Evaluation of anti-tumor activity of metformin against Ehrlich ascites carcinoma in Swiss albino mice

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ABSTRACT
In many types of malignancy, ascites is a prognostic sign of advanced stage, with a survival rate of 11% for patients with ascites more than 6 months. Currently, combination therapy has become the base of cancer treatment. However, cytotoxicity to normal tissue is the major limitation of current combined drugs. In this study, Ehrlich ascites carcinoma (EAC) inoculated into mice was targeted with three consecutive doses of metformin, a safe drug with an anti-cancer effect. To test its suitability as a potential safe candidate against EAC cells for later combination therapy in comparable with cisplatin as a reference anti-neoplastic drug. The group that received metformin developed less malignant ascites than the control group. Metformin induced cellular quiescence in the EAC cells by upregulation of cyclin-dependent kinase inhibitor 1 (p21) expressions as cisplatin acted. Cell cycle analysis confirmed the quiescence state of the EAC cells treated with metformin or cisplatin. Furthermore, metformin-induced toxicity to EAC cells through elevation of reactive oxygen species levels (ROS). Therefore, metformin can be a suitable candidate for future combination with a low dose of cisplatin to treat the aggressiveness of EAC cells.

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Introduction
By 2040, 27.5 million new cases of malignancy are expected annually if the current prevalence of cancer and global population continue to grow at the same rate [1]. In many types of cancer, ascites is a prognostic indication of advanced stage; just of cancer patients who are diagnosed with ascites live beyond a further six months [2]. Combination therapy has become the base of cancer therapy [3]. Fundamentally, the combined agents work in a synergistic or additive manner, and thus, the required therapeutic dose of each agent is low [4]. The treatment with multiple agents enhances the possibility to target all cancer cells including cancer stem cells that is responsible for drug resistance and cancer recurrence [5]. Conversely, the treatment with a constant single therapeutic agent triggers alternative salvage pathways in the cancer cell which confers a subsequent drug resistance [6]. Unfortunately; most of the existing combined chemotherapy drugs for cancer are still relatively limited due to the toxicity effect on healthy cells [7]. The pursuit of safe and alternative chemo-adjuvants work in different anti-cancer mechanisms for combination therapy becomes necessary.

Currently, there is a tendency to recruit the anti-cancer effect of some pharmaceutical agents although they are primarily prescribed for other therapeutic purposes [8]. Fortunately, a great advantage is associated with such an approach because marketed drugs would have already...
passed by FDA procedures of drug safety and have known pharmacokinetic properties [9]. Metformin, the first line drug for diabetes type 2, can be used as a safe anti-cancer agent by suppressing glucose uptake, the fuel for cancer initiation and growth [10]. There are indications of activating cellular quiescence by metformin, through reducing glucose utilization by malignant cells leading to cell cycle arrest [11]. Cisplatin is the alkylating agent that has been used for a long time to treat many cancer types such as neck, ovary, lung, breast, testis, head and cancer. However, it is toxic to bone marrow, hair and stomach [12]. The main dose-limiting toxicity of cisplatin is nephrotoxicity [13]. Furthermore, drug resistance has been observed in many cisplatin-treated patients who have relapsed in later years after remission [6]. Correspondingly, the combination of cisplatin with other anticancer drugs has become the mainstream of cancer treatments to reach the required therapeutic effect with low toxicity and resistance possibility [14].

Targeting cancer cells with nontoxic therapeutic agents differ in the mechanisms of action enhances the treatment efficacy. Herein, metformin was tested as a safe anticancer agent to induce quiescence in EAC cells, through targeting EAC-bearing mice with intraperitoneal three subsequent doses over three days compared to a single dose of cisplatin as a reference anti-cancer drug. In an attempt to find a safe anticancer chemo-adjuvants for future combination with cisplatin to halt the EAC progression.

Materials and methods

Drugs and chemical reagents

Metformin from (Sigma-Aldrich, St. Louis, MO, USA) was solubilized in sterile water to prepare a 0.15 M solution to be used in the experiment. Cisplatin was purchased from Mylan (10 mg/10 mL vial; Saint-Priest, France). All other chemicals/reagents were of analytical grade.

Animal care and handling

A total of 36 female Swiss albino mice aged 6–8 weeks and weighed 18–25 g were purchased from National Research Center (Cairo, Egypt). In cages supplied with standard pellet diet and water ad libitum, mice were acclimatized for 1 week at identical conditions (70–80% humidity; 27 ± 2°C; 12-h light/darkness cycle). All performed experiments were in accordance with the guidelines for the care and use of laboratory animals approved by the University Animal Ethical Committee.

Tumor cell line

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 tested for 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 [15].

Plantation and experimental design

On day zero, mice were inoculated with 2.5 × 106 EAC cells (0.2 ml PBS/mouse). Twenty-four hours later, the inoculated mice with Ehrlich cells were divided (12 mice/group) according to the treatment mode as following: EAC control group was tumorized mice. EAC+Met group treated with metformin for three consecutive days. EAC+Cis group was injected one dose of cisplatin. All tested therapeutic agents are injected intraperitoneally. Metformin dose was 200 mg/kg while cisplatin was given at a dose of 3.5 mg/kg. All groups treated daily with a 0.2 ml saline solution after 24 h of the last therapeutic dose until 14th day post-tumor inoculation.
**Sampling**

On 15th day, mice 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 direct measurement of tumor progression parameters (volumes of ascitic fluids and cells viability), and then EAC cells were isolated by centrifugation (2,000 rpm for 10 min at 4°C) and divided for later experiments.

