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Research Article
Metaformin-Based Regimen Inhibits Glucose Uptake and G6PD Activity: A *de novo* Anti-cervical Cancer Strategy Tackles HeLa and its Derivative Hep2 Cells

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Abstract

Background and Objective: Cervical cancer is the second major cancer in women around the word, with an increasing rate of mortality reported in Egypt. Metformin (MT), a first therapeutic line against type 2 diabetes, inhibits various cancer cell proliferations. The signal transduction trails that control the Warburg effect during tumourigenesis remain critical to be discovered. For this aim, metformin's aptitude to inhibit glucose metabolism in cancerous cells may provide a likely profit by restriction of energy capitals and thus affecting cancer cell propagation and maintenance. **Materials and Methods:** Due to cancer is not only a metabolic disease but also a genetic aliment, recently approved safe and potent anticancer candidates have been added, in the current study to the arsenal tackling cervical carcinogenesis, raloxifene (RX) and cytosine β-D-arabinofuranoside hydrochloride (CYT). Cytotoxic screenings of metformin-based regimens against human cervical cancer HeLa cells and its derivative Hep2 cells were performed. The mechanistic effects of these regimens on glucose uptake rate throughout glucose transporters and glucose-6-phosphate dehydrogenase (G6PD) activity upon these cell lines were investigated. **Results:** It is resulted that metformin-combinatorial regimens significantly decrease glucose uptake and inhibit G6PD in HeLa and Hep2 cells, which in turn induce cancer cell death through bioenergetic deprivation and nucleotide biosynthesis defection. **Conclusion:** Metformin-based therapeutic regimens with RX and CYT synergistically work together to tackle cervical cancer *in vitro* via glycolytic blackout, thus we augmented these regimens could provide a *de novo* strategy to overcome cervical cancer chemo-resistance, helping us get closer to the era when cervical cancer is not an pestilence aliment.

Key words: Metformin, glucose uptake, G6PD, HeLa cell line, Hep2 cell line

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cervical cancer is the second supreme distributed cancer in women globally and is also a key element of morbidity and mortality[1]. In many developing nations, cervical cancer, if not eradicated in appropriate time, has been considered as one of the major hostile gynecological cancers[2]. It is mostly perceived during advanced phases (IIB and IIIB) in these nations. Metastasis-associated recurrence is seen in those who have advanced phases of the disease throughout the initial 2 years following therapy completion[3].

Human papillomavirus (HPV) has been planned as an etiological element in the pathogenesis of cervical cancer[2]. Annually, there are unbelievable newly diagnosed cervical cancer cases (approximately 11,000) in the US and approximately 40% of this number undergoes mortality as a consequence of this aliment[4]. The mainstream of cervical cancer-related mortality occurs in developing nations like Egypt, where 25.76 million women over 15 years of age are at peril of evolving cervical cancer. Indeed, it has been assessed that approximately 514 women are spotted with cervical cancer and annually there are 299 cases flit gradually owing to this aliment in Egypt, thus epidemic cancer lines as the second greatest recurrent cancer among women in Egypt[5].

Metformin (MT) is a first therapeutic line used worldwide for type 2 diabetes[6]. Several evidences have suggested that long-term management of metformin may diminish tumor in numerous organs and may inhibit breast, colon and glioma cancer cell proliferation[7]. There have been few researches debating the metformin efficacy against cervical cancer. However, based on its effects on tumor inhibition generally, metformin is prospective to inhibit cervical cancer cell growth[8].

The signal transduction trails that control the Warburg effect during tumourigenesis remain critical to be discovered. For this aim, metformin's aptitude to inhibit glucose metabolism in cancerous cells may provide a likely profit by restriction of energy capitals and thus affecting cancer cell propagation and maintenance[9].

Due to cancer is not only a metabolic disease but also a genetic disease, recently approved safe and potent anticancer candidates have been added, in the current study to the arsenal tackling cervical carcinogenesis, raloxifene (RX) and cytosine β-D-arabinofuranoside hydrochloride (CYT). The RX, a selective estrogen receptor modulators (SERMs) has been described to be a mixed estrogen agonist/antagonist[10], depending on the expressions of Estrogen Receptors (ER), their mediators and regulation of signaling pathways[11]. The RX proved to be a safe candidate for the inhibition of cancer in addition to osteoporosis[12]. The RX inhibits breast and colon cancer-induced animal models[13].

Cytosine β-D-arabinofuranoside hydrochloride (CYT), which is universally branded as cytarabine, is a chemotherapy mediator utilized principally in the cure of cancers of white blood cells such as Acute Myeloid Leukemia (AML) and non-Hodgkin lymphoma, being an efficacious antimetabolite in leukemia therapy. Synonyms of this drug are cytosine and arabinoside, IUPAC name is 4-amino-1-β-D-arabinofuranosylpyrimidin-2(1H)-one. The CYT is the first serious cancer drugs that altered the sugar component of nucleosides. It has a primary amino group on the pyrimidine ring acting as a functional group in ring opening reactions for maleic anhydride containing copolymers. The presence of amino and phenol groups gives both amphoteric and polar character to the molecule. Due to unique chemical structure, CYT can be considered as potent antimetabolite, antiviral, immunosuppressive and antitumor agent[14].

Overall, the current study aimed at cytotoxic screening of metformin-based regimens against human cervical cancer HeLa cells and its derivative Hep2 cells. These therapeutic regimens basically consist of raloxifene (RX) and cytosine β-D-arabinofuranoside hydrochloride (CYT) in combination or not with metformin (MT). In addition, the mechanistic effects of these regimens were studied on glucose consumption rate through glucose transporters and glucose-6-phosphate dehydrogenase (G6PD) activity using HeLa and Hep2 cancer cell lines.

