy of free neoplastic cells in peritoneal ascites fluid to a solid neoplasm leading to extended survival time. Ascitic fluid is the direct source of nutrients for tumor cells and as the tumor cells proliferate rapidly, the volume of ascites fluid increases at the same levels [29]. In the present study, the volume of ascitic fluid reflected the low proliferation rate and viability of cells treated with metformin and cisplatin. The therapeutic potential of metformin and cisplatin in combination was observed in human ovarian cancer cells [30] and hepatocarcinoma cells [31].

Cell quiescence is a reversible G0 phase from which cells may back to the cell cycle under physiological stimuli[32]. The quiescent cancer stem cells protect cancer cells from anti-proliferating agents. Awakening of these dormant cancer cells leads to tumor recurrence which may occur after long periods [33]. It is generally believed that *mTOR* inhibition maintains quiescence and suppresses senescence (geroconversion). High level of p53 response inhibits mTOR, which favors quiescence over senescence [34]. p21 is a downstream target gene of the tumor suppressor p53 [35]. It promotes quiescent state by blocking G1 progression under serum stimulation [36]. Our RT-

PCR results revealed that one dose cisplatin alone caused a long-term inhibition of the *mTOR* expression in malignant ascites cells. In addition, the results demonstrated that cisplatin increases the expression of *p21*, the matter that confirmed by previous publications [37,38]. Metformin acts as anticancer by its downregulation of *mTOR* and activation of AMPK [39].

In the present study metformin upregulated *p21* expression. This is in agreement with Cai, who observed the upregulation of *p53, p27* and *p21* in a xenograft model of esophageal squamous cells carcinoma treated with metformin [40]. Thus, the cell cycle arrest at G0/G1 induced by metformin and cisplatin in combination was through inhibition of *mTOR* and activation of *p21*. Also, the results provided an explanation for the reason of delayed tumor development by the combined treatment.

ΔNp63 isoforms are a class of p63, which bind to TAp63, p53, p21, and TAp73 and repress their functions, thus acting as oncogenes [41, 42]. Recently, it has been reported that ΔNp63 expression can deregulate tumor cell migration and tumor invasiveness [43,44]. In the present study, the development of solid tumors instead of ascitic fluids confirmed the combined effect of cisplatin and metformin to inhibit ΔNp63, leading to restriction of cancer progression. This is similar to the results of Yun-Feng He et al., that revealed that the ΔNp63 silencing can promote the adhesiveness of the human bladder carcinoma cell line 5637 cells by activating F-actin cytoskeleton synthesis [45].Cancer cells are known for their high level of reactive oxygen species (ROS) which play a pivotal role in cancer progression [46]. However, excessive ROS level can be toxic to cancer cells, the reason behind many trails for developing many ROS generating agents and antioxidant inhibitors [47] .Herein, EAC cells treated with

cisplatin had low SOD and GSH levels.

This agreed with previous studies [48,49]. Also, metformin depleted GSH and SOD levels. In accordance with this result, it was found that metformin acts as a pro-oxidant via downregulation of intracellular glutathione, inhibition of proliferation and induction of apoptosis of esophageal squamous cancer cells [50]. Therefore, the combined effect of both drugs caused further accumulation of ROS leading to kill EAC cells.

Previously, it has shown that EAC cells are metastasized to liver, kidney, lungs, spleen, diaphragm, bone, blood and adrenal glands [51]. In this work, metastasis increased among the treated groups as the cytotoxicity of the drug increased EAC cells invasion to liver, kidney, and lungs were marked with the combined therapy treated group. This can be attributed to chemotherapy increasing the invasion of cancer cells [52, 53]. Herein, as the Ehrlich cells metastasized to the surrounding tissues, they lost their malignant capacity. The histopathology results did not show increasing numbers of EAC cells after 40 days post tumor inoculation. Also, it has observed that most EAC cells infiltrating into the liver may die or may become "dormant in the liver [53].

Although cisplatin is known for its nephrotoxicity, which is dose-limiting toxicity. In the present investigation, there were no acute toxicity or histopathological complications in the lungs, liver, and kidney even after 40 days. On other words, the tissue damages were reversible and associated with significant changes in biochemical parameters in serum of the dual therapy. These findings supported that the tested combination treatment in the aforementioned doses is safe.

In summary, the value of this work was the restriction of a malignant ascites in a solid tumor in EAC xenografts by inhibition of p63 level, induction of chemo-quiescence by inhibition of mTOR and upregulation of p21expression besides accumulation of ROS by inhibition of antioxidant system. These effects achieved by non-toxic and existing drugs in combination involved one dose of cisplatin and subsequent doses of metformin results in extending survival time.

Conclusion

SLICER score was not validated in our series since it underestimates the RFS. In order to be applied in daily practice, it must be tested and validated on a larger cohort of patients with different demographic and clinical characteristics and for a longer period of time.

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