**qPCR analysis**

4 × 10^6 cells were isolated from groups had ascitic fluid, washed and suspended in a cold 1 ml PBS for immediate gene expression analysis. Total RNA was extracted cells using a TRIzol™ Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA, cat. no. 12183555) according to the manufacturer’s instructions and quantified by measuring the optical density at 260 nm using a spectrophotometer. The expression of p21 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). 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’; GAPDH forward 5’-ATGGTGAAGGTCGGTGTGAAC-3’ and reverse, 5’-TTGATGTTAGTGGGGTCTCGC-3’. The transcript levels of target genes were calculated using the comparative method (2^-ΔΔCt) [16].

**Cell cycle analysis by flow cytometry**

2.5 × 10^6 EAC cells were collected from groups that had ascitic fluids fixed in 1 ml ice cold absolute alcohol and preserved at +4°C for cell cycle analysis according to the method of Vindeløv [17]. The samples were placed in a FACS Calibur system (BD, Sunnyvale, CA, USA). Data analysis was performed using DNA analysis program MODFIT (verity software and CELLQuest software (version 3.3; Becton Dickinson)).

**Biochemical assays in serum and tumor homogenate**

Glutathione (GSH), superoxide dismutase (SOD) levels and malondialdehyde (MDA) were measured in the harvested EAC cells using assay kits (Biodiagnostic Company for Laboratory Services, Giza, Egypt).

**Statistical analysis**

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

**Results**

**Metformin has anti-tumor potential against EAC cells**

To assess the anti-tumor effects of metformin against EAC cells, changes in tumor volume and tumor cells viability of treated groups were observed (Figure 1). EAC-bearing mice treated with metformin showed a significant low ascitic fluid volume on the day 15 post-tumor inoculation compared to EAC control group but not to cisplatin (Figure 1(a)). Regarding cells viability, cisplatin and metformin displayed a significant decrease in EAC cells (P < 0.05) reached 83.39 ± 1.64% and 97.16 ± 1.15%, respectively, compared to the EAC control group (Figure 1(b)).
Effect of metformin p21 gene expression in EAC cells

Three consecutive doses of metformin (200 mg/kg) and cisplatin (3,5 mg/kg) that was injected as one dose caused a long-term significant increase of p21 expression compared to EAC control group (Figure 2).

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

Metformin and cisplatin 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 3(b,c)).

Metformin and cisplatin effect on redox status in EAC cells Cisplatin and metformin as single drugs inhibited the antioxidant enzymes SOD and GSH levels significantly (P < 0.05) in the EAC cells compared to that of EAC control group. Malondialdehyde (lipid peroxidation product) level increased after treatment with metformin or cisplatin (Figure 4).

Discussion

Malignant ascites develops in the advanced stage of several types of cancer as an indicator...
of the treatment failure to control cancer progression [18]. Nowadays, combination therapy is a solution to enhance treatment efficacy and inhibit drug resistance [19,20]. However, the toxicity to healthy tissue and drug resistance associated by regular doses of the existing anti-cancer drugs hinder the combination treatment benefit [21]. Regimen of a low effective dose of the anti-neoplastic drug with safe agents that have anti-cancer effect may provide the optimum mode of treatment. In our study, metformin-induced cytotoxic effect against EAC cells without acute damage to surrounding tissues. That is to say, metformin can be a nontoxic chemo-adjuvant for subsequent combination with low dose of cisplatin to treat EAC aggressiveness.

Ascitic fluid is the direct nutritional source for tumor cells and as the tumor cells proliferate rapidly, the volume of ascites fluid increases at the same levels [22]. In the present study, the volume of ascitic fluid indicated the low viability and proliferation rate of cells treated with metformin or cisplatin.

p21 is a downstream target gene of the tumor suppressor p53 [23]. It promotes cellular quiescence by arresting G1 progression under serum stimulation [24]. Metformin increased p21 expression. This is in agreement with [25], who observed that p53, p27 and p21 upregulation

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). Numeric data show the proportions of cells in different cell-cycle phases.
esophageal squamous cells carcinoma treated with metformin. Similarly, a single dose cisplatin upregulated p21 expression, the matter that confirmed by previous publications [26].

The present cell cycle results indicated that metformin arrested EAC cells at G0/G1 as cisplatin did. Previous reports suggest that cisplatin can upregulate the expression of p53, which activates p21 to arrest the cell cycle in the G0/G1 phase in HHC cells [27]. A study by EA Queiroz et al. has shown that metformin decelerates MCF-7 cells proliferation by promoting G0-G1 cell cycle arrest, inhibiting cyclin D1 and inducing cell apoptosis [28]. Cancer cells are characterized by their high level of reactive oxygen species (ROS) which is considered as an important factor in cancer progression [29]. However, excessive ROS level can be toxic to cancer cells, the reason for developing many ROS generating agents and antioxidant inhibitors [30]. Herein, EAC cells treated with cisplatin had low SOD and GSH levels. This agreed with previous reports [31,32]. Also, metformin
inhibited 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 [33]. As a result of treatment with metformin or cisplatin, malondialdehyde level increased although in accordance with studies demonstrated that cisplatin or metformin-induced toxicity to cancer cells [34,35].

In summary, the anti-cancer properties of metformin in addition to cisplatin resulted in inhibition of proliferation of EAC cells, induction of cell cycle arrest and initiation excessive oxidative stress in EAC cells, suggesting that metformin a good candidate for future combination with a low dose of cisplatin to synergize its effect.

Disclosure statement

No potential conflict of interest was reported by the authors.

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