MATERIALS AND METHODS

Cell culture: Human cervical cell line (HeLa) and its derivative (Hep2) were supported by vaccines, sera and drugs (VACSERA, Egypt) which purchased them from American Type Culture Collection (ATCC, USA). The base medium for these cell lines are Eagle's minimum essential medium. To make the complete growth medium, the following components were added to the base medium: 10% fetal bovine serum for growth and 1% penicillin/streptomycin. Cells were propagated in the proper conditions (at 37°C and 5% CO_2) and maintained in the complete growth medium. When the cells are approximately 70% confluent, they were sub-cultured using Trypsin-EDTA. Corning® T-75 flasks were used for subculturing. All cell culture media and reagents were purchased from (Lonza, USA).

Cytotoxicity assay: The cell viabilities of the metformin (MT), raloxifene (RX) and cytosine β-D-arabinofuranoside (CYT) individually or combined were measured by MTT assay using HeLa and Hep2 cells. The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) is based on the

conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity[15]. Briefly, the cells were cultured in 96-well plates at a density of 1×10^4 cells well^{-1}. 0, 20, 40, 60, 80, 100 μM of the chemotherapies were added per well in Eagle's minimum essential medium over cells. Also, media without drug was added as a control. After 24 h incubation, MTT dissolved in PBS was added to each well at a final concentration of 5 mg mL^{-1} and the samples were incubated at 37°C for 4 h. Water-insoluble crystals of formazan that formed during MTT cleavage in actively metabolizing cells were then dissolved in dimethyl sulfoxide (DMSO). Absorbance was measured at 455 nm, using a microplate reader (Model 500, BIORed Instrument Inc., USA). The mitochondrial activity (%) was calculated and compared with the control.

IC_{50} and fold change measurements: The half maximal inhibitory concentrations (IC_{50}) values, the concentrations inhibit 50% of cell viability were obtained by plotting the percentages of cell viability versus the concentrations of the sample using polynomial concentration-response curve fitting models (OriginPro 8 software). Finally, the fold change of the combinatorial chemotherapies; RX+MT, CYT+MT and RX+MT+CYT versus the individual doses of MT in HeLa and Hep2 cells were measured as well.

Glucose consumption rate: Glucose levels in Eagle's minimum essential medium of HeLa and Hep2 cells were measured following treatment with metformin-combinatorial regimens against the individual drugs via glucose detection kit (Spectrum Diagnostics, Egypt). HeLa and Hep2 cells were cultured in 96-well plates at a density of 1×10^4 cells well^{-1}. In the following day, 5 mM glucose (Sigma, USA) was added in the media after 2 h of cells starvation. About 0, 20, 40, 60, 80, 100 μM of the chemotherapies were incubated with HeLa and Hep2 cell lines overnight. Absorbance was measured at 450 nm using a microplate reader (Model 500, BIORed Instrument Inc., USA). The change (%) in glucose uptake levels versus control was calculated.

Glucose-6-phosphate dehydrogenase (G6PD) activity: About 1×10^4 HeLa and Hep2 cells were seeded in 96-well plates and incubated at CO_2 incubator overnight. After 24 h incubation, 0, 20, 40, 60, 80, 100 μM of the chemotherapies (MT, RX and CYT) were added per well in Eagle's minimum essential medium over cells. After 24 h incubation, glucose-6-phosphate dehydrogenase (G6PD) activity was measured upon treated and untreated cervical cancer cells according to the manufacturer's instructions (BioMed Diagnostics, Germany). Briefly, G6PDH working reagent (R1) was added to the sample after its reconstitution in 1 mL of distilled water. Solution was mixed well and incubated for 10 min at room temperature. After adding starter reagent (R2), the solution was mixed again and incubated for 5 min at 37°C. Absorbance was measured at 340 nm using a microplate reader (Model 500, BIORed Instrument Inc., USA). The change (%) in G6PD activity versus control was calculated. The absorbance detection was repeated every 1-3 min.

Bio-statistics: All assays were repeated three times. Comparisons between groups versus controls were made using ANOVA test and values of $p<0.05$ were considered statistically significant using SPSS program. Moreover, the IC_{50} were made using a polynomial fitting of the OriginPro 8 program.

RESULTS

Individual RX and combinational MT+RX therapies significantly reduce cell growth capacity compared to individual MT regimen: The cytotoxicity of metaformin (MT) and raloxifene (RX) individually and in combination were tested on cervical cancer cell lines (HeLa and Hep2) at different concentrations (0, 20, 40, 60, 80 and 100 μM) using MTT assay, as an average of 3 independent runs, in as representative bright fields of cell growth capacity upon IC_{50} treatments, as water insoluble-formazan crystals of MTT upon IC_{50} treatments. Data in Fig. 1a revealed a significant dose dependent decrease ($p<0.05$) in cell viability of HeLa and Hep2 cells upon the individual MT therapy with a high significant dose dependent decrease ($p<0.01$) in cell viability of HeLa and Hep2 cells upon the individual RX as well as combinatorial RX+MT treatment (around 80% cancer cell death). It was noticed that the IC_{50} was undetectable (i.e., >100 μM) upon the MT treatment in both cell lines, while in the IC_{50} of RX was 28.46 in HeLa cell line and 24.74489 in Hep2 cell line. Intriguingly, combining RX to MT decreases the IC_{50}, which in turn, increases cervical cancer cell death (IC_{50} of RX+MT was 46.2 in HeLa cell line and 38.09 in Hep2 cell line) as shown in Table 1. All of these results were confirmed by the bright field and the water insoluble-formazan crystals images.

RX+MT+CYT triple therapeutic regimen presents a promising anti-cervical cancer upon HeLa and Hep2 cell lines: The cytotoxicity of cytosine β-D-arabinofuranoside (CYT) alone, MT combined with CYT and CYT+RX+MT were measured on the two cell lines (HeLa and Hep2). Each regimen was examined at different concentrations (0, 20, 40, 60, 80 and 100 μM) using MTT method, represented in Fig. 2 as an

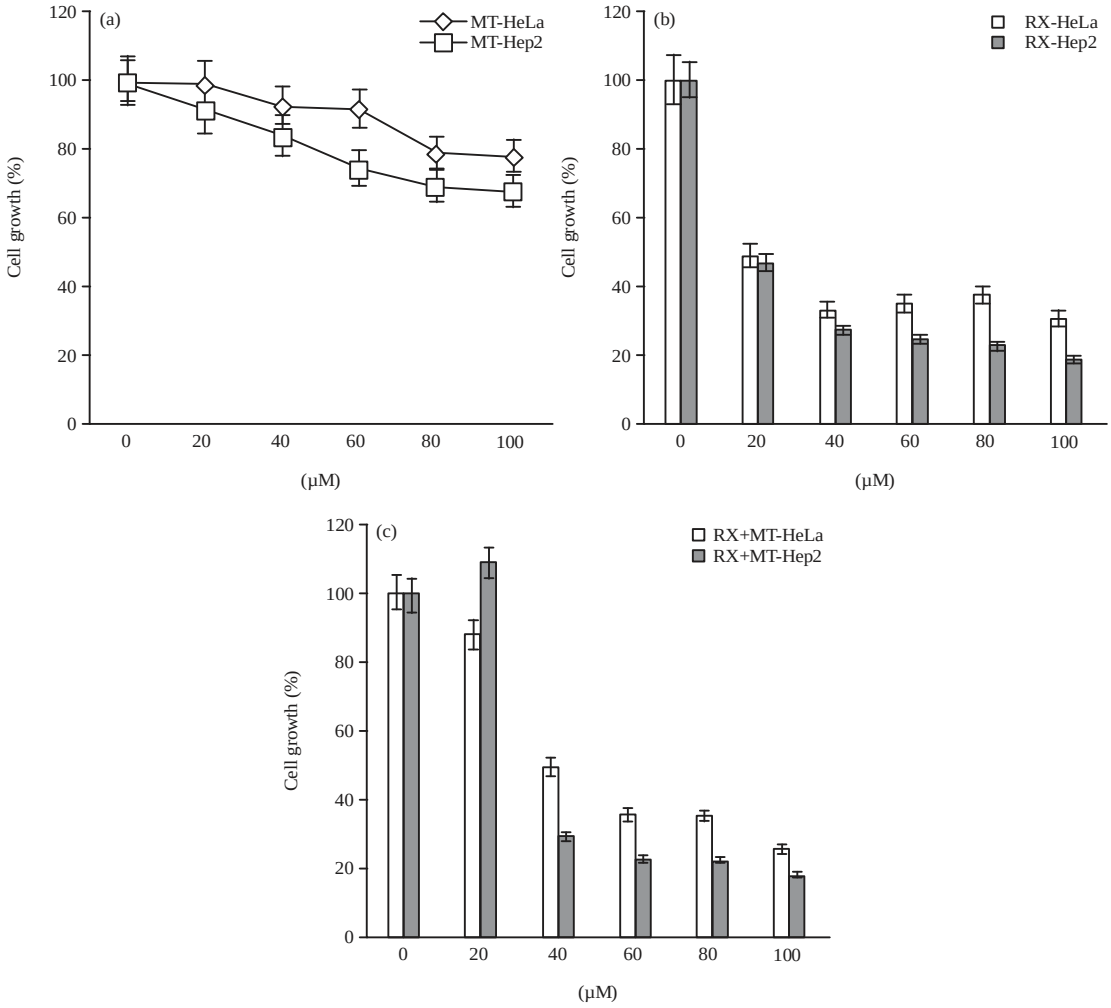

Fig. 1(a-c): Mitochondrial-based cytotoxicity reflects cell growth capacity of HeLa and Hep2 cell lines upon, (a) Metaformin (MT), (b) Raloxifene (RX) and (c) Their combinational therapeutic regimen. Doses in the range of 0-100 μM were used in a 3 independent runs (n = 3)

Table 1: Cytotoxicity IC_{50} and fold changes of the metaformin-based regimens on HeLa and Hep2 cell lines

Drugs	HeLa cells	Fold	Hep2	Fold cells
MT	>100	-	>100	-
RX	28.46135	-	24.74489	-
CYT	>100	-	>100	-
RX+MT	46.20405	2.164312	38.09195	2.625227
CYT+MT	27.03634	3.698725	32.20131	3.105464
RX+MT+CYT	25.13594	3.978367	36.02496	2.775853

Cells were treated for 24 h, IC_{50} for three experiments is shown. Fold change was calculated by dividing the IC_{50} of the individual compound (MT, RX or CYT) by that of the respective combinatorial regimen (RX+MT, RX+CYT or RX+MT+CYT), p-values show statistically significant differences between mean IC_{50} of the combinatorial regimen and their individual counterparts, MT: Metaformin, RX: Raloxifene, CYT: Cytosine β-D-arabinofuranoside, IC_{50}: Half maximal inhibitory concentration of cell growth

average of 3 independent runs in Fig. 3 as representative bright fields of cell growth capacity upon IC_{50} treatments and in Fig. 4 as water insoluble-formazan crystals of MTT upon IC_{50} treatments. Data in Fig. 2 illustrate the percentage of viability of both HeLa and Hep2 cervical cancer cells after 24 h incubation with the proposed treatments. The results revealed a significant dose dependent decrease ($p<0.05$) in cell viability of HeLa and Hep2 cells upon CYT alone with a high significant dose dependent decrease ($p<0.01$) in cell viability of HeLa and Hep2 cells upon the combinatorial CYT+MT treatment. While, there was an intriguing effect of the triple therapy CYT+MT+RX on cell viability, where this synergistic combination killed more than 80% of the cervical cancer cells after 24 h. The IC_{50} of CYT was >100 in case of both cell lines, while the IC_{50} of CYT+MT was 27.03 in HeLa cell line and 32.20 in Hep2 cell line. Upon triple therapeutic regimen (RX+MT+CYT), the IC_{50} were 25.13 in HeLa cell line and 36.02 in Hep2 cell line (Table 1). The RX+CYT+MT as a triple therapy has the most effective cytotoxicity against Hep2 cells

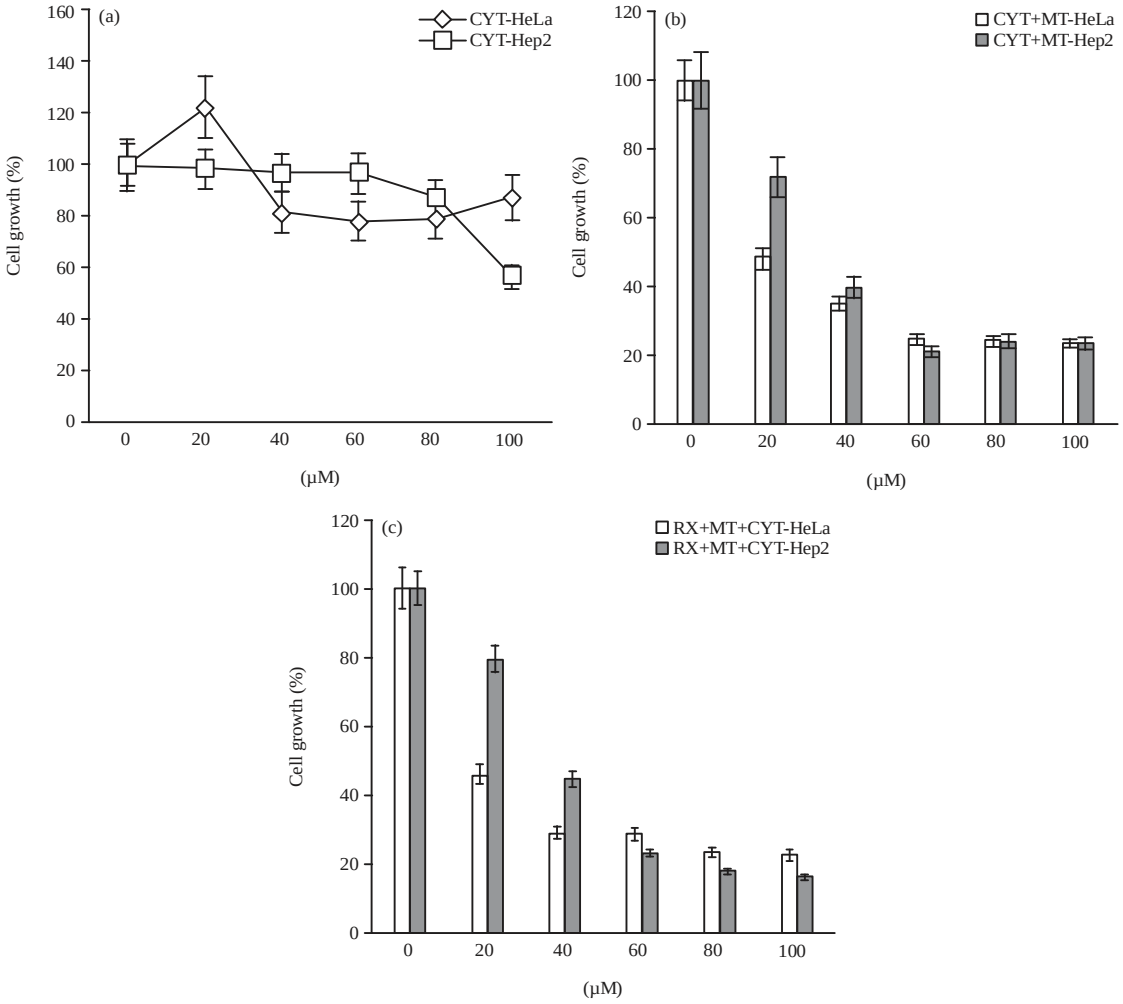

Fig. 2(a-c): Mitochondrial-based cytotoxicity reflects cell growth capacity of HeLa and Hep2 cell lines upon, (a) Cytosine β-D-arabinofuranoside (CYT), (b) CYT+MT and (c) The triple combinational therapeutic regimen (RX+MT+CYT). Doses in the range of 0-100 μM were used in a 3 independent runs (n = 3)

at all doses and kills approximately 83% of the cells, moreover, it gives the best output on HeLa cell line with around 80% cell death at the highest concentration. All of these results were confirmed by the bright field and the water insoluble-formazan crystals images as illustrated in Fig. 3 and 4.

Metformin-based regimen selectively inhibits glucose uptake and G6PD activity in HeLa cell line: The treatments of cervical cancer cells with RX, MT and CYT as individual and combinational therapies (RX, MT, CYT, RX+MT, CYT+MT and RX+MT+CYT), at different concentrations (0, 20, 40, 60, 80 and 100 μM) were investigated by measuring glucose uptake rate (Fig. 5a) and G6PD activity (Fig. 5b) in HeLa cell line. The experiments of the represented column bars in Fig. 5 was repeated 3 independent times (n = 3). By testing the change of glucose level in media over HeLa cells, we found that any metformin-based regimen i.e., MT, RX+MT, CYT+MT and RX+MT+CYT not the individual RX and CYT treatments, had the highest inhibitory effect significantly on glucose transports (i.e., these regimens inhibited glucose uptake through HeLa cells, $p<0.001$). Regarding he individual RX and CYT treatments, there was an inhibition in glucose uptake as well, but in a weak pattern compared to the metformin-based regimens, noting that CYT inhibits glucose uptake in HeLa cells more than RX compared to the untreated HeLa cells (Fig. 5a). In addition, as illustrated in Fig. 5b, the triple therapy with RX+MT+CYT was the most effective regimen inhibiting G6PD activity at 100 μM, where this triple regimen inhibited around 40% of G6PD activity in HeLa cells ($p<0.01$). Hypothetically, the tremendous effects of this triple therapy (RX+MT+CYT) in inhibiting G6PD activity and glucose uptake (Fig. 5a, b), mechanistically lead to HeLa cell bioenergetics deprivation

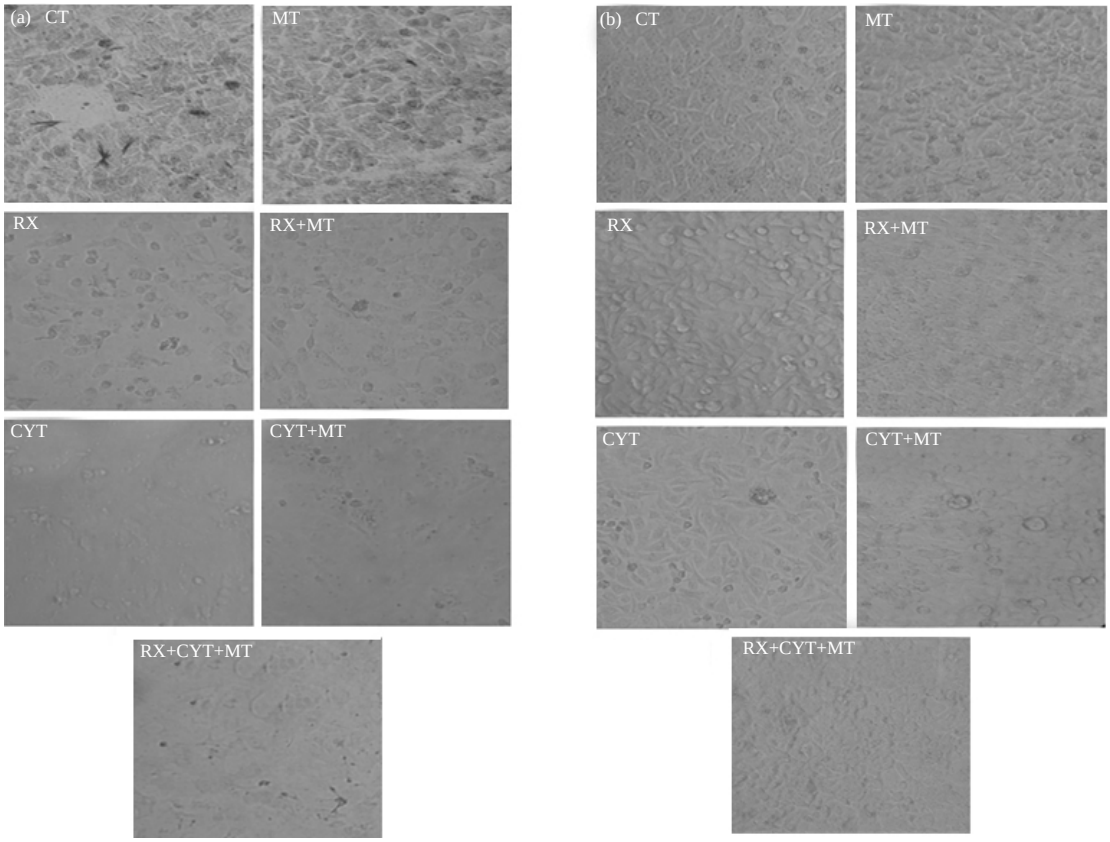

Fig. 3(a-b): Bright field images of cell growth experiments on (a) HeLa and (b) Hep2 cell lines post-treatments using the IC_{50} of different individual and combinational therapeutic regimens

and in turn cervical cancer cytotoxicity and death after arresting of nucleotide biosynthesis (Fig. 2).

Triple (RX+MT+CYT) therapeutic regimen decrease glucose uptake and G6PD activity in Hep2 cells: The treatments of Hep2 cancer cells with each RX, MT and CYT as individual and combinational therapies were examined by measuring their inhibitory effects on glucose uptake through Hep2 glucose transports and G6PD activity, using different concentrations (0, 20, 40, 60, 80 and 100 μM) incubating with Hep2 cells for 24 h. The experiments of the represented column bars in Fig. 6 was repeated 3 independent times (n = 3). By testing the change of glucose level in media over Hep2 cells, we found that the triple (RX+MT+CYT), the double (RX+MT) and the individual MT decrease glucose uptake by Hep2 cell line, especially the triple therapy at 100 μM by inhibiting approximately 22% of the glucose uptake through Hep2 cells as well as around 15% of the G6PD activity in the same cells ($p<0.05$). This inhibition cascade of this unique triple therapy (RX+MT+CYT) will, in turn leads to Hep2 cell death. On the contrary, RX, CYT and even CYT+MT had differences on glucose uptake rate, but not statistically significant ($p>0.05$), compared to the untreated Hep2 cells. With the same line, all regimens, except the triple one had variations over different concentrations, but not statistically significant ($p>0.05$).

DISCUSSION

Cervical cancer remains a major health dilemma for women, especially in developing countries with around 470 thousand new diagnosed patients and 233 thousand mortalities annually. The high mortality rate is basically owing to the privation of powerful treatment for this aliment with high-risk of alimental occurrence, in addition to the lack of chemo-response of unachievable aliment[16].

HeLa cell line, a model for cervical cancer research, is the oldest and the first continuous cancer cell line, most widely distributed, permanent human cell line and have been a mainstay of cancer studies ever since their seclusion from the hostile glandular cervical cancer of a young woman over 50 years ago[17]. Moreover, Hep2 cell line, a HeLa contaminant, contains HeLa marker chromosomes. This Hep2 cell line was originally derived from an epidermoid carcinoma of the larynx. Based on isoenzyme analysis, HeLa marker

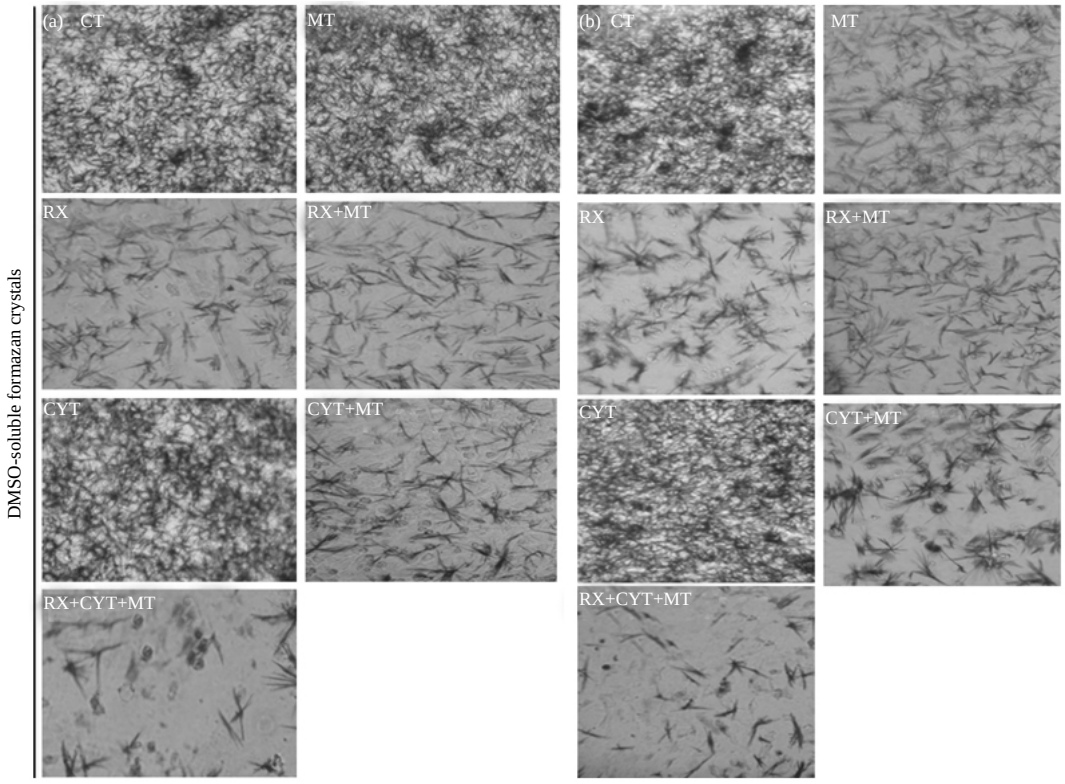

Fig. 4(a-b): Bright field images of the DMSO-soluble formazan crystals before solubilization on, (a) HeLa and (b) Hep2 cell lines post-treatments using the IC_{50} of different individual and combinational therapeutic regimens

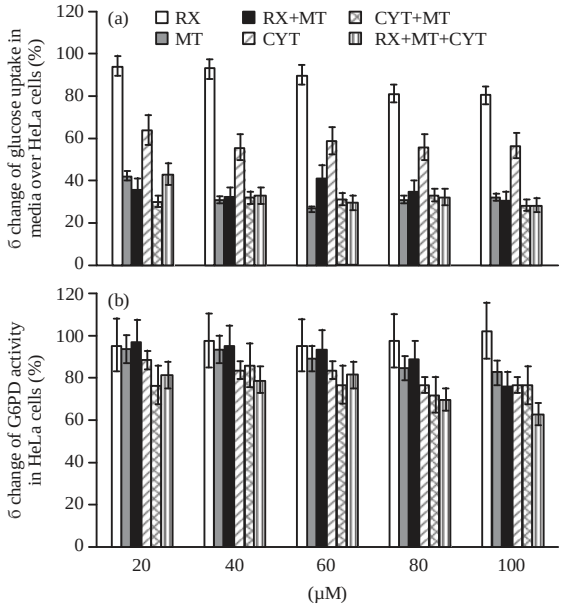

Fig. 5(a-b): (a) Glucose uptake and (b) G6PD activity in HeLa cell line post-treatments using different individual and combinational therapeutic regimens. Doses in the range of 0-100 μM were used in a 3 independent runs (n = 3)

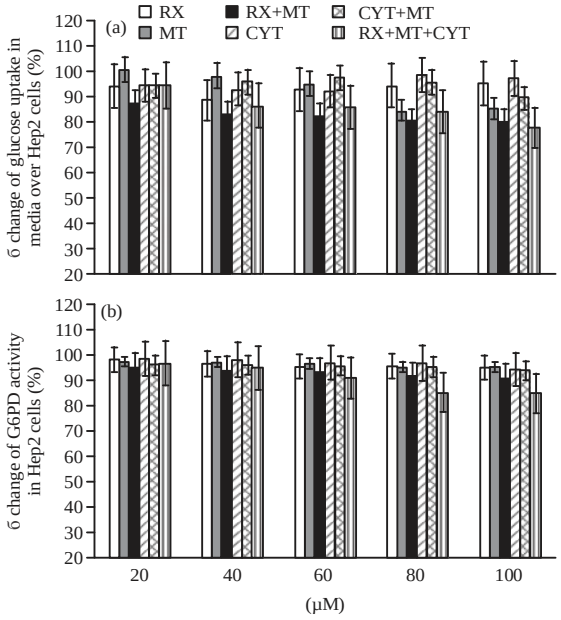

Fig. 6(a-b): (a) Glucose uptake and (b) G6PD activity in Hep2 cell line post-treatments using different individual and combinational therapeutic regimens. Doses in the range of 0-100 μM were used in a 3 independent runs (n = 3)

chromosomes and DNA fingerprinting were found in Hep2 cell line. The American Type Culture Collection (ATCC) confirmed these cell lines are positive for the presence of human papilloma viral DNA sequences via polymerase chain reaction[18,19].

Treatment for cervical cancer depended on surgery and/or radiation and cisplatin-based chemoprevention. However, a large scale of cases consequently develops chemo-resistance during chemotherapy. In-depth investigations concerning the molecular approaches of cervical cancer are required to promote the development of novel effective molecular targeted drugs[1].

The GLUT, an insulin-mediated glucose transporter, facilitates the passive diffusion of circulating glucose down its concentration gradient into cells[20]. Due to cancer cells are dependent on glycolysis, which end-up with only 2 adenosine triphosphate "ATPs" not mitochondrial respiration, which produce up to 38 ATPs for each glucose molecule, they have to compensate for this 18-fold lower machine-efficacy of energy production. Upregulation of GLUTs 1, 2, 3 and 4 to uptake more glucose is one of the prospective solutions. Thus, glucose uptake increment is a fundamental character distinguishing cancer cells from healthy cells. Some glycolytic metabolites such as glucose-6-phosphate (G6P) and dihydroxyacetone phosphate could be sidetracked into other metabolic passageways. For example, G6P is often consumed by pentose phosphate pathway to synthesize nucleotides and NADPH, a major reducing agent important for redox homeostasis and drug detoxifying reactions[21].

Using of metformin, in the current study, as a therapeutic candidate against gynecological cancer may represent a good model of the drug repositioning concept, following investigating the metformin's anticancer impact, which approved previously in terms of risk performance, drug screening and safety validation[22]. The mechanistic way of metformin to act as a key regulator of cellular metabolism has been reported, where it inhibits proliferation and induces apoptosis in cancer cells as a result of decreased energy disposition due to an increased AMP:ATP ratio and AMP-activated protein kinase (AMPK) activation. Thus, metformin up regulates glucose transport in normal cells, where decreases mitochondrial respiration chain activity and ATP production that, in turn activates AMP-activated protein kinase, which regulates energy homeostasis[23].

The signal transduction ways that control the Warburg effect during tumourigenesis remain important points to be explored. For this reason, metformin's ability to inhibit glucose metabolism in cancer cells may offer a possible benefit by limiting energy resources and consequently affecting cancer cell proliferation and growth. One of the most relevant clinical uses of the Warburg effect relies on the fact that the high rate of deregulated glucose consumption can be used for cancer diagnosis and prognosis. Mapping of glucose consumption by monitoring ^{18}F-fluoro-deoxy-glucose (FDG) using Positron Emission Tomography (PET) scanner is of great interest to study the effect of metformin. This glucose analogue enters the cell via the glucose transporters (GLUTs). Then, it is phosphorylated to FDG6P and remains stuck within the cytosol, being a false substrate for all further reactions of G6P to alternative passageways. The evaluation of metformin's effect *in vitro* on glucose uptake has been carried out in our study and in different cancer cellular models[9].

The underlying machinery triggering this antitumor effect of metformin is deliberated to involve the stimulation of adenosine monophosphate-activated protein kinase (AMPK) and embarrassment of mammalian target of rapamycin (mTOR), which inhibit cell progression[24].

Metformin therapeutic regimen and caloric restriction increase the AMP:ATP ratio and stimulate AMPK, switching cells from an anabolic to a catabolic state. Mechanistically, through AMPK pathway, metformin persuades the phosphorylation of CREB-regulated transcription co-activator 2 (CRTC2 or TORC2) sequestering CRTC2 from the nucleus to the cytoplasm[25]. This machinery constrains the transcriptional activation of gluconeogenic genes such as phosphoenolpyruvate (PEP) carboxykinase (PEPCK) (PCK2) and glucose-6-phosphatase (G6Pase). In addition, there is a debate in whether AMPK is mandatory to decrease circulating glucose concentration[26].

Basically, glucose-6-phosphate dehydrogenase (G6PD) has been enrolled in the regulation of cellular antioxidative machineries. Cancer cells often lose the balance of oxidation and antioxidation, but the role of G6PD in such an imbalance is still largely obscure. To examine the related function of G6PD in cancer cells, Li *et al.*[27] established a stable human melanoma A375 cell line with silenced G6PD using RNA interference technology called "A375-G6PD delta cells", accompanied by an 88.83% suppression of the endogenous G6PD expression and a 78.47% decrease in G6PD activity. In comparison with the A375 wild cells, they were characterized by a reduced proliferation with the MTT proliferation assay, a 25% decrease in colony-forming efficiency and an up to 40% increase of apoptotic rate with flow cytometric analysis. These results are in compatible line demonstrating that G6PD inhibition rendered tumor cells more susceptible to diamide-induced oxidative stress. Together, our and other data are in agreement to support the important functions of G6PD in the regulation of cell growth and antioxidative capacity of tumor cells.

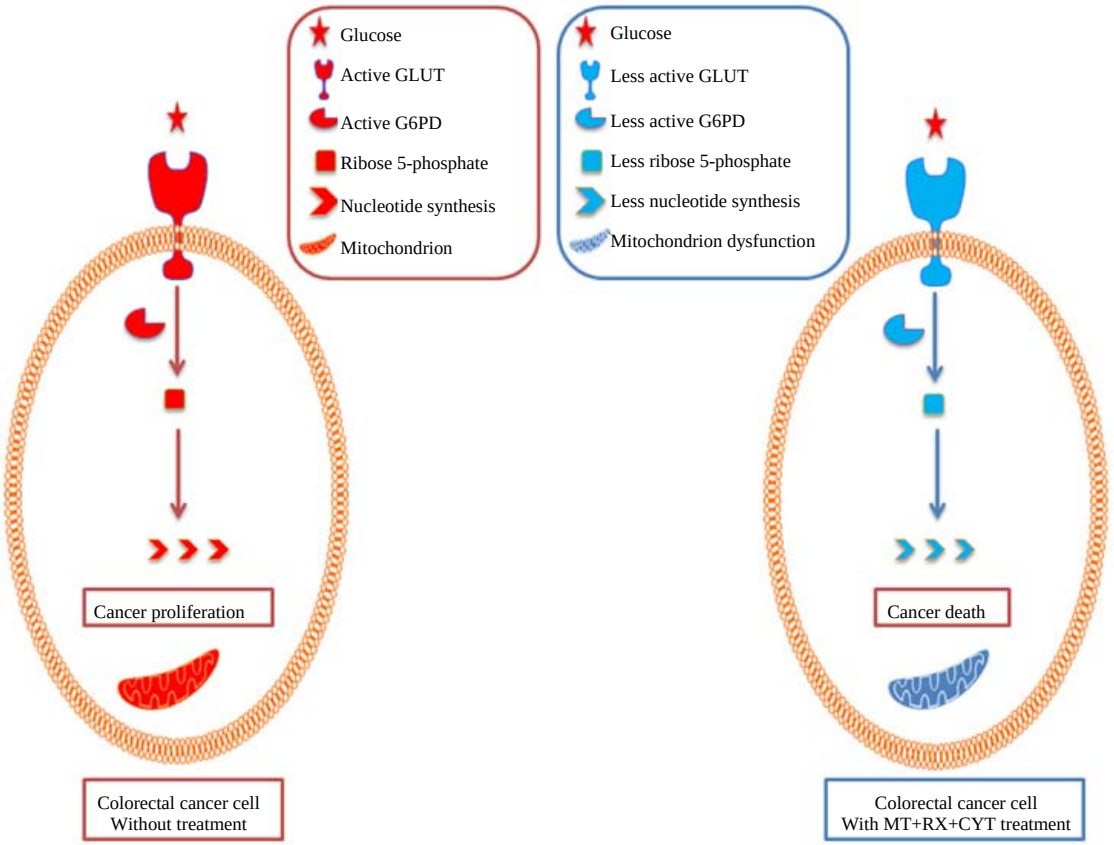

Fig. 7: Differential effects of metaformin-based regimens (right part) against control (left part) on cervical cancer cells (Example: HeLa cells). Right part (blue shapes) shows a remarkable cancer death associated with low glucose uptake (inactive glucose transporters), low G6PD activity and nuclear DNA synthesis, which in turn led to bioenergetics deprivation-mediated HeLa cell death. Left part (red shapes) shows a significant cancer growth (HeLa control) mediated with glucose uptake increment (active glucose transporters), high G6PD activity and normal DNA synthesis, which in turn led to bioenergetics induction linked with triggering HeLa cell proliferation

Furthermore, liver cancer-induced model is featured by elevated activity of the first irreversible enzyme of the oxidative branch of the Pentose Phosphate Pathway (PPP), G6PD for production of NADPH[28]. In the current study, the G6PD activity was high in HeLa and Hep2 cell lines, which decreased after metformin-based therapeutic regimens. These high activities of G6PD in cancer cells are in a harmonic line with what observed by Frederiks *et al.*[28] who indicating that G6PD activities in preneoplastic lesions were increased 25 times compared with extra-lesional parenchyma. It is concluded that NADPH in preneoplastic lesions is mainly produced by G6PD.

Xiao *et al.*[29] inspected that the metformin kinetics in cervical cancer cells and estimated liver kinase B1 (LKB1) activity and activating AMPK in these cells. They observed that metformin reticent the cervical cancer cell growth of the ME180, C33A and CaSki cells, but exhibited the reduced capacity of the HeLa, HT-3 and MS751 cell growth, which is in a agreement with our observation on HeLa cells. Moreover, raloxifene (RX) was able to induce apoptosis in both androgen-dependent (LNCaP) and -independent (PC3 and DU145) cell lines[30,31], as well as caused colorectal cancer cell growth arrest *in vitro*[32] and prostate cancer growth arrest *in vivo*[33]. Shibata *et al.*[13] found that RX inhibited cancer proliferation and metastasis to lymph nodes in a mouse immunocompetent metastatic mammary cancer model expressing cytoplasmic ERα. In addition, tumor tissues from the RX-treated mice illustrated induction of apoptotic markers, cell cycle arrest at S phase and inhibition of angiogenesis.

Figure 7 illustrated that, as a conclusion scheme representing a summary of these results gained from the current study. This scheme showed the differential effects, mechanistically-wise, of metaformin-based regimens (right part) against control (left part) on cervical cancer cells, taking

HeLa cells as an example, because both glucose uptake and G6PD activity were significantly inhibited by the most of these regimens on HeLa cell line. Right part of this scheme (blue shapes) showed a detectable cancer death associated with low glucose uptake (Due to the inactivated glucose transporters), low G6PD activity, DNA synthesis defect and mitochondrial dysfunction (based on the mitochondrial-related MTT assay), which in turn led to bioenergetics deprivation-mediated HeLa cell death. On the contrary, in left part of this scheme (red shapes), we illustrated that there was a well cancer HeLa cell growth-mediated with glucose uptake increment (Due to activated glucose transporters in cancer cells without treatments), high G6PD activity and normal mitochondrial respiration (based on the mitochondrial-related MTT assay), which in turn led to bioenergetics induction linked with triggering HeLa cell proliferation.

CONCLUSION

We concluded that there was a significant dose dependent decrease in cell proliferation of cervical cancer cell lines, HeLa and Hep2, upon metformin-based treatments with a high significant decrease upon the individual RX and the combinatorial RX+MT treatment (around 80% cancer cell death). Additionally, there was a high significant dose dependent decrease upon the combinatorial CYT+MT treatment. Intriguingly, there was a potent effect of the triple therapy CYT+MT+RX on cancer cell growth, where this synergistic wave killed more than 80% of the cervical cancer cells. Furthermore, we observed that any metformin-based regimen, not the individual RX and CYT treatments had the highest inhibitory effect on glucose uptake through HeLa cells. The triple therapy was the most effective regimen inhibiting G6PD activity at 100 μM, where this triple regimen inhibited approximately 40% of G6PD activity in HeLa cells. This will mechanistically deprive the HeLa cell bioenergetics and, in turn, tackle cervical cancer cells. Again 100 μM of triple therapy decreases approximately 22% of glucose uptake and 15% of the G6PD activity in Hep2 cells. This inhibitory cascade of this unique metformin-based triple therapy could provide a promising therapeutic arsenal against cervical cancer.